Taxonomy and population biology of selected *Ceratocystis* spp. with hat-shaped ascospores.

by

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DECLARATION

I, the undersigned, hereby declare that this thesis submitted herewith for the degree Magister Scientiae to the University of Pretoria, contains my own independent work and has not been submitted for any degree at any other University.

Marelize van Wyk

February 2003

I dedicate this thesis to my two late grandmothers Elisabeth Marè and Marie Wilkie

"To laugh often and much; to win the respect of intelligent people and the affection of children; to earn the appreciation of honest critics and endure the betrayal of false friends; to appreciate beauty, to find the best in others; to leave the world a little better; whether by a healthy child, a garden patch or a redeemed social condition; to know even one life has breathed easier because you have lived. This is the meaning of success."

Ralph Waldo Emerson

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PREFACE

The genus *Ceratocystis* includes some of the most devastating pathogens particularly of woody plants. In forestry, *C. fimbriata* is a well-known pathogen of *Eucalyptus* spp.; *C. albofundus* is a serious pathogen of *Acacia* spp., while *C. polonica* and *C. laricicola* infect *Picea* and *Larix* spp., respectively. During the past 10 years, reports of *Ceratocystis* spp. from commercial hardwood forestry species have doubled. These fungi thus represent a serious threat to forestry globally and they require further study.

In the first chapter of this dissertation, a broad overview of the literature pertaining to the genus *Ceratocystis* is presented. A retrospective consideration of the controversial taxonomic history establishes the background for the research in this dissertation. Morphological, biochemical as well as phylogenetic studies have been reviewed to illustrate the basis for best discriminating between species. The mode of reproduction as well as the distribution, host range, infection, dispersal, disease development and symptoms are also presented. This chapter has a special focus on *Ceratocystis* spp. with hat-shaped ascospores because the research in this dissertation is predominantly on members of this group of *Ceratocystis* spp.

Discovering and describing new species is fundamental to understanding fungi. In chapters two, three and five of this dissertation, I consider the taxonomy of three new *Ceratocystis* spp. from Bhutan, Sulawesi and Sumatra, respectively. These fungi were discovered on *Picea spinulosa*, *Syzigium aromaticum* and *Quercus* trees, respectively. In these chapters the new species are characterized and described using morphological and DNA sequence data. In the studies using sequence data, three different gene regions; the Internal Transcription Spacer regions (ITS), the beta-tubulin (β-tubulin) region and the Transcription Elongation Factor-1α region (EF1-α) are used to compile multi gene genealogies. The isolates requiring characterisation were compared with other *Ceratocystis* spp. in order to determine their identity.

One of the fastest growing DNA-based tools used to derive and understanding of the origin, mode of reproduction and other population based characteristics in plant

pathology is the use of polymorphic DNA markers or microsatellites. Understanding the population structure of pathogens represents an important aspect of disease management. Microsatellites are often very specific and can only be used on a single species. However, in some cases, closely related species include similar repetitive regions and the primers developed for one species can be used on others. A new species discovered as a result of research presented in Chapter 3 is phylogenetically closely related to *C. fimbriata*. The primers developed for the amplification of repetitive regions in *C. fimbriata,* were thus applied to a population of isolates of this fungus in Chapter 4.

In chapter five of this dissertation, the non-pathogenic species *Ceratocystis moniliformis* was studied to determine whether it is a monophyletic or polyphyletic. Historically, a variety of descriptions exist for this species, suggesting that it probably represents a species complex. DNA sequence and morphological characteristics of a wide range of *C. moniliformis* isolates were, thus used to compare them. For the DNA sequence studies, data from three different gene regions were used to provide strong support for the comparisons.

Studies presented in this dissertation were undertaken during the course of approximately two years. They have relied on an important collection of isolates from diverse parts of the world and I believe that they represent a useful milestone in *Ceratocystis* taxonomy and ecology. Chapters are presented as manuscripts intended for publication. The fact that they have been written sequentially has meant that some redundancy between chapters has been unavoidable.

CHAPTER 1

The genus *Ceratocystis* with special reference to species with hat-shaped ascospores occurring on forest trees

1. INTRODUCTION

Globally, commercial forestry plantations cover about 30 % or up to 3,934,890,000 ha of land (Anonymous 2003). The trees most extensively planted are softwoods, eucalypt-, and wattle-trees (Evans 1982, Kanowski 1997, Anonymous 2003). Trees from these planting programmes are used for structural timber, pulp and paper production, utility poles, fuel and mine props (Sutton 1995, Anonymous 2003).

Diseases of forest trees are known to have a substantial influence on fibre production resulting not only in tree death, but greatly influencing quality, growth and yield. In commercial plantation forestry, *Ceratocystis* Ellis & Halst. spp. were first reported early in the 1900's (Halsted 1890, Hedgcock 1906). The first *Ceratocystis* sp. to be reported on a forest tree, *Liquidambar styraciflua* L., was *C. moniliformis* Hedgcock (Hedgcock 1906). *Ceratocystis polonica* (Siemaszko) Moreau and *C. laricicola* Redfern & Minter are two species that cause substantial damage to conifers (spruce) (*Picea* spp.) and Larch (*Larix deciduas* Mill.) (Redfern *et al.* 1987, Christiansen & Solheim 1990). These fungi are associated with the bark beetles *Ips typographus* L. (Coleoptera: Scolytidae) and *Ips cembrae* Heer (Coleoptera: Scolytidae), respectively.

The genus *Ceratocystis* resides in the phylum Ascomycota Whittaker (Hunt 1956), and in the order Microascales (Luttrell) Malloch (1970). This order includes genera with long necked ascomata and sticky ascospore masses adapted to insect dispersal (Hunt 1956, Upadhyay 1981). The ascospores are mostly dextrinoid (yellowish brown or reddish brown in colour) when young, without germ pores. The thin walled asci are evanescent and produce eight ascospores that are often formed in chains (Benny & Kimbrough 1980, Kirk *et al.* 2001). *Ceratocystis* further groups within the class Sordariomycetes (Eriksson & Winka 1997). This class of fungi is characterised by the production of perithecioid ascomata, and the presence of unitunicate asci, often with apical annuli (Kirk *et al.* 2001). Species of *Ceratocystis* are generally treated in their own family, Ceratocystidaceae Locq., which is characterised by the absence of stromata and dark, usually long necked ascomatal perithecia with ostiolar hyphae (Kirk *et al.* 2001).

The genus *Ceratocystis* includes several economically important plant pathogens of trees, agricultural crops and herbaceous plants (Alexopoulos 1962, Seifert, Wingfield & Kendrick 1993). Collectively, species in this genus infect a wide variety of commercially

important food crops (e.g. pineapple, banana, sweet potato, sugarcane, nut and stone fruits, beans, mangoes, dates, coffee and cacao), agronomic crops (e.g. cotton, tobacco, rubber), timber (e.g. eucalypt-, wattle- and plane trees), as well as amenity species (e.g. plane, myrtle beech, sugar maple, aspen, white beach) (Grylls & Seifert 1993, Kile 1993). Species of *Ceratocystis* are responsible for a wide range of disease symptoms including wood stain, canker diseases, vascular wilts and root diseases. There are also a few species to which no particular economic importance has been attributed (Hunt 1956, Seifert *et al.* 1993).

Until recently, *Ceratocystis* spp. have not been considered a problem in commercial hardwood plantations. However, in the 1980's, *C. fimbriata* Ellis & Halst., was reported as the causal agent of canker and death of *Acacia decurrens* de Wild. plantations in Brazil (Ribeiro *et al.* 1985). Shortly after this, *C. albofundus* De Beer, Wingfield & Morris, was described as the causal agent of wilt and death of *Acacia mearnsii* de Wild. in South Africa (Morris, Wingfield & De Beer 1993, Wingfield *et al.* 1996). In 1999, *C. fimbriata* was reported to cause rapid wilt and death of *Eucalyptus* spp. in the Republic of Congo and Brazil (Laia, Alfenas & Harrington 1999, Roux *et al.* 2000). The same fungus was also reported killing *Eucalyptus* trees in Uganda (Roux, Wingfield & Byabashaija 2001) and more recently in Uruguay (Barnes *et al.* 2003a). Four other *Ceratocystis* spp. are known to occur on *Eucalyptus* trees. These include *C. moniliformis* (Hedgcock 1906), *C. eucalypti* Yuan & Kile (Kile *et al.* 1996), *C. moniliformopsis* Yuan & Mohamm. (Yuan & Mohammed 2002) and *C. pirilliformis* Barnes & Wingfield (Barnes *et al.* 2003b). The former three are not considered to be pathogens, while *C. pirilliformis* has not been tested for its pathogenicity.

Species of *Ceratocystis* have a worldwide distribution and are being reported from an increasing number of hosts and geographical areas. The reports of *C. fimbriata* from *Eucalyptus* spp. and *C. albofundus* from *Acacia* have, led to increased concern regarding their threat to commercial forestry, especially in the tropics and the Southern Hemisphere (Roux *et al.* 2003). The aim of this review is to summarize the taxonomic history of *Ceratocystis* and to provide an overview of knowledge pertaining to *Ceratocystis* spp. with hat-shaped ascospores, associated with forest trees.

2. TAXONOMIC HISTORY

The history of *Ceratocystis* dates back more than a century. The genus was first established in 1890, in association with black rot of sweet potato (*Ipomoea batatas* Lam.) in New Jersey, USA (Halsted 1890). The causative fungus was named *C. fimbriata,* which is also the type species of this genus (Halsted 1890, Halsted & Fairchild 1891). The first description of *C. fimbriata* was, however, inaccurate, as Ellis and Halsted mistook the ascospores for conidia, as they failed to observe asci. In 1892 Saccardo, unaware of the oversight of Ellis & Halsted, transferred *C. fimbriata* to *Sphaeronaema fimbriatum* Fr. (Saccordo 1892). Von Hönel (1918) divided the genus *Ceratostomella* Sacc., which was established for fungal species with colourless ascospores by Saccardo (1878), into two groups based on morphology. The one group, residing in *Ceratostomella*, represented species with fleshy perithecia and cylindrical asci. The other group was provided with the name *Linostoma* Hönel and consisted of species with carbonaceous perithecia with long necks and ovoid asci containing spores arranged in several rows (Von Hönel 1918). *Sphaeronaema fimbriatum* was transferred to *Linostoma* (Von Hönel 1918). Unfortunately the name *Linostoma* had been assigned to a genus of flowering plants in the *Thymeleacaeae* Juss. Von Sydow and Sydow (1919), therefore, established the genus *Ophiostoma* Syd. & Syd. and relegated all the species previously in *Linostoma* to this newly established genus, separated from the genus *Ceratostomella.*

Almost thirty years after *Ceratocystis* was first discovered, Elliott (1923) found that the presumed pycnidia of the fungus were in fact perithecia with ascospores emerging from deliquescent asci. He thus transferred *S. fimbriatum* to *Ceratostomella* Sacc. (Elliot 1923). The work of both Von Hönel (1918) and Von Sydow & Sydow (1919) was, however, not accepted until Melin and Nannfeldt (1934) reduced the genus *Endoconidiophora* Münch, which was established for species that formed conidia endogenously, to *Ceratostomella.* They transferred *Ceratostomella fimbriata*, *C. paradoxa* (Dade) C. Moreau and *C. adiposa* Butl. to *Ophiostoma*, due to the fact that they had *Chalara* (Corda) Rabenh. conidial stages (Melin & Nannfeldt 1934). Melin & Nannfeldt (1934) stated that the oldest genus name *Endoconidiophora* should be used, but this name would be confusing and thus suggested the genus name *Ophiostoma* until such a time that a more suitable name could be suggested. Melin and Nannfeldt (1934) divided *Ophiostoma* into two sections, *Brevirostrata* Nannfeldt and *Longirostrata* Nannfeldt. Short, conical perithecial necks characterised the section *Brevirostrata*, while longer filiform necks characterised

Longirostrata. The latter was further subdivided into two groups, those with endogenous *Chalara*-type conidia, and those with exogenous conidia.

In 1950, some species of the genus *Endoconidiophora* were transferred back to *Ceratocystis* (Bakshi 1950). Bakshi (1951) published a significant paper separating *Ceratocystis* and *Ophiostoma*. Although Bakshi's (1951) division of *Ceratocystis* and *Ophiostoma* was generally accepted (Moreau 1952, Hunt 1956), some authors chose to treat the two genera as one (Von Arx 1952, Von Arx & Műller 1954, Upadhyay 1981). This resulted in considerable confusion in later taxonomic classification of species in these genera.

Distinct differences between the anamorphs of *Ceratocystis* and *Ophiostoma* have been observed (Moreau 1952, Hunt 1956, Nag Raj & Kendrick 1975, Upadhyay 1981). Hunt (1956), separated *Ceratocystis* into two groups based on anamorph characteristics. The first group consisted of species, that produced conidia endogenously while the other group had conidia developing both endogenously and exogenously. The second group was then subdivided into two categories, those with mycelial conidia and those with *Graphium* Corda or *Leptographium* Lagerb. & Melin conidial types.

Olchowecki & Reid (1974), recognized four groups in *Ceratocystis* based on ascospore morphology. These were referred to as the Minuta, Ips, Fimbriata and Pilifera groups. Upadhyay and Kendrick (1975) accepted *Ceratocystis* but established the new genus, *Ceratocystiopsis*, to accommodate species with falcate generally aseptate (1-septate in one species), ascospores with hyaline, gelatinous sheaths with attenuated ends, and asci fusiform or clavate. The Minuta group of Olchowecki & Reid (1974) was more or less equivalent to the genus *Ceratocystiopsis* Upadhyay & Kendr. (Upadhyay & Kendrick 1975, Harrington 1981).

It is accepted today that species of *Ceratocystis* have *Thielaviopsis* Went (*Chalara*) (Paulin-Mahady, Harrington & McNew 2002) anamorphs with enteroblastic conidiogenesis, while species of *Ophiostoma* have anamorphs in a number of genera, most commonly *Sporothrix* Hektoen & Perkins, *Leptographium*, *Pesotum* Crane and *Hyalorhinocladiella* Upadhyay & Kendr., with holoblastic conidiogenesis (Von Arx 1974, De Hoog & Scheffer 1984, Samuels 1993, Wingfield, Viljoen & Wingfield 1999,

Upadhyay 1981). Species of *Ceratocystiopsis* have anamorphs that are similar to those of *Ophiostoma* (Wingfield 1993).

The three genera, *Ceratocystis*, *Ophiostoma* and *Ceratocystiopsis* have collectively been known as *Ceratocystis sensu lato* (De Hoog & Scheffer 1984, Wolfaardt, Wingfield & Kendrick 1992, Wingfield *et al.* 1994). Species of *Ceratocystis* are very different to those of *Ceratocystiopsis* and *Ophiostoma* based on morphology, physiology and molecular data (Smith, Patik & Rosinski 1967, Weijman & De Hoog 1975, Harrington 1981, De Hoog & Scheffer 1984, Hausner, Reid & Klassen 1993a). *Ceratocystiopsis* and *Ophiostoma*, however, often share characteristics (De Hoog & Scheffer 1984, Hausner *et al.* 1993a, Wingfield 1993). Due to the close relationship and similarities of *Ceratocystiopsis* and *Ophiostoma*, various authors have proposed their synonymy (Wingfield, Van Wyk & Marasas 1988, Hausner *et al.* 1993a,b, Wingfield 1993), which is now widely accepted.

Ceratocystis and *Ophiostoma* can also be separated at the ultrastructural level. In-depth studies of the development of teleomorph structures revealed that young asci in *Ceratocystis* line the periphery of the inner perithecium while in *Ophiostoma*, the young asci are produced from the base of the inner perithecium (Van Wyk, Wingfield & Van Wyk 1991). The centrums of *Ceratocystis* and *Ophiostoma* also differ with regard to the type of cells present. *Ceratocystis* spp. have filamentous cells while *Ophiostoma* spp. have pseudoparenchymatous cells (Benny & Kimbrough 1980).

Biochemical studies on *Ceratocystis* and *Ophiostoma* have clearly shown that they should reside in separate genera. *Ceratocystis* spp. are sensitive to the antibiotic cycloheximide while *Ophiostoma* spp. are highly tolerant (Harrington 1981). The composition of the cell walls of *Ceratocystis* and *Ophiostoma* are also different. *Ophiostoma* spp. contain cellulose and rhamnose in their cell walls while *Ceratocystis* spp. contain neither of these compounds (Smith *et al.* 1967, Jewell 1974, Weijman & De Hoog 1975). *Ceratocystis* spp. are thus more like other ascomycetous fungi with chitin in their cell walls.

Contention regarding the separation between *Ceratocystis* and *Ophiostoma* has been resolved largely in the last decade, based on DNA sequence analysis. The two genera form distinct clades when their DNA sequences are compared (Hausner *et al.* 1992, 1993a,c, Spatafora & Blackwell 1994, Wingfield *et al.* 1994, Witthuhn *et al.* 1998, 1999). Some of the genes used to separate these two fungal groups initially have been the large sub-unit

(LSU) ribosomal RNA (rRNA) (Hausner *et al.* 1993a,b, Wingfield *et al.* 1994) and the small sub-unit (SS) rRNA (SSrRNA) (Hausner *et al.* 1992, 1993a,b). The separation of *Ophiostoma* and *Ceratocystis* is now widely accepted. The two genera are clearly phylogenetically distinct from each other. Phylogenetically, *Ceratocystis* resides in the Microascales and *Ophiostoma* in the Ophiostomatales (Hausner *et al.* 1993a,c, Wingfield *et al.* 1996, Witthuhn *et al.* 1998, 1999).

3. MORPHOLOGY

In the early days of fungal taxonomy, morphological characteristics represented the primary means to distinguish between mycelial fungi. Consequently, many oversights emerged and these have led to considerable taxonomic confusion. In this regard, species in *Ceratocystis* have been no exception. As mentioned earlier in this review, the ascospores were mistakenly identified as conidia because the rapidly deliquescing asci were not seen (Halsted 1890). Twenty-five years later, it was observed that these "conidia" were in fact ascospores and the fungus was placed in the genus thought to be correct at that time (Elliott 1923).

3.1 Teleomorph

In *Ceratocystis*, the teleomorph state provides the primary morphological characteristics to distinguish between most species. Important characteristics include the shape, size, colour and ornamentation of the ascocarp bases, the attachment of the neck to the base, the length and ornamentation of the neck and the shape and size of the ascospores.

The ascomatal bases of *Ceratocystis* spp. are enlarged and have a characteristic bulbous form, which is either globose e.g. *C. moniliformis* (Hedgcock 1906), globose but flattened or flask-shaped, e.g. *C. fimbriata* (Upadhyay 1981) or pear shaped, e.g. *C. pirilliformis* (Barnes *et al.* 2003b). The bases can be ornamented or unornamented. When ornamented, it is in the form of short conical spines, which can be brown to black, thin or thick-walled, or they can be in the form of septate, unbranched hyphal hairs (Hunt 1956, Upadhyay 1981). *Ceratocystis moniliformis* (Hedgcock 1906) and *C. moniliformopsis* (Yuan & Mohammed 2002) are the only species with perithecial bases that are covered with short conical spines. Almost all *Ceratocystis* spp. have pale brown to black bases, with the exception of *C. albofundus*, that has hyaline bases (Morris *et al.* 1993, Wingfield *et al.* 1996).

The ascomatal necks of *Ceratocystis* spp. are dark brown to black, except for the apices that are pale brown to hyaline. This characteristic is, therefore, not useful for identification. The length of the necks can, however, be used as a means for discrimination between species. *Ceratocystis moniliformis*, for example, has necks of 500–900 µm long (Hedgcock 1906), while *C. fagacearum* (Bretz) Hunt (Upadhyay 1981) and *C. moniliformopsis* (Yuan & Mohammed 2002) have necks of 265–500 µm and 480–780 µm in length, respectively. The tips of the necks of most *Ceratocystis* spp. are ornamented with hyaline to pale brown ostiolar hyphae. The orientation of the ostiolar hyphae at the apices of the necks can also be used for diagnostic purposes. Some species, for example *C. albofundus* (Wingfield *et al.* 1996) have convergent ostiolar hyphae, while others have divergent ostiolar hyphae such as those found in *C. moniliformis* (Hedgcock 1906). The bases of the necks are also useful in distinguishing between species of *Ceratocystis*, due to a small number of species that have a disc-shaped attachment to the bases of the ascomata. These disc-shaped attachments are found in *C. moniliformis* (Davidson 1935) and *C. moniliformopsis* (Yuan & Mohammed 2002).

The time of extrusion of the ascospore masses in *Ceratocystis* varies between species as well as temperature. Three days after a spore drop has been inoculated onto artificial agar and incubated, *C. moniliformis* already produces mature ascomata with ascospores (Hedgcock 1906). *Ceratocystis fimbriata*, for example, only produces ascomata with ascospores after 7 days of incubation at 25 °C (Hunt 1956).

Ceratocystis spp. have diverse ascospore morphologies. Some are hat-shaped in side-view due to flanged appendages such as in the case of *C. moniliformis* (Hedgcock 1906) and *C. pirilliformis* (Barnes *et al.* 2003b). *Ceratocystis autographa* Bakshi was described as having oblong-ellipsoidal ascospores (Bakshi 1951, Grylls & Seifert 1993) although this species has an unclear taxonomic description. *Ceratocystis stenospora* Griffin was described with fusiform to falcate ascospores (Griffin 1968, Grylls & Seifert 1993) but the validity of this species is also not certain. All the ascospores in these species are hyaline in colour, one-celled, smooth, sheathed and produced in a gelatinous matrix (Hunt 1956, De Hoog 1974, Van Wyk *et al.* 1991).

3.2 Anamorph

The anamorphs of *Ceratocystis* spp. have traditionally been accommodated in *Chalara* (Von Arx 1974, Harrington 2000). A recent phylogenetic study by Paulin-Mahady *et al.* (2002) showed that the anamorphs of these fungi should most appropriately be treated in *Thielaviopsis*. Their study, based on DNA sequence data, also emphasised the fact that, aside from *C. moniliformis* and *C. fagacearum*, most anamorph species of *Ceratocystis* produce thick-walled aleuroconidia from the tips of specialized hyphae. The genus *Thielaviopsis* is based on *T. ethacetia* Went (Nag Raj & Kendrick 1975), which was later synonymized with *T. paradoxa* (de Seynes) Höhn, the anamorph of *C. paradoxa* (Dade) Moreau. *Chalaropsis* Pyronel., which was characterised as an endogenous and an enteroblastic conidial phase was reduced to synonymy with *Thielaviopsis* (Nag Raj & Kendrick 1975).

Thielaviopsis spp. are characterised by the formation of aleuroconidia on specialised hyphae (Paulin-Mahady *et al.* 2002). These aleuroconidia are thick walled and are usually dark in colour. They can be formed in chains or can occur singly. Aleuroconidia are not a prerequisite for species to be placed into this genus (Paulin-Mahady *et al.* 2002). Phialides produce cylindrical conidia through ring wall building, which can be extruded in chains or singly. The conidia may remain hyaline or become thick-walled and dark with maturity (Nag Raj & Kendrick 1975, Minter, Kirk & Sutton 1982, 1983, Nag Raj & Kendrick 1993). When known, teleomorphs of *Thielaviopsis* most appropriately reside in the genus *Ceratocystis* (Paulin-Mahady *et al.* 2002).

A distinguishing characteristic of the anamorphs of *Ceratocystis*, is the presence or absence of aleuroconidia, also known as chlamydospores. These are known to enable species to survive in soil. For example, *C. fimbriata,* which is known to be able to survive in soil for many years, has chlamydospores (Rossetto & Riberio 1990, Marin *et al.* 2003), while *C. moniliformis*, which is not known to survive is soil does not produce chlamydospores (Hedgcock 1906, Upadhyay 1981). The size of these structures is also taxonomically useful e.g. *C. fimbriata* being 9–15 x 7.5–11 µm (Upadhyay 1981), while those in *C. pirilliformis* are 8–12 x 5.5–8 µm (Barnes *et al.* 2003b).

The size of *Thielaviopsis* conidia can be used to distinguish between species of *Ceratocystis*. For example, the length of the cylindrical conidia in *C. fimbriata* ranges from 9–21 µm (Upadhyay 1981), while those of *C. moniliformis* are 6–8 µm (Hedgcock

1906). The barrel-shaped conidia in *C. fimbriata* are 9–14 x 5–15 µm in diameter (Upadhyay 1981), while those of *C. pirilliformis* are 4–6 x 3–4 µm (Barnes *et al.* 2003b).

The size of the phialidic conidiogenesis cells differs between species of *Ceratocystis* and can be used to distinguish between some species. *Ceratocystis fimbriata*, for example has phialides that are 35-130 x 2.5-6 µm, at the base (Upadhyay 1981), while those of *C. moniliformopsis* range between 5-32.5 x 4-5 µm, at the base (Yuan & Mohammed 2002).

4. PHYLOGENETIC PLACEMENT

The use of DNA sequence data has revolutionized the taxonomic placement of fungal species. Phylogenetic relationships between distantly related taxa, such as genera of fungi, can be inferred from slowly evolving gene regions, for example, the LSU rRNA gene. In contrast closely related taxa such as species can be distinguished by using rapidly evolving gene regions (e.g. Internal Transcribed Spacer regions {ITS}) (Bruns, White & Taylor 1991, Messner 1995, Mitchell, Roberts & Moss 1995). The rRNA genes consist of highly conserved regions interspersed with variable regions and can, therefore, be used to study the relatedness between both distantly and closely related species (Jorgenson & Cluster 1988, Taylor *et al.* 1993). DNA sequence data from rRNA genes have been used effectively to determine the phylogenetic relationships among ophiostomatoid fungi and have contributed significantly to understand the differentiation between *Ophiostoma* and *Ceratocystis* (Hausner *et al.* 1992, 1993a,b,c, Spatafora & Blackwell 1994, Wingfield *et al.* 1994).

The genes used to distinguish between *Ophiostoma* and *Ceratocystis* have been used to show that *Ceratocystis* spp. group within the Microascales, while *Ophiostoma* spp. reside in the Ophiostomatales (Spatafora & Blackwell 1994). Partial rDNA sequence data have also contributed to reducing *Ceratocystiopsis* spp. to synonymy with *Ophiostoma* (Hausner *et al.* 1993c). It has thus been with the aid of DNA sequence data, that *Ophiostoma* and *Ceratocystis* have been unequivocally separated and shown to be phylogenetically distantly related.

Various gene regions have been used to study the taxonomic placement and relationships among species of *Ceratocystis*. The ITS region of the rRNA genes were used by Witthuhn *et al.* (1999) to show that the genus can be divided into two discrete clades. One of these

clades is more commonly known as the *C. coerulescens* clade, while the other is referred to as the *C. fimbriata* clade. The ITS region has also been used to distinguish between *Ceratocystis* spp. that are morphologically similar and thought to be the same species. It was proved by DNA sequence data, from the ITS regions, that there were in fact five distinct species within the *C. coerulescens* group (Witthuhn *et al.* 1998). *Ceratocystis laricicola* and *C. polonica* are morphologically difficult to distinguish and are often identified based only on their different hosts and insect associations (Yamaoka *et al.* 1997). Comparison of ITS sequences for these two species has, however, not supported the notion that they are distinct species (Harrington & Wingfield 1998, Witthuhn *et al.* 1998). Other genes that are frequently used to distinguish between species are; MAT genes (Witthuhn *et al.* 2000, Marin *et al.* 2003) and β-tubulin genes (Loppnau & Breuil 2003).

Various molecular markers, such as Restriction Fragment Length Polymorphisms (RFLPs) and microsatellites have been used in the identification of *Ceratocystis* spp. and in studies pertaining to populations of these species. RFLPs have been used to distinguish between closely related *Ceratocystis* spp. such as *C. coerulescens*, *C. pinicola* Harrington & Wingfield, *C. douglassi* (Davidson) Wingfield & Harrington, *C. resinifera* Harrington & Wingfield, *C. rufinipenni* Wingfield Harrington & Solheim, *C. polonica* and *C. adiposa* (Witthuhn *et al.* 1999). This technique uses the principle that nucleotides at a given place in the DNA of different species might differ, and thus, the restriction sites might be different. As a result, different sized fragments are seen on an agarose gel if there are differences in the nucleotide sequence. When a PCR-RFLP was done on the β-tubulin gene DNA, *Ceratocystis* spp. causing sapstain of conifer trees showed different bands, resulting in a rapid method whereby species can be distinguished (Loppnau & Breuil 2003).

Simple Sequence Repeats (SSRs) or microsatellites are more specific than RFLP's and can detect up to five times more variation (Tautz 1989). Microsatellites are typically used to study populations rather than single species, as they have a high level of polymorphism and can distinguish between individuals of the same species. They are co-dominant markers, compared to the dominant RFLP markers. Microsatellites consist of runs of short repeating sequences that can be variable in number between individuals (Jarne & Lagoda 1996) and can also identify different alleles within a single locus in individuals. By designing DNA primers flanking these repeat regions, the polymorphisms between

individuals can be studied easily as the microsatellites are inherited in a Mendelian fashion (Schlötterer & Pemberton 1988, Tautz 1989). Microsatellites have been used in a study of *C. fimbriata* to reflect intra-specific relationships between isolates and to compare the results of the population comparison with those emerging from phylogenies based on DNA sequencing (Barnes *et al.* 2001). The microsatellite markers developed for *C. fimbriata* have also been used for *C. albofundus* (Barnes 2002, Nakabonge 2002), which also provides an indication that microsatellite markers can sometimes be used to study population structures in closely related species.

5. BIOCHEMISTRY

The biochemistry of fungi plays an important role in the expression of its morphology and development. As early as 1921, biochemistry was used to differentiate between fungi. Von Wettstein (1921) divided the fungal Kingdom into two groups based on the carbohydrate constitution of the cell walls. The one group of fungi contained cellulose while the other group, including the ascomycetes contained chitin in their cells. In terms of the *Ophiostomatoid* fungi, the presence or absence of cellulose and chitin, as well as rhamnose in their cell walls has been used to distinguish *Ceratocystis* spp. from *Ophiostoma* spp. (Smith *et al.* 1967, Jewel 1974).

Most *Ceratocystis* spp. are deficient in their ability to produce vitamins such as thiamine, pyridoxine, and biotin (Robbins & Ma 1942). These vitamins can be added to artificial media to induce growth. Thiamine, as well as calcium, enhance the production of sexual structures, and thus facilitate sexual reproduction of these fungi (Robbins & Ma 1942, Hawker 1966, Upadhyay 1981).

Species of *Ceratocystis* produce intense fruity odours in young cultures, due to the production of monoterpenes (Hunt 1956, Lanza & Palmer 1977, Hanssen 1993). Monoterpenes are produced only when the nitrogen source is very low (Lanza & Palmer 1977). Odours tend to differ when different media are used to culture these fungi (Lanza, Ko & Palmer 1976, Lanza & Palmer 1977). The fruity aroma produced by these fungi is important in attracting insects that facilitate their dispersal.

Species of *Ceratocystis* can be placed in two groups based on the production of fruity aromas. The one group consists of species that do not produce the fruity aromas, for

example, *C. polonica* (Upadhyay 1981, Yamaoka *et al.* 1997). These fungi tend to have very specific insect vectors such as bark beetles (Yamaoka *et al.* 1997). The other group consists of species, which produce strong fruity odours to attract a wide variety of insects, for example *C. fimbriata* (Upadhyay 1981). The latter group have loose associations with insects such as flies (Diptera) and picnic beetles (Coleoptera: Nitidulidae).

6. REPRODUCTION

In general, ascomycetous haploid fungi have three basic reproductive strategies. They can either reproduce vegetatively, asexually or sexually (Alexopoulos 1962). When a fungus reproduces vegetatively, an unspecialised part of the original culture becomes detached from the main body of mycelium to form independent individuals. In asexual reproduction, specialized reproductive bodies are formed from which the new individuals are released, independent of nuclear fusion. Both of these forms of reproduction lead to progeny that are identical to their parents, both phenotypically and genotypically. Sexual reproduction relies on the fusion of two compatible nuclei (n) and a subsequent reduction division follows which produces new haploid individuals (Hawker 1966).

Ceratocystis spp. have a number of reproductive strategies that contribute to differing levels of genetic diversity. Most *Ceratocystis* spp. are self-fertile (homothallic), thus there is no need for two different individuals to reproduce via meiosis. This is found, for example, in *C. fimbriata* and *C. virescens* (Davids.) Moreau (Olson 1949, Webster & Butler 1967a,b, Harrington & McNew 1997). Species of *Ceratocystis* can also be heterothallic, and therefor need two separate individuals with opposite mating structures or genes to reproduce. Some heterothallic species in *Ceratocystis* are: *C. paradoxa*, *C. radicicola* Bliss, *C. fagacearum* and *C. eucalypti* (Heptig, Toole & Boyce 1952, Kile *et al.* 1996).

Sexual compatibility in heterothallic ascomycetes is determined by two alleles (idiomorphs), MAT-1 and MAT-2, at a single mating type locus. Thus, two strains must be of opposite mating types to produce ascospores (Glass & Kuldau 1992). Some *Ceratocystis* spp. show uni-directional mating type switching where self-fertile or self-sterile progeny can be produced from single ascospores, e.g. *C. coerulescens* and *C. albofundus* (Harrington & McNew 1997). MAT-2 strains can switch to MAT-1 and self,

but MAT-1 progeny cannot switch to MAT-2 and therefore remain self-sterile (Harrington & McNew 1997).

7. DISTRIBUTION & HOST RANGE

The genus *Ceratocystis* has a worldwide distribution, and has been reported from all continents except Antarctica (Table 1 & 2) (Kile 1993). *Ceratocystis moniliformis,* for example, has been found in Africa (Luc 1952, Witthuhn *et al.* 1999), North America (Von Schrenk 1903, Davidson 1935), Europe (Bakshi 1951) and Asia (Kitajima 1936, Roldan 1962, Witthuhn *et al.* 1999) (Table 1). *Ceratocystis fimbriata* has also been found in Africa (Roux *et al.* 2000), the Americas (Sharples 1936, Pontis 1951, Riberio *et al.* 1985, Barnes *et al.* 2003a), Asia (Halsted 1890, Kile 1993) and Europe (Kile 1993) (Table 2). There are species of *Ceratocystis*, however, that have a limited distribution such as *C. pirilliformis*, which has been found only in Australia (Barnes *et al.* 2003b), while *C. albofundus* has been found only in Africa (Wingfield *et al.* 1996, Roux *et al.* 2001).

The host range of *Ceratocystis* spp. differs between species and geographical areas. Some *Ceratocystis* spp. such as *C. moniliformis* are found on many plants including fruit trees, forest trees, fruits and crops (Table 1). Others, such as *C. polonica* (Table 3), are strongly associated with a particular host and are carried by a specific insect, in this case *Picea* infested with *Ips typographus* (Yamaoka *et al.* 1997). Certain species are pathogenic to some hosts while non-pathogenic to others. *Ceratocystis fimbriata* is considered as a species with a wide host range. However, an increasing number of studies indicate that this species might represent host specific forms (Leather 1966, Webster & Butler 1967b, Harrington 2000).

8. INFECTION, DISEASE DEVELOPMENT AND SYMPTOMS

Most *Ceratocystis* spp. that infect woody plants require wounds for infection (Seifert *et al.* 1993). On trees, the spores germinate, and the mycelium enters through wounds and moves through the xylem into the ray parenchyma cells. The fungus subsequently causes dark reddish brown to purple discolouration of the tissues. Chlamydospores, the survival structures, produced by some *Ceratocystis* spp., are known to survive in the soil for long periods of time (Moutia & Saumtally 1999). Under optimal conditions, these spores can germinate and infect susceptible crops via the roots (Kile 1993, Moutia & Saumtally 1999). Disease symptoms caused by *Ceratocystis* spp. include fruit rots, sapstain of

timber, vascular wilts, vascular stains, cankers and root and stem rots (Kile 1993, Seifert *et al.* 1993). Some of the more economically important species of *Ceratocystis* are listed in Table 3.

9. DISPERSAL

Ceratocystis spp. employ a variety of dispersal mechanisms. They are dispersed via root grafts, as seen between *Platanus* trees (Skelly & Merril 1968, Accordi 1986), as well as by means of infested soil (Rossetto & Riberio 1990, Moutia & Saumtally 1999, Marin *et al.* 2003). *Ceratocystis fimbriata* has been reported to have the capacity to survive in soil for years (Rossetto & Riberio 1990, Moutia & Saumtally 1999, Marin *et al.* 2003). The fungus can also be dispersed by air, as an indirect association with insects and in the frass that insects produce when they tunnel through the wood, which can be dispersed by wind or water (Iton 1960). There have been no reports on the spread of these fungi from or within seeds (Upadhyay & Kendrick 1975, Harrington 2000). Agricultural practices as well as thinning of trees with the aid of infected machetes or pruning tools also aid in dispersal (Grosclaude, Olivier & Romiti 1995, Marin *et al.* 2003). In Colombia for example, the soil borne *C. fimbriata* commonly infects wounds made by the shoes of farm workers who use planted trees as support when climbing steep hills (Marin *et al.* 2003).

Insect transmission is the main method of dispersal for *Ceratocystis* spp., especially in living trees (Leach 1940, Himelick & Curl 1958, Upadhyay 1981). *Ceratocystis* spp. are well adapted for insect dispersal. The ascospores are collected in slimy masses at the apices of long ascomatal necks, (also described as a stalked spore-drop) (Ingold 1961), that stick to the bodies of insects (Leach, Orr & Christensen 1934, Griffin 1968). Some species of *Ceratocystis* also emit fruity odours that attract insects (Lanza & Palmer 1977). Spores that are ingested by the insects pass unharmed through their intestinal tracts (Leach 1940).

There are two distinct categories of association between *Ceratocystis* spp. and insects. Some species have a casual relationship with insects. These are the species that tend to produce fruity odours that attract many species of flies (Diptera) and sap beetles (Nitidulidae) (Himelick & Curl 1958). Species of *Ceratocystis* that have this form of adaptation include *C. fimbriata*, *C. albofundus*, *C. pirilliformis*, *C. fagacearum* and *C. moniliformis*. Another group of *Ceratocystis* spp. tend to not produce fruity odours. These

species rely on very close relationships with specific insects and particularly bark beetles for their dispersal. Thus, *C. polonica* is specifically carried by the bark beetle, *Ips typographus* that infests spruce (*Picea* spp.) in Europe and Asia (Siemaszko 1938, Christiansen & Solheim 1990), *C. laricicola* is carried by *I. cembrae* that infests larch in Europe and Asia (Redfern *et al.* 1987, Yamaoka *et al.* 1998) and C. *rufipenni* Wingfield, Harrington & Solheim is carried by *Dendroctonus rufipennis* Kirby that infests Engelman spruce (*Picea engelmannii* Parry) in Western North America (Solheim & Safranyik 1997, Wingfield, Harrington & Solheim 1997).

10. *CERATOCYSTIS* SPP. WITH HAT-SHAPED ASCOSPORES THAT ARE ASSOCIATED WITH HARDWOOD TREES IN PLANTATIONS

In recent years there has been increased concern regarding the number of *Ceratocystis* spp. that affect exotic plantation forests. *Ceratocystis* spp. group into two distinct clades, namely the *C. coerulescens* and *C. fimbriata* clades (Witthuhn *et al.*1999). There are three species, *C. fimbriata* (Upadhyay 1981), *C. albofundus* (Morris *et al.* 1993) and *C. pirilliformis* (Barnes *et al.* 2003b) in the *C. fimbriata* clade that have hat-shaped ascospores. Of these, *C. fimbriata* and *C. albofundus* are highly pathogenic and cause considerable losses to commercial forestry plantations (Wingfield *et al.* 1996, Harrington 2000). The steadily increasing number of reports of these and other pathogens associated with commercial plantation trees have caused considerable concern amongst forestry companies over the last 10 years. The following section of this review focusses on *Ceratocystis* spp. with hat-shaped ascospores that have been reported from commercial plantations of hardwood trees (Table 4, 5). These fungi are also related to those treated in the research chapters of this thesis.

10.1 ***Ceratocystis fimbriata*** Ellis & Halsted, Bull. N. J. Agric. Sta. 76: 14. 1890.

≡ *Sphaeronaema fimbriatum* (Ell. & Halst.) Sacc., Syll. Fung. 10: 125. 1892.
≡ *Ceratostomella fimbriata* (Ell. & Halst.) Elliott, Phytopath. 13: 56. 1923.
≡ *Ophiostoma fimbriatum* (Ell.& Halst.) Nannf., Svenska Skogsfor. Tidskr.32: 408. 1934.
≡ *Endoconidiophora fimbriata* (Ell. & Halst.) Davidson, J. Agric. Res. 50: 800. 1935.
= *Rostrella coffeae* Zimmermann, Medded. s'lands plantentuin 37: 24. 1900.
= *Endoconidiophora variospora* Davidson, Mycologia 36: 303. 1944.
= *Ceratocystis variospora* (Davids.) Moreau, Rev. Mycol. (Paris) Suppl. Col.17:22. 1952.

= *Ophiostoma variosporum* (Davids.) von Arx. Antonie van Leeuwenhoek 18: 212. 1952.

Ceratocystis fimbriata was first discovered as the causal agent of black rot of sweet potato in 1890 (Halsted 1890) and it is the type species for the genus *Ceratocystis*. It has globose to flask shaped ascomatal bases without ornamentation. The bases, as well as the necks of the ascomata, are dark in colour and almost black (Halsted & Fairchild 1891, Hunt 1956, Upadhyay 1981). *Ceratocystis fimbriata* produces hat-shaped ascospores and abundant chlamydospores (Hunt 1956, Upadhyay 1981). *Ceratocystis fimbriata* grows slowly, 15 mm in 12 days, with an optimal temperature of 22 °C (Hunt 1956, Upadhyay 1981). It is also characterised by the production of fruity, banana like odours in young cultures (Hunt 1956, Upadhyay 1981). Two types of conidia are produced; one cylindrical and the other more barrel-shaped (Hunt 1956, Upadhyay 1981).

Forest trees that are associated with infection by *C. fimbriata* include *Eucalyptus* spp. (Roux *et al.* 2000), *Populus tremula* L. (Aspen) (Wood & French 1963), *Platanus* spp. (Plane) (Jackson & Sleeth 1935) and *Gymnocladus* spp. (Pontis 1951) (Table 2). *Ceratocystis fimbriata* is pathogenic to many different tree crops and infects through wounds (Kile 1993). The symptoms usually seen after infection of *C. fimbriata* on forestry trees range from cankers to wilt and dieback (Pontis 1951, Roux *et al.* 2000). *Ceratocystis fimbriata* has "races" or "strains" that are host specific (Webster & Butler 1967b, Wellman 1972, Harrington 2000). This host specificity seems to correlate with geographic regions (Webster & Butler 1967b, Harrington 2000, Barnes *et al.* 2001, Baker *et al.* 2003).

There has been considerable debate regarding the origin of *C. fimbriata*. It was originally thought to have originated in South America and to have spread on infected plant material to the rest of the world. These hypotheses were, however, based on host specialization, range and DNA sequence data. Recently, Barnes *et al.* (2001) developed microsatellite markers for *C. fimbriata*. These authors compared three populations of *C. fimbriata*; a population from Congo and Uruguay from commercial *Eucalyptus* plantations and a population from Colombia from coffee trees. The study showed a high genetic differentiation between these three populations with minimal gene flow. There was little evidence of recombination within the populations. The population study done by Baker & Harrington (2000), which used microsatellites, nuclear DNA fingerprints & mitochondrial DNA RFLP's concluded that *C. fimbriata* f. *platani* is native to North America.

10.2 ***Ceratocystis pirilliformis*** Barnes & Wingfield, Mycologia 95: 865-871. 2003.

Ceratocystis pirilliformis is a recently discovered species that infects *Eucalyptus* trees in Australia (Barnes *et al.* 2003b). It is phylogenetically closely related to *C. fimbriata* but it has pear-shaped ascomatal bases with thick necks, producing hat-shaped ascospores. The anamorph is typical of *Thielaviopsis* producing both cylindrical and barrel-shaped conidia. It is a slow growing fungus, growing 22 mm in 12 days at an optimal temperature of 25 °C. The cultures appear white at first, becoming grey to olive green (Barnes *et al.* 2003b). *Ceratocystis pirilliformis* produces an aroma similar to that of *C. fimbriata*, which is fruit-like and resembling banana like when cultures are actively growing.

Ceratocystis pirilliformis was isolated from artificial wounds on *Eucalyptus nitens* Dean et Maiden. It causes a sapstain that discolours the wood and may decrease timber value (Barnes *et al.* 2003b). No other reports of this fungus are known and its possible role in disease development must still be determined.

10.3 ***Ceratocystis albofundus*** De Beer, Wingfield & Morris Syst. Appl. Microb. 19: 191-202. 1996.

= *Ceratocystis fimbriata* (Ell. & Halst.) Elliot, Gorter, Morris *Plant Path.* 42 : 814-817. 1993.

Ceratocystis albofundus has hat-shaped ascospores and is the second species to be described as a pathogen of commercial plantation forest trees. It is characterised by light ascomatal bases compared to the dark bases of *C. fimbriata* and *C. pirilliformis* (Morris *et al.* 1993). The ostiolar hyphae of *C. albofundus* are convergent compared to the divergent ostiolar hyphae of *C. fimbriata* and *C. pirilliformis*. Colonies of *C. albofundus* are light in colour and have an optimum growth temperature of 30 °C. *Ceratocystis albofundus* has a typical *Thielaviopsis* anamorph producing both cylindrical and barrel-shaped conidia (Morris *et al.* 1993, Wingfield *et al.* 1996). A sweet smelling aroma, similar to that of *C. fimbriata*, is produced by *C. albofundus*.

Ceratocystis albofundus was first identified in 1977 as *C. fimbriata*, occurring on *Protea* spp. (Gorter 1977). Thereafter, it was reported as the cause of wilt and dieback of a few

Acacia spp. (Morris *et al.* 1993, Wingfield *et al.* 1996). The species name was corrected by Wingfield *et al.* (1996) who recognised it as a new species *C. albofundus*. This fungus is geographically restricted and has been reported only from the African continent.

Ceratocystis wilt caused by *C. albofundus* is characterised by the formation of cankers and lesions on the bark of affected trees and the exudation of gum from the lesions. Internally, the affected trees show signs of discolouration. The result of infection by this fungus is a rapid wilt and dieback of trees, often leading to death within a few weeks after infection (Morris *et al.* 1993).

It has been hypothesised that *C. albofundus* is native to South Africa. This is based on the fact that the fungus is known only from South Africa and occurs on native *Protea* spp. (Roux *et al.* 2001). It has been supported by a population genetic study using the oligonucleotide marker CAT5 as well as restriction digests of the mitochondrial DNA. Recently, it has been shown that microsatellite markers developed for *C. fimbriata* also function for studies on *C. albofundus* (Barnes 2002). Nakabonge (2002), therefore, compared the South African population used by Roux *et al.* (2001) with a Ugandan population of the fungus. They found that the Ugandan population was slightly more diverse than the South African population with very little gene flow detected. *Ceratocystis albofundus* is probably native to Africa and not only to South Africa as has previously been suggested (Nakabonge 2002). *Ceratocystis albofundus* is of great quarantine importance, as it does not occur outside Africa and for example might threaten Australian *Acacia* spp., both in countries where they are grown as exotics or in their native environment.

10.4 ***Ceratocystis moniliformis*** (Hedgcock) Moreau, Rev. Mycol. (Paris) Suppl.Col.17: 22, 1952.

≡ *Ceratostomella moniliformis* Hedgcock, Mo. Bot. Gard. Ann. Rept. 17: 78, 1906.
≡ *Ophiostoma moniliforme* (Hedgcock) Davidson, J. Agric. Res. 50: 800, 1935.
≡ *Endoconidiophora bunae* Kitajima, Bull. Imp. For. Exp. Sta., Meguro, Tokyo 35: 126.
= *Ceratocystis bunae* (Kitajima) Moreau, Rev. Mycol. (Paris) Suppl. Col. 17: 22, 1952.
= *Ceratocystis wilsonii* Bakshi, Mycol. Pap. 35: 4, 1951.
= *Ceratocystis filiformis* Roldan, Phillip. J. Sci. 91: 418, 1962.

Ceratocystis moniliformis was discovered in 1903 on gumwood (*Liquidambar styraciflua)* in Texas (Von Schrenk 1903). Hedgcock (1906) provided the first description of this fungus as *Ceratostomella moniliformis*. Although there have been a number of morphological descriptions of *C. moniliformis*, these differ quite markedly. *Ceratocystis moniliformis* has a few distinguishing characteristics that separate it from other species in this genus. One of these characteristics is the ornamentation on the ascomatal bases, which are covered in conical spines (Hedgcock 1906, Luc 1952, Hunt 1956, Upadhyay 1981). Another characteristic is the disciform shape of the base of the ascomatal neck (Bakshi 1951). This fungus is renowned for its rapid growth. Within three to five days, ascomata are formed from a single spore isolate and they mature within another one to two days (Hedgcock 1906, Davidson 1935). A characteristic banana-oil odour is produced when the cultures are young (Davidson 1935). Both cylindrical and barrel-shaped conidia are formed (Davidson 1935, Kitajima 1936, Bakshi 1951, Luc 1952).

Ceratocystis moniliformis infects wounds on many woody plants. Due to the fact that it is not a pathogen and only causes sapstain, the occurrence of the fungus has not been well documented. *Ceratocystis moniliformis* has been isolated in areas such as South Africa on *Macaranga capensis* Baill. (Van Wyk *et al.* 1991) and from much cooler climates such as Poland where it occurs on *Quercus robur* L. (Kowalski & Butin 1989).

10.5 *Ceratocystis moniliformopsis* Yuan & Mohammed *Austr. System. Bot.* 15: 125, 2002.

Ceratocystis moniliformopsis was described in 2002 from infected *Eucalyptus obliqua* L'Herit. logs in Australia (Yuan & Mohammed 2002). It is morphologically similar to *C. moniliformis*, as indicated by its name. The ascomata as well as ascospores are similar to those of *C. moniliformis* but it has a unique conidiogenous cell morphology, not found in other *Ceratocystis* spp. The pathogenicity of this fungus is still unknown.

10.6 *Ceratocystis acericola* Griffin *Can. J. Bot.* 46: 694, 1968.

Ceratocystis acericola was described in 1968 as a new species of *Ceratocystis* isolated from *Acer saccharum* Marsh in Ontario, Canada. The ascomatal bases are black, globose and ornamented with thick-walled hyphae (Griffin 1968). The ascomatal necks have a characteristic swelling or "annulus" below the apex with no ostiolar hyphae present

(Griffin 1968). The ascospores were first described as being orange-section shaped with a sheath (Griffin 1968) but later it was described as having hat-shaped ascospores (Grylls & Seifert 1993). No description of the anamorph was given.

11. CONCLUSIONS

The genus *Ceratocystis* includes fungi with variable ecological and pathogenic properties ranging from non-pathogenic staining fungi to highly aggressive pathogens (Wingfield *et al.* 1993). During the course of the last ten years, reports of *Ceratocystis* spp. associated with commercial plantation forestry in the Southern Hemisphere have increased dramatically. At least two of these species, *C. fimbriata* and *C. albofundus* are pathogenic to *Eucalyptus* spp. (Roux *et al.* 2000, Barnes *et al.* 2003b), and *A. mearnsii* (Morris *et al.* 1993, Roux, Dunlop & Wingfield 1999, Roux *et al.* 2001), respectively. The role of other *Ceratocystis* spp., as the causal agents of disease of exotic plantation forest trees, remains to be determined.

There are still many unanswered questions pertaining to the origin and dispersal of *Ceratocystis* species between countries and continents. Obtaining knowledge on these aspects could significantly contribute to the way the forestry industry could restrict further distribution of these pathogens. This also will impact on how disease management strategies could be formulated in future. The aim of this thesis is to study some *Ceratocystis* spp. in more detail that are important to the forestry industry. This study concentrates mainly on the morphology, phylogeny, pathogenicity and population structure of these fungi. Isolates included for this study are all characterised by having hat-shaped ascospores as was discussed in this review.

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13. APPENDIX

Table 1. Distribution and host range of *Ceratocystis moniliformis* causal agent of vascular stain.

Geographical distribution	Host	Reference
South Africa	*Macaranga capensis*	Van Wyk *et al.* 1991
France, Madagascar	*Theobroma* spp.	Luc 1952, Paulin-Mahady *et al.* 2002
Poland	*Quercus robur*	Kowalski & Butin 1989
USA	*Liquidamber styraciflua*	Von Schrenk 1903
"	*Pinus ponderosa*	Hedgcock 1906
"	*Pinus palustris, P. echinata, P. taeda, Liquidamber styraciflua, Liriodendron tulipifera, Nyssa aquatica, Fagus grandifolia, Magnolia* sp., *Quercus* sp.	Davidson 1935
Tokyo	*Fagus crenata, Quercus glandulifera, Magnolia hyplleuca, Kalopanax ricinifolius, Ptercarya rhoifolia, Cercidiphyllum japanicum*	Kitajima 1936
Scottland	*Quercus* sp.	Bakshi 1951
Cameroon	*Pycnanthus kombo*	Luc 1952
Madagascar	*Theobromae* spp.	Luc 1952
Philippines	*Calamus maximus, Endospermum peltatum, Parkia javanica*	Roldan 1962
China	*Hevea* sp.	Witthuhn *et al.* 1999
South Africa	*Erythrina* sp.	Witthuhn *et al.* 1999

Table 2. Distribution and host range of *Ceratocystis fimbriata*.

Geographical distribution	Host	Reference
AFRICA		
Republic of Congo, Uganda, Kenya	*Eucalyptus* spp., *Ipomoea batas*	Khiurani, Carey & Narla 2000, Roux *et al.* 2000
ASIA		
Taiwan, South East Asia, China	*Crotolaria juncea, Ipomoea batas, Punica granatum, Mangifera indica*	Chun Yee *et al.* 1997, Clark & Moyer 1988, Huang *et al.* 2003
AMERICAS		
Brazil, Colombia, USA, Central and South America, Venezuela, Canada, Uraguay	*Crotolaria juncea, Ipomoea batas, Populus* spp., *Hevea brazilienis, Prunus* spp., *Citrus* spp. *Platanus* spp., *Coffea arabica, Theobroma cacao, Acacia* spp., *Gwemelina arborea, Eucalyptus* spp., *Mangifera indica*	Halsted 1890, Beeley 1929, Sharples 1936, Costa & Krug 1935, Pontis 1951, Walter, Rex & Schreiber 1952, Iton 1959, Zalasky 1965, Manion & French 1967, DeVay *et al.* 1968, Muchovej, Albuquerque & Riberio 1978, Vigouroux 1979, Panconesi 1981, Riberio *et al.* 1985, Toviotdale & Harper 1991, Mourichon 1994.
AUSTRALIA		
Australia	*Syngonium* spp.	Walker *et al.* 1988
EUROPE		
Europe, France, Italy, Spain, Switzerland	*Populus* spp., *Platanus* spp.	Walter *et al.* 1952, Panconesi 1981

Table 3. Important species of *Ceratocystis* pathogenic to trees, crops, and ornamental shrubs.

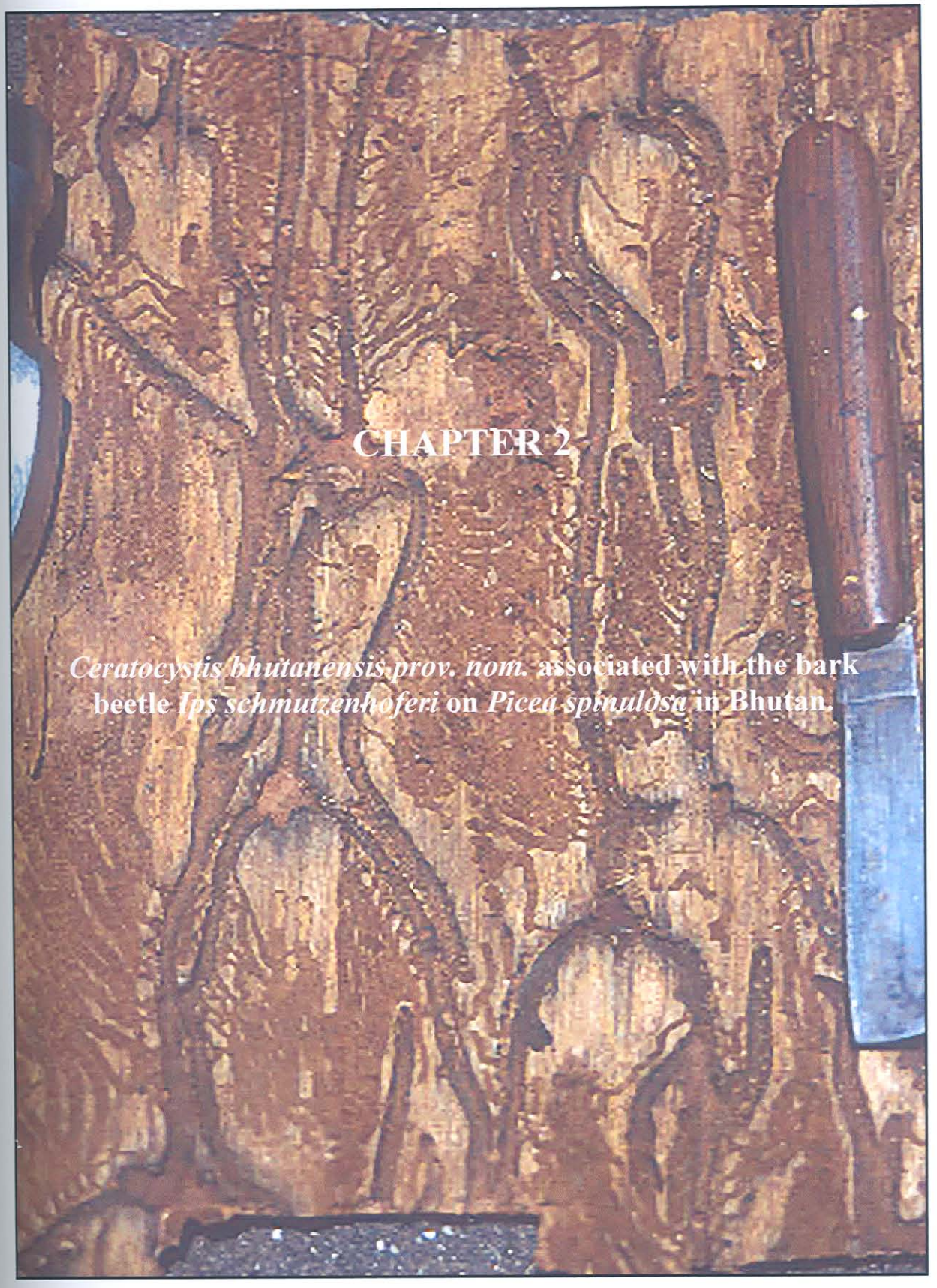
Ceratocystis species	Host	Disease	Reference
C. fimbriata	Sweet potato	Rot	Halsted 1890
"	Coffee & Rubber trees	Wilt	Pontis 1951,
"	Eucalypt	Canker	Roux *et al.* 2000
C. paradoxa	Palms & Sugarcane	Root- and stem rot	Moreau 1952, Hausner *et al.* 1993a
C. adiposa	Sugarcane	Root rot	Moreau 1952, Hausner *et al.* 1993a
C. fagacearum	Oak trees	Vascular wilt	Hunt 1956, Olchowecki & Reid 1974, Hausner *et al.* 1993a
C. polonica	Spruce trees	Vascular wilt	Yamaoka *et al.* 1997
C. virescens	Maple trees	Sap streak	Olchowecki & Reid 1974
C. albofundus	Wattle trees	Vascular wilt	Morris *et al.* 1993. Roux *et al.* 1999

Table 4. Morphology of the teleomorph of *Ceratocystis* spp. with hat-shaped ascospores.

PERITHECIAL	*C. moniliformis* (Hedgcock 1906)	*C. fimbriata* (Upadhyay 1981)	*C. pirilliformis* (Barnes *et al.* 2003)	*C. moniliformopsis* (Yuan & Mohammed 2002)	*C. albofundus* (Wingfield *et al.* 1996)	*C. acericola* (Griffin 1968)
BASE:						
Colour	Brown → black	Dark brown →black	Black	Dark brown →black	Yellowish brown	Black
Diameter	90 – 180 µm	950 – 1200 µm	115 – 187 µm	200 – 320 µm	416 – 848 µm	75 – 100 µm
Ornamentation	Spares conical spines, 12 – 16 µm	Few hyphal hairs	Hyphae	Pigmented setae, hyphae (base)	Unornamented	Hyphae
Form	Globular	Globose	Pear-shaped	Oviod	Globose	Globose
NECK:						
Colour	Black	Black (base) → lighter (tip)	Black	Black	Black (base) → light (tip)	-
Base	Disciform	-	-	Disciform	Collar	-
Length	500 – 900 µm	950 µm	327 – 683 µm	480 – 780 µm	208 – 840 µm	100 – 400 µm
Width: base	20 – 40 µm	18 – 35 µm	19 – 33 µm	40 – 50 µm	20 – 32 µm	20 – 35 µm
Width: tip	10 – 15 µm	10 – 18 µm	12 – 21 µm	18 – 22 µm	16 – 24 µm	8 – 12 µm
Ostiolar hyphae	Brown, strait, 12 – 18 µm	Hyaline, straight or flexuous, tapered, blunt or subulate, 18 – 75 µm	Hyaline, strait, convergent	Hyaline, aseptate, unbranched, convergent 25 – 45 x 1.5 – 2 µm	Hyaline, divergent 40 – 60 µm	None
ASCUS:	Evanescent	Not seen	Evanescent	Not seen	Evanescent	Not seen
ASCOSPORES:						
Colour	Hyaline	Hyaline	Hyaline	Hyaline	Hyaline	Hyaline
Shape: face view	Oval	Elliptical	Elliptical	Oblong	Elliptical	Cylindrical
Shape: side view	Flat on 1 side	Reni-form	Hat-shaped	Reniform / Hat-shaped	Hat-shaped	Orange section
Shape: end view	-	Oval	-	-	-	Globose to elliptical
Length	4 – 5 µm	3.5 – 8 µm	8 – 11.5 µm	4 – 5 µm	4 – 6 µm	2.5 – 4.0 µm
Width	3 – 4 µm	2 – 2.5 µm	4 – 6 µm	2 – 2.5 µm	3.5 – 5 µm	1.5 – 2.5 µm
Texture	Slimy, grey mass	Tawney, mucilaginous cap	Hyaline, gelatinous sheath	Hyaline, gelatinous sheath	Slimy droplet, double brim	Hyaline, gelatinous sheath

Table 5. Morphology of the anamorph of *Ceratocystis* spp. with hat-shaped ascospores.

	C. moniliformis (Hedgcock 1906)	*C. fimbriata* (Upadhyay 1981)	*C. pirilliformis* (Barnes *et al.* 2003)	*C. moniliformopsis* (Yuan & Mohammed 2000)	*C. albofundus* (Wingfield *et al.* 1996)
CONIDIOPHORES:					
Length	-	35 – 130 µm	62 – 147 µm	5 – 32.5 µm	24 – 104 µm
Width	-	2.5 – 6 µm (base)	-	4 – 5.3 µm	3 – 5 µm
CONIDIA:					
Shape	Cylindrical	(1) Cylindrical (2) Barrel	(1) Cylindrical (2) Barrel	(1) Cylindrical (2) Barrel	(1) Cylindrical (2) Barrel
Length	6 – 8 µm	(1) 9 – 21 µm (2) 9 – 14 µm	(1) 12 – 25 µm (2) 4 – 6 µm	(1) 13 – 21 µm (2) 12 – 17.5 µm	(1) 8 – 24 µm (2) 6 – 10 µm
Width	1.8 – 2.2 µm	(1) 2 – 6.5 µm (2) 5 – 15 µm	(1) 2 – 4 µm (2) 3 – 4 µm	(1) 2 – 3 µm (2) 5 – 7.5 µm	(1) 3 – 4 µm (2) 4 – 8 µm
CHLAMYDOSPORES:	No	Yes	Yes	No	No
Length	-	9 – 15 µm	8 – 12 µm	-	-
Width	-	7.5 -11 µm	5 – 8 µm	-	-
CULTURE:					
Growth rate	Perithecia in 2 – 3 days	45 – 55 mm in 12 days	22 mm in 12 days	6 – 7.5 mm per day	27 mm in 8 days
Colour	Hyaline → grey → black	Hyaline → grey/brown → brown/black	Hyaline → grey → green	Hyaline → green/brown	White → creamy
Mycelium	Coarsely granular, 2 – 8 µm dia.	Smooth, 1.5 – 5 µm dia.	-	-	Hyaline, smooth, 2 – 5 µm dia.



CHAPTER 2

Ceratocystis bhutanensis prov. nom. associated with the bark beetle *Ips schmutzenhoferi* on *Picea spinulosa* in Bhutan.

ABSTRACT

The Eastern Himalayan spruce bark beetle, *Ips schmutzenhoferi*, is a serious pest of *Picea spinulosa* and *Pinus wallichiana* in Bhutan. In 2001 a study was initiated that aimed to identify the ophiostomatoid fungi associated with this conifer bark beetle. During this survey, a *Ceratocystis* sp. was isolated from individuals of *I. schmutzenhoferi* collected from galleries on *P. spinulosa.* Morphological characteristics and comparisons of DNA sequence data were used to identify this fungus. Based on morphology, the *Ceratocystis* sp. from Bhutan resembled *C. moniliformis* and *C. moniliformopsis*, but was distinct from these species in colony morphology, micro-morphology, growth profiles at different temperatures, as well as the odour that it produces in culture. DNA sequence data of the ITS regions of the rDNA operon, β-tubulin and Elongation Factor 1-α genes, confirmed that this fungus represents a taxon distinct from *C. moniliformis*, *C. moniliformopsis* and all other species of *Ceratocystis*. Based on morphological characteristics, comparisons of DNA sequence data and its unique ecology, we, therefore, describe this fungus as new and provide the name *C. bhutanensis prov. nom.* Currently this fungus is only known from one locality in Western Bhutan and its geographical distribution, ecology, pathogenicity and vector relationships require further study.

INTRODUCTION

The Kingdom of Bhutan is renowned for its intact forest resources, which are of immense socio-economic and ecological importance for this Himalayan country. Sixty-four percent of Bhutan is covered by forests (FAO 1999, 2001). Conifer forests form the natural vegetation in most parts of the mountainous areas at elevations above 1800 m a.s.l. (FAO 1999). Eastern Himalayan spruce (*Picea spinulosa* (Griffith) A.) and Himalayan blue pine (*Pinus wallichiana* Jackson) are important tree species in these forests, forming either pure stands or mixed species stands together with other conifers.

Bark beetles (Coleoptera: Scolytidae) are amongst the most damaging agents affecting conifer forests, worldwide. Some of the most aggressive of these insects are species within the genus *Ips* de Geer (Postner 1974, Wood & Bright 1992). The best known of these is the eight-spined European spruce bark beetle, *I. typographus* L., that can cause extensive mortality of Norway spruce (*Picea abies* (L.) Karst.) in Europe (Postner 1974, Christiansen & Bakke 1988). In Bhutan, the Eastern Himalayan spruce bark beetle, *I. schmutzenhoferi* Holzschuh is a serious pest in conifer forests at elevations between 2500 and 3800 m a.s.l. (Schmutzenhofer 1988). This scolytid mainly attacks living trees and logs of Eastern Himalayan spruce and Himalayan blue pine, but logs of Himalayan larch (*Larix griffithiana* (Lindl. & Gord.) Carrière) are occasionally also infested (Schmutzenhofer 1988, Tshering & Chhetri 2000). During the 1980s, this insect caused a destructive outbreak in Western and Central Bhutan, during which 3000 ha of forest were affected and losses of approximately 2 million m^3 of timber occurred (Schmutzenhofer 1988).

Conifer infesting bark beetles are well known to carry blue-stain fungi belonging to the ascomycete genera *Ceratocystis* Ell. & Halst and *Ophiostoma* Von Syd. & Syd. and related anamorph genera (Francke-Grossman 1967, Upadhyay 1981, Whitney 1982, Webber & Gibbs 1989, Jacobs & Wingfield 2001). Members of these genera have also been referred to as the ophiostomatoid fungi (Wingfield, Seifert & Webber 1993). These fungi cause blue, grey or black discolouration in the sapwood of living trees, logs and lumber, mostly on conifers. This kind of damage results from the presence of pigmented fungal hyphae in the ray parenchyma cells and tracheids of the sapwood (Münch 1907, Liese & Schmid 1961, Seifert 1993). Damage due to sapstain is cosmetic rather than structural, and results in substantial financial losses, because markets prefer non-stained

wood (Münch 1907, Seifert 1993, Uzunovic *et al.* 1999). Some bark beetle associated blue-stain fungi also cause vascular stain diseases on living conifer trees and are suspected to aid their insect vectors to exhaust the defence mechanisms of their host trees (Whitney 1982, Paine, Raffa & Harrington 1997).

Most fungal associates of bark beetles belong to the genus *Ophiostoma*. In contrast, *Ceratocystis* spp. usually have loose relationships with insects (Harrington 1987). However, there are three *Ceratocystis* spp. that are consistently associated with conifer bark beetles (Harrington & Wingfield 1998). The first of these, *C. polonica* (Siem.) Moreau, is associated with *I. typographus* and other species of *Ips* on *Picea abies* in Europe (Solheim 1986, 1992, Krokene & Solheim 1996, Kirisits, Grubelnik & Führer 2000), and with *I. typographus* f. *japonicus* Niijima on *Picea jezoensis* (Sieb. & Zucc.) Carr. in Japan (Yamaoka *et al.* 1997). The second, *C. laricicola* Redfern & Minter, is associated with the larch bark beetle *I. cembrae* Heer on *Larix* Miller spp. in Europe and Japan (Redfern *et al.* 1987, Yamaoka *et al.* 1998, Stauffer *et al.* 2001). The third, *C. rufipenni* Wingf., Harr. & Solh., is associated with the spruce bark beetle *Dendroctonus rufipennis* Kirby on *Picea engelmannii* Parry and *Picea glauca* (Moench) Voss in Western North America (Solheim 1995, Wingfield, Harrington & Solheim 1997).

The small number of *Ceratocystis* spp. that are associated with bark beetles display high levels of pathogenicity, when compared to *Ophiostoma* spp. from the same niche (Solheim 1988, Solheim & Safranyik 1997, Kirisits 1998, Krokene & Solheim 1998). *Ceratocystis polonica* is highly pathogenic to Norway spruce and contributes to tree death following attack by *I. typographus* (Christiansen 1985, Solheim 1988, 1992, Kirisits & Offenthaler 2002). Likewise, *C. laricicola* is considered to play an important role in the death of *Larix* spp. infested by *I. cembrae* (Redfern *et al.* 1987, Yamaoka *et al.* 1998, Kirisits 2001, Harrington *et al.* 2002) and *C. rufipenni* can kill Sitka spruce (*Picea sitchensis* (Bongard) Carrière) in mass inoculation experiments (Solheim & Safranyik 1997).

During a recent survey of ophiostomatoid fungi associated with *I. schmutzenhoferi* in Bhutan a *Ceratocystis* sp. resembling *C. moniliformis* Hedgc. and *C. moniliformopsis* Yuan & Mohammed was isolated. Despite its similarity with these *Ceratocystis* sp., the association of the *Ceratocystis* sp. from Bhutan with a conifer bark beetle aroused suspicion that it might be a separate, hitherto undescribed taxon. This study compares the *Ceratocystis* species from Bhutan with *C. moniliformis* and *C. moniliformopsis* and

assesses their phylogenetic relationships based on the ITS region of the rDNA operon, β-tubulin and EF1-α gene sequences.

MATERIALS AND METHODS

Collection of material for fungal isolation

A survey of ophiostomatoid fungi associated with *I. schmutzenhoferi* in Bhutan was conducted in July 2001. Samples for fungal isolation were collected at several locations in Western and Central Bhutan (Fig. 1) where *I. schmutzenhoferi* attacked living trees or logs of *Picea spinulosa* and/or *Pinus wallichiana*. The collection sites included mixed conifer forests at Jelekha (3300 m a.s.l.), Changaphug (3600 m a.s.l.), Phobjikha valley (3100 m a.s.l.), and near the Renewable Natural Resources Research Center (RNR-RC) in Yusipang (2700 m a.s.l.) as well as wood depots at Gidakom (2200 m a.s.l.) and Ramtokto (2100 m a.s.l.) (Fig. 1). Logs and standing trees, infested by *I. schmutzenhoferi* were examined for suitable material to conduct fungal isolations. Galleries of the insects occurring in the bark or on the surface of the sapwood on logs and standing pine and spruce trees were inspected on site, with the aid of a 10x magnification hand lens, for the occurrence of sexual and asexual stages of ophiostomatoid fungi. At the research station in Yusipang adult beetles of *I. schmutzenhoferi* (2nd generation) were collected from a pheromone trap installed specifically for the purpose of insect specimen collection.

Adult and juvenile beetles, breeding galleries, stem discs and stem sections from beetle-infested *P. spinulosa* and *P. wallichiana* trees and logs were collected for further investigation. All samples were stored in plastic bags and transported to the laboratory at RNR-RC in Yusipang. Dry bark samples were sprayed with distilled water and the bags sealed for a few days to create a moist environment, conducive to sporulation of fungi within the beetle galleries. Reference specimens of *I. schmutzenhoferi* were stored in ethanol and are maintained at the Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Universität für Bodenkultur Wien (BOKU), Vienna, Austria. In addition to material obtained from pine and spruce infested by *I. schmutzenhoferi*, wood samples were collected from broken *Cassia fistula* L. trees near Punakha (*ca* 1300 m a.s.l.) and Wangdi (*ca* 1100 m a.s.l.). The purpose of these collections was to search for *Ceratocystis moniliformis* on this subtropical hardwood species.

Fungal isolations

Fungi were isolated on 2 % Malt Extract Agar (MEA) (20 % w/v) (Biolab, Midrand, South Africa) or on 2 % Malt Agar (MA) (20 % w/v) (DiaMalt, Hefe Schweiz AG, Stettfurt, Switzerland) (W. Behrens & Co., Hamburg, Germany), both supplemented with 100 mg/L streptomycin sulphate (SIGMA, Steinheim, Germany, or VWR International GmbH, Vienna, Austria). In order to get a comprehensive view of the fungi associated with *I. schmutzenhoferi* various isolation methods were applied. Fungi were isolated directly from adult beetles (2^{nd} generation) collected from two spruce logs at Jelekha, from young beetles (1^{st} generation) obtained from a pine log at Ramtokto and from swarming beetles (2^{nd} generation) collected from a pheromone trap at Yusipang. To obtain isolates directly from the insects, their body parts were dissected and spread onto 2 % MA.

Fungi were also isolated from the sapwood of one spruce tree from Jelekha. Six stem discs (*ca* 10-15 cm wide, with a diameter of 18-21 cm), were cut from the upper part of this tree. These were split vertically and isolations from the sapwood were done along radii underneath female galleries of *I. schmutzenhoferi*, following a similar procedure as that described by Solheim (1992). Three radii per disc were sampled, resulting in a total of 18 radii. Small pieces of sapwood were transferred onto 2 % MA plates. From each radius, samples were taken 2, 5 and 10 mm apart from the cambium into the sapwood.

Most isolations were made from ascospores and conidia taken directly from sexual and asexual fungal structures occurring in and around female and larval galleries and pupal chambers of the insects. Bark and sapwood samples from spruce and pine collected at the localities Jelekha, Gidakom, Ramtokto, Changaphug and Phobjikha valley were examined with a dissecting microscope at magnifications ranging from 10x to 40x. With a fine needle, ascospores and conidia accumulating at the apices of perithecia and on conidiophores, respectively, were carefully removed and transferred to 2 % MA or 2 % MEA plates. Isolation of *C. moniliformis* from *C. fistula* collected at Punakha and Wangdi was done in a similar manner, from ascospores obtained from perithecia occurring on the wood surface.

A selective method for the isolation of *Ceratocystis* spp. was also used (Moller & De Vay 1968). Fresh carrots were washed and lightly sprayed with 70 % ethanol. Carrot discs (5-10 mm thick) were cut and four to eight discs were placed in plastic Petri dishes (90 mm). Beetles, larvae and pupae of *I. schmutzenhoferi* were dissected and spread over the surface

of the carrot discs. Larval frass, collected from the insect galleries was also put onto the carrots. The discs were examined for the incidence of perithecia after 5-10 days incubation at *ca* 20 °C.

Pure cultures were obtained by transferring ascospore or conidial masses as well as small pieces of mycelium from the primary isolation plates onto fresh 2 % MA or 2 % MEA plates. Fungal cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, the Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Universität für Bodenkultur Wien (BOKU), Vienna, Austria and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Holotype material of the new *Ceratocystis* sp. from Bhutan, consisting of a dried culture of isolate CMW 8217 on 2 % MEA is kept at the National Fungal Herbarium (PREM), Pretoria, South Africa (Table 1).

Culture characteristics and morphology

The growth of isolates CMW 8217, CMW 8241 and CMW 8244 representing the *Ceratocystis* sp. obtained from *I. schmutzenhoferi*, was determined on 2 % MEA (Fig. 2). Three isolates of *C. moniliformis* (CMW 9590, CMW 8238 & CMW 10134) and *C. moniliformopsis* (CMW 9986, CMW 10214 & CMW 10215) were used for comparisons in the growth study (Fig. 2). Prior to the growth assays, the isolates were grown in culture for two weeks at 25 °C (Fig. 3). Mycelial plugs were taken from actively growing cultures using a 5 mm cork borer and a single mycelial plug was transferred to the centre of a 90 mm Petri dish containing 2 % MEA. Five plates for each isolate were incubated at 4 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C and 35 °C, respectively. Colony diameter for each culture was assessed by taking two measurements at right angles to each other, every day, for four days or until the plates were almost completely covered by mycelium. Averages were computed separately for each isolate and each test temperature. The entire experiment was repeated once.

Morphological characteristics were described from 10-day-old cultures, on 2 % MEA supplemented with streptomycin sulphate (0.001 g vol^{-1}, SIGMA, Steinheim, Germany) and Thiamine (0.001 g vol^{-1}, SIGMA, Steinheim, Germany). Fungal structures were mounted in lactophenol containing cotton blue. Fifty measurements for each taxonomically relevant structure were made from isolate CMW 8217, and to correlate that these values are representative for the *Ceratocystis* sp. from Bhutan 10 further

measurements were made for each of five other isolates (Table 1). Ranges, averages, and standard deviations of the corresponding measurements were calculated. The microscopic observations were made using a Carl Zeiss microscope and the photographic images were made with a Zeiss Axio Vision camera system. Colour descriptions were determined using the colour charts of Rayner (1970). The measurements and morphological characteristics of the *Ceratocystis* sp. from Bhutan were compared with descriptions of *C. moniliformis* (Hedgcock 1906, Upadhyay 1981) and *C. moniliformopsis* (Yuan & Mohammed 2002) (Table 2).

DNA extraction

Representative isolates of the *Ceratocystis* sp. from *I. schmutzenhoferi* in Bhutan as well as isolates of *C. moniliformis*, *C. moniliformopsis* and *C. virescens* (Davids.) Moreau (Table 1) were selected for DNA extraction and sequencing. From each culture a single ascospore mass was transferred, using a sterile needle, from actively growing and sporulating cultures, to 50 ml 3 % ME broth, in Erlenmeyer flasks, and incubated at 25 °C. After two weeks, the thick mycelial mats were filtered from the broth and lyophilised for two days. The freeze-dried mycelium was placed in liquid nitrogen and ground to a powder using a glass rod, and DNA was extracted using the method described by Barnes *et al.* (2001).

PCR amplification

The two ITS regions (ITS1 and ITS2) and the 5.8S gene of the rDNA operon were amplified using primers ITS1 and ITS4 (White *et al.* 1990) at an annealing temperature of 55 °C. A part of the β-tubulin gene was amplified using primers βt1a and βt1b at an annealing temperature of 55 °C (Glass & Donaldson 1995) and the EF1-α gene of the rDNA operon was amplified using primers EF1-728F and EF1-986R at an annealing temperature of 56 °C (Carbone & Kohn 1999).

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 x 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 Sabax water (Adcock Ingram Scientific, Johannesburg, South Africa). The PCR programme was set at 96 °C for 2 min, followed by 10 cycles at 94 °C for 20 s, x °C (x = the annealing temperature specified for each set of primers) for 40 s and 72 °C for

45 s. 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 °C completed the programme. Amplification of the respective genes was confirmed on a 2 % agarose (Roche diagnostics, Mannheim, Germany) gel supplemented with ethidium bromide. PCR amplicons were purified using the Magic PCR Preps, Purification System (Promega, Madison, USA).

Sequencing and data analysis

PCR amplicons were sequenced in both directions using the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California). The same primers as those in the PCR reactions were used for sequencing of the respective gene areas. Sequence reactions were run on an ABI PRISM™ 3100 Autosequencer (Applied BioSystems, Foster City, California, U.S.A) and sequences were analysed using Sequence Navigator version 1.0.1 (Applied BioSystems, Foster City, California).

The sequences obtained for the *Ceratocystis* sp. from *I. schmutzenhoferi* were compared with those of morphologically similar *Ceratocystis* spp. (Table 1). Sequences were aligned manually and analysed using PAUP version 4.0b10* [Phylogenetic Analysis Using Parsimony (and other methods)] (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 based on parsimony with tree bisection reconnection was used to obtain the phylogram. Confidence intervals using 1000 bootstrap replicates were calculated. The out-group taxon *C. virescens* was rooted as a midpoint with respect to the in-group. All sequences derived from this study have been deposited in GenBank (Table 1). A partition homogeneity test (Swofford 2002) was used to determine whether the sequence data sets for the three different genome 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 were generated using the MCMC procedure, sampling every 100th tree and printing every 10th tree. To avoid including trees that might have been sampled before convergence of the Markov chain, 8600 trees were discarded. For the combined analysis of the three gene sequences, gamma rate heterogeneity was set, and no codon specific sites were included for the ITS gene. For β-

tubulin and EF1-α sequences, codon specific sites were specified with a site-specific substitution rate and the site partition was treated as a by-codon.

RESULTS

Collection of material for fungal isolations

Conspicuous blue-stain was observed on the surface of the sapwood and in the bark around nuptial chambers and female and larval galleries of *I. schmutzenhoferi* on inspected spruce and pine trees and logs. However, intensive blue-stain, deeply penetrating into the sapwood was not seen on any of the inspected trees and logs, neither on spruce nor on pine. On the stem discs of the spruce tree from which isolations were made, a narrow zone of desiccation, extending 5 to 8 mm deep into the sapwood and recognizable by its white to yellowish colour, occurred.

Fungal isolations

From the isolations made, the *Ceratocystis* sp. was only isolated directly from beetles of the second generation collected from galleries on *P. spinulosa* at the locality Jelekha. In the sample of 20 beetles from this collection site which were used for fungal isolations, 16 (80 %) yielded growth of the *Ceratocystis* sp. and this fungus was thus among the dominant species recovered from this niche. Fourteen isolates of the *Ceratocystis* sp. representing isolations from separate beetles were initially maintained and 11 of these strains were used for phenotypic, morphological and molecular characterization of this fungus in the present study.

The *Ceratocystis* sp. mentioned above was neither isolated from beetles obtained from Ramtokto and Yusipang nor from desiccated sapwood of the spruce tree collected at Jelekha. Likewise, perithecia and conidiophores were never observed in galleries of *I. schmutzenhoferi* on spruce and pine. Subsequent attempts to isolate this fungus again from adult and juvenile beetles, larvae, and pupa or from larval frass on carrot discs, known to be selective for *Ceratocystis* spp., were unsuccessful.

Ascomata resembling those of *C. moniliformis* were common on the surface of the wood of broken *C. fistula* trees near Punakha and Wangdi. It was easy to isolate *C. moniliformis* by transferring ascospores from the perithecial tips to MA and MEA plates. Two isolates

of this fungus from Bhutan were included for morphological and molecular comparisons with the *Ceratocystis* sp. from *I. schmutzenhoferi* (Table 1).

Cultural characteristics and morphology

All three *Ceratocystis* spp. tested grew very rapidly in culture, at least near their temperature optimum. *Ceratocystis bhutanensis prov. nom.*, *C. moniliformis* and *C. moniliformopsis* differed considerably in their growth profiles at different temperatures (Fig. 2). Only the results of the first study were used for the graphs (Fig. 2), the results of the second study were used to determine if the cultures had the same growth rate. The optimum temperature of the *Ceratocystis* sp. from *I. schmutzenhoferi* in Bhutan varied between 20 °C (Isolates CMW 8244 and CMW 8241) and 25 °C (Isolate CMW 8217), while for *C. moniliformis* and *C. moniliformopsis* it was 30 °C and 20 °C, respectively (Fig. 2). At 4 °C the *Ceratocystis* sp. from Bhutan grew, while no growth was observed for either *C. moniliformis* or *C. moniliformopsis*. There was diminished growth at 10 °C for all the isolates, while two of the *C. moniliformis* isolates (CMW 8238 and CMW 10134) had no growth at this temperature (Fig. 2). While *C. moniliformopsis* did not grow from 25 °C to 35 °C, *C. moniliformis* grew very fast at 25 °C and 30 °C and also showed no growth at 35 °C. The *Ceratocystis* sp. from Bhutan displayed fast growth at 25 °C, diminished growth at 30 °C for two of the isolates (CMW 8244 and CMW 8217) and no growth for one isolate (CMW 8241) and also did not grow at 35 °C (Fig. 2).

Within the species, considerable variation was observed (Fig. 2). For the three *C. moniliformis* isolates there was major differences observed at four critical temperatures (15 °C – 30 °C). The greatest variation was at 30 °C where one isolate (CMW 9590) had a growth rate of 90 mm in 3 days, while the other two isolates (CMW 8238 and CMW 10134) had a growth rate of 60 mm and 18 mm, respectively. For the isolates used to study *C. moniliformopsis* little variation was observed. One of the isolates for *C. moniliformopsis* (CMW 10214) seemed to have a better growth rate than the other two isolates (CMW 9986 and CMW 10215) at all temperatures studied. For the isolates used for *C. bhutanensis prov. nom.* variation was observed at two temperatures. One isolate (CMW 8241) had a very fast growth rate reaching 78 mm in just three days at 20 °C but the growth rate dropped dramatically at 25 °C and no growth was observed at 30 °C for this isolate. The other two isolates (CMW 8217 and CMW 8244) also had good growth rates at 20 °C but their growth tempo did not differ significantly at 25 °C but they still grew at 30 °C.

On MEA, cultures of the *Ceratocystis* sp. from *I. schmutzenhoferi* were light in colour when young, but turned grey and finally black as they became older. In young cultures, the submerged mycelium had a honey colour (19"b) and the aerial mycelium was cream-buff (19"d). In older cultures (> 14 days), the submerged mycelium was umber (15 m) with the aerial mycelium ecru-drab (13""""d) (Fig. 3). In even older cultures (> 28 days), the submerged mycelium tended to be black (7""""k) and the abundant aerial mycelium dark olive (21"m) (Rayner 1970). This is very different to *C. moniliformis* that produced white to cream-buff (19"d) mycelium (Fig. 3) and the *C. moniliformopsis* isolates that were described as having a colourless to white grey appearance with the centre becoming greenish brown due to sporulation of the ascomata (Yuan & Mohammed 2002) which was observed by us to be more brownish in colour (Fig. 3). Isolates of the unknown species of *Ceratocystis* from *I. schmutzenhoferi* produced an unpleasant rotten fruity odour. This was in contrast to the pleasant banana-oil odour typically produced by cultures of *C. moniliformis* (Davidson 1935) and little to no odour produced by *C. moniliformopsis* (Yuan & Mohammed 2002). The *Ceratocystis* sp. from Bhutan produces ascomata within a few (3-4) days, which are then overgrown by dense aerial mycelium and make the detection of ascomata difficult. *Ceratocystis moniliformis* and *C. moniliformopsis* also produce perithecia within a few days, but they can be clearly seen in older cultures.

The ascomatal bases of the *Ceratocystis* sp. isolated from *I. schmutzenhoferi* were black, globose and covered with short conical spines, resembling those of *C. moniliformis* (Fig. 4a-b). The bases of the necks also resembled *C. moniliformis* in being disc shaped and detaching from the bases of the ascomata when disturbed (Fig. 4b). The ostiolar hyphae were divergent (Fig. 4c), exuding sticky masses of hat-shaped ascospores (Fig. 4d). Two types of hyphae were present, one smooth-walled, and the other rigid and granular (Fig. 4e-f). The anamorph was typical of *Thielaviopsis* Went, with the phialidic conidiogenous cells producing both cylindrical and barrel-shaped conidia (Fig. 4g-i). No chlamydospores are present in the *Ceratocystis* sp. from Bhutan. When older cultures are examined with the dissecting microscope, accumulations of pigmented, thick-walled cells become obvious as black dots. These structures were originally suspected to represent chlamydospores, but were later identified as old conidia produced by the *Thielaviopsis* anamorph.

Care has to be taken when the cultures of the *Ceratocystis* sp. from Bhutan are to be preserved. There is a tendency of some cultures to degenerate on MEA plates. This phenomenon has also been observed in *C. moniliformis* and in *C. moniliformopsis*. The cultures that degenerate become white in colony morphology, they have a slower growth rate and no longer produce ascomata in culture. One alternative to preserve these cultures is to scrape the mycelium and fungal structures into an Eppendorf tube and freeze dry it. The culture can then be grown from there by placing a small amount of the freeze-dried material onto a MEA plate.

PCR amplification

Amplification of the ITS regions and the 5.8S gene of the rDNA resulted in amplification products of ~500 bp. Amplification of the β-tubulin gene resulted in amplification products of ~500 bp, while the amplification of the EF1-α gene resulted in amplification products of ~300 bp.

Sequencing and analyses

Partition homogeneity tests for the three sequence data sets gave a P-value of P = 0.46 for the ITS and β-tubulin, P = 1.00 for the ITS and EF1-α and P = 0.14 for the β-tubulin and EF1- α combinations. All datasets had a value greater than the minimum required value of P = 0.05 and they could thus be combined. The combined sequences of the three gene areas, resulted in a dataset that was 1491 bp long (Appendix), had a single most parsimous tree, with a consistency index (CI) of 0.9532, a homoplasy index (HI) of 0.0468, a retention index (RI) of 0.9291 and a rescaled consistency index (RC) of 0.8757. The posterior probability of the branch nodes of the combined tree, generated with the Bayesian inference programme supported the bootstrap values. The posterior probability for the branch nodes for the three clades representing *C. moniliformis*, *C. moniliformopsis* and the *Ceratocystis* sp. isolated from *I. schmutzenhoferi*, respectively, was 100 %.

A heuristic search resulted in a single well-resolved tree (Fig 5). Species of *Ceratocystis* included in this tree formed three distinct sub-clades (Fig. 5). One of these sub-clades included the *Ceratocystis* sp. isolated from *I. schmutzenhoferi* in Bhutan, supported by a bootstrap value of 100 %. The other sub-clades included isolates of *C. moniliformis* and *C. moniliformopsis*, respectively (Fig. 5).

TAXONOMY

Comparison of DNA sequence data confirmed morphological observations that the *Ceratocystis* sp. from *I. schmutzenhoferi* in Bhutan is related to *C. moniliformis* and *C. moniliformopsis*. The data, however, provided robust support for the view that this fungus represents a new and previously undescribed species of *Ceratocystis*. The fungus is, therefore, described as a new taxon.

Ceratocystis bhutanensis Van Wyk, Wingfield & Kirisits, *prov. nom.*

(Fig.3,4)

Etymology: Bhutanensis referring to the country where this species has been discovered, in Bhutan.

Stat.conid.: *Thielaviopsis*

Coloniae juvenes cremeo-fulvidae, infra mellinae, seniores griseo-mustellinae, infra umbrinae, dein atro-olivaceae, infra nigrae. *Mycelium* plerumque in medio immersum; mycelium album aerium adest. *Crescit* optime ad 25 °C, nullo incremento supra 35 °C, deminuto ad 4 °C. *Hyphae* leves vel granulatae, in septis non constrictis, 1-3.5 µm latae. *Bases ascomatum* atrobrunneae vel nigrae, globosae, spinis hyphisque ornatae, spinis atrobrunneis vel nigris, (4.5-) 8-19 (-27) µm longis, bases (112-) 138-178 (-206) µm diametro. *Colla ascomatum* basin versus atrobrunnea vel nigra, apicem versus laetescentia, (450-) 453-519 µm longa, basi, 34-42 (-44) µm lata, apice (11-) 12-14 (-17) µ lata, apice discoideo. *Hyphae ostiolares* divergentes, hyalinae, (13-) 18-26 (-34) µm longae. *Asci* non visi. *Ascosporae* lateraliter visae cucullatae, aseptatae, hyalinae, in vagina investitae, cum vagina 4-6 x 2-5 µm, sine vagina 2-5 x 2-5 µm. Ascospora*e* in massis mucilagineis fulvo-luteis in apicibus collorum ascomatum cumulantes. *Anamorpha Thielaviopsis*: conidiophora in mycelio singula, hyalina, basi tumida, apicem versus angustata, (15-) 23-39 (-51) µm longa, basi (3-) 4-6 (-9) µm lata, apice 1–3 µm lata. Evolutio *conidii* phialidici per parietes annulares faciendas, *conidia* in catenis biformibus facta: conidia primaria hyalina, aseptata, cylindrica, (6-) 7-9 (-10) x 1-3 µm, conidia secondaria hyalina, aseptata, doliiformia, 3-5 x (1.5-) 2-3 (-3.5) µm.

Typus: **Bhutan**: *Thimphu dzongkhag*, Jelekha, isolated from *Ips schmutzenhoferi* collected from *Picea spinulosa*, 4 July 2001, *T. Kirisits and D. B. Chhetri*, (PREM 57804 - holotypus, living culture: CMW 8217).

Colonies that are young in culture had a honey colour for the submerged mycelium (19"b) the aerial mycelium being cream-buff (19"d). In older cultures (> 14 days), the submerged mycelium was umber (15 m) with the aerial mycelium ecru-drab (13""""d) (Fig. 3). In older cultures (> 28 days), the submerged mycelium was black (7""""k) and the abundant aerial mycelium dark olive (21"m). *Mycelium* submerged in medium, abundant white aerial mycelium present. *Optimal temperature* 20 °C, no growth at 35 °C, diminished growth at 4 °C and 30 °C. Isolates can grow up to 20 mm per day at the optimum temperature. *Hyphae* smooth or granulated, not constricted at septa, 1-3.5 µm wide. *Ascomatal bases* dark brown to black, globose, ornamented with spines and hyphae, spines dark brown to black, (4.5-) 8-19 (-27) µm long, bases (112-) 138-178 (-206) µm in diameter. *Ascomatal necks* dark brown to black at base, becoming light brown towards the apex, (450-) 453-519 µm long, 34-42 (-44) µm wide at the base, (11-) 12-14 (-17) µm wide at the apex, with a disc-like (disciform) base. *Ostiolar hyphae* divergent, hyaline, (13-) 18-26 (-34) µm long. *Asci* not observed. *Ascospores* cucullate in side view, aseptate, hyaline, invested in sheath, 4-6 x 2-5 µm with sheath, 2-5 x 2-5 µm without sheath. Ascospores accumulating in originally white and later buff-yellow (19d) mucilaginous masses on the apices of ascomatal necks. *Thielaviopsis anamorph*: conidiophores occurring singly on mycelium, hyaline swollen at the base, tapering towards the apex, (15-) 23-39 (-51) µm long, (3-) 4-6 (-9) µm wide at base, 1-3 µm wide at the apices. Phialidic *conidium* development through ring wall building, *conidia* formed in chains of two types: primary conidia hyaline, aseptate, cylindrical (6-) 7-9 (-10) x 1-3 µm, secondary conidia hyaline, aseptate, barrel-shaped 3-5 x (1.5-) 2-3 (-3.5) µm.

Additional specimens examined: **Bhutan**: *Thimphu dzongkhag*, Jelekha, isolated from *Ips schmutzenhoferi* collected from *Picea spinulosa*, 4 July 2001, T. Kirisits and D. B. Chhetri, (culture CMW 8241, PREM 57808; culture CMW 8242, PREM 57809; culture CMW 8108; culture CMW 8244, PREM 57811; culture CMW 8243, PREM 57810).

DISCUSSION

Very little is presently known about the occurrence of ophiostomatoid fungi in the Himalayas and their role as tree pathogens and agents of blue-stain in this part of the world. Prior to this study, only one ophiostomatoid fungus, *Ophiostoma himal-ulmi* Brasier & Mehrotra has been reported from the Western Himalayas, where it occurs on *Ulmus wallichiana* Planchon and is associated with elm bark beetles (Brasier & Mehrotra 1995). The discovery of *C. bhutanensis prov. nom.* and the detection of *C. moniliformis* in Bhutan represent the first reports of species of *Ceratocystis* from this country and the entire Himalayas. To determine wheter this fungus will have an impact on the forestry industry in Bhutan pathogenecity tests have to be conducted. *Ceratocystis bhutanensis prov. nom.*, described in this study is also the first fungus to be recorded as an associate of bark beetles, specifically of *I. schmutzenhoferi* in Bhutan. Many other ophiostomatoid fungi, including species of *Ophiostoma*, *Ceratocystiopsis* Upadhyay & Kendr., *Leptographium* Lagerb. & Melin and *Pesotum* Crane have been detected in the survey in Bhutan in 2001 (Kirisits, Wingfield & Chhetri 2002). Examination of these fungi is currently underway in order to unambiguously identify them and to provide formal names for the taxa that are considered as new to science. The survey in Bhutan was conducted in a very dry year and the dry weather conditions during spring and early summer 2001 may have accounted for the limited amount of stain observed on trees and logs infested by *I. schmutzenhoferi*. In a more humid year, more stain would be observed due to the more favorabile conditions for fungal growth.

Ceratocystis bhutanensis prov. nom. is morphologically very similar to *C. moniliformis* and *C. moniliformopsis*, but the occurrence of the fungus from Bhutan on a conifer tree in association with a bark beetle appeared to be untypical for the latter species and provided first evidence that the isolates obtained from *I. schmutzenhoferi* might represent a new species. This view was supported by the colony morphology and the distinct odour of the *Ceratocystis* sp. from Bhutan. Micro-morphological comparison of *C. bhutanensis prov. nom.* with *C. moniliformis* and *C. moniliformopsis* revealed small differences between these fungi and DNA sequence analyses of three nuclear genes finally provided unequivocal evidence that the isolates from Bhutan represent a new *Ceratocystis* sp. *Ceratocystis moniliformis*, *C. moniliformopsis* and *C. bhutanensis prov. nom.* are additional examples of morphologically almost indistinguishable, yet genetically and ecologically distinct species in the genus *Ceratocystis*. Other well-known examples are *C.*

polonica and *C. laricicola* that are morphologically identical, but show differences in their habitat and vectors, display host preference and can be differentiated by molecular markers (Harrington & Wingfield 1998, Kirisits 2001, Harrington *et al.* 2002).

Based on morphology, *C. bhutanensis prov. nom.* most closely resembles *C. moniliformis*. *Ceratocystis moniliformis* is typified by ascomatal bases covered with short conical spines, disc-shaped bases on its necks and hat-shaped ascospores (Davidson 1935, Nag Raj & Kendrick 1975, Upadhyay 1981). *Ceratocystis bhutanensis prov. nom.* shares all three of these characteristics. Morphologically, *C. bhutanensis prov. nom.* and *C. moniliformis* can be distinguished from each other by the much darker colour of the culture and the considerably more abundant production of aerial mycelium in the former species. *Ceratocystis bhutanensis prov. nom.* also produces an aroma very different to that of *C. moniliformis*. The two fungi have distinct types of hyphae; *C. bhutanensis prov. nom.* has smooth and granular hyphae, while *C. moniliformis* has only smooth hyphae. These morphological differences, as well as the different ecologies of the two fungi enable easy recognition of *C. bhutanensis prov. nom.* They also provide strong justification for the description of a new species.

Ceratocystis bhutanensis prov. nom. can be distinguished from *C. moniliformis* based on growth rate in culture. The former species grows considerably faster at temperatures below 15 °C than *C. moniliformis*, which hardly grows at temperatures below 15 °C. This ability to grow at low temperatures is consistent with the ecology of *C. bhutanensis prov. nom.* This growth at low temperatures of *C. bhutanensis prov. nom.* acts as a good distribution barrier. This fungus is associated with an insect that occurs at relatively high altitudes in the Himalayan mountain ranges, where it would be adapted to low temperatures. This is in contrast to *C. moniliformis,* which is common in the sub-tropics and tropics (Davidson 1935, Upadhyay 1981) where it is associated with temperatures much higher than those in the area where *C. bhutanensis prov. nom.* was found. Indeed, the two isolates of *C. moniliformis* from Bhutan examined in this study were collected near Punakha and Wangdi, at areas where sub-tropical climate and vegetation reach far north into the Himalayas. The growth-temperature relationships and the relatively low temperature optimum of *C. bhutanensis prov. nom.* resemble those of *O. himal-ulmi* that is also adapted to relatively low temperatures and has a temperature optimum between 22-25 °C (Brasier & Mehrotra 1995).

Another species very similar to *C. moniliformis* is *C. moniliformopsis* (Yuan & Mohammed 2002). *Ceratocystis bhutanensis prov. nom.* can easily be distinguished from *C. moniliformopsis.* The two fungi share the same characteristics with each other as they do with *C. moniliformis* (Davidson 1935, Yuan & Mohammed 2002). *Ceratocystis bhutanensis prov. nom.* can, however, be distinguished from *C. moniliformopsis* based on the culture morphology, as *C. bhutanensis prov. nom.* has a much darker mycelial growth than *C. moniliformopsis*. There is no description of aroma production of *C. moniliformopsis* but a similar, faint, fruity banana-oil odour that *C. moniliformis* produces was found, and not the fermenting fruit odour of *C. bhutanensis prov. nom.* The hyphae of *C. moniliformis* and *C. moniliformopsis* are the same and thus different to *C. bhutanensis prov. nom.* as described above. *Ceratocystis moniliformopsis* has convergent ostiolar hyphae in contrast to the divergent ostiolar hyphae of *C. moniliformis* and *C. bhutanensis prov. nom.* The ascomatal bases of *C. bhutanensis prov. nom.* and *C. moniliformis* are globose in shape while *C. moniliformopsis* has an ovoid ascomatal base shape. The ornamentation on the ascomatal base for *C. moniliformis* was described as conical spines (Hedgcock 1906) while *C. moniliformopsis* was described as having two types of ornamentation present on the ascomatal base. The first of these is the ampliform to conical, rostrate or obtuse at the apex spines. The second type is the hyphal hairs that are said to be distinct for this species, but hyphae have been observed on the bases of *C. bhutanensis prov. nom.* as well as on *C. moniliformis*. Yuan and Mohammed (2002) described two types of conidiogenous cells, both being phialidic only differing at the apices. In 1951, Bakshi also observed two types of conidiogenous cells for *C. moniliformis* only differing in width (Bakshi 1951). For *C. bhutanensis prov. nom.* only one type of conidiogenous cell was observed.

Other than *C. bhutanensis prov. nom.*, there are six *Ceratocystis* spp. known to have hat-shaped ascospores. These include *C. fimbriata* Ell. & Halst. (Upadhyay 1981), *C. moniliformis* (Davidson 1935), *C. albofundus* De Beer, Wingf. & Morr. (Wingfield *et al.* 1996), *C. moniliformopsis* (Yuan & Mohammed 2002), *C. pirilliformis* Barnes & Wingfield (Barnes *et al.* 2003b) and *C. acericola* Griffin (Griffin 1968). Of these species, only *C. moniliformis* and *C. moniliformopsis* have spines on their ascomatal bases and both have very characteristic disc-shaped bases on their ascomatal necks. *Ceratocystis bhutanensis prov. nom.* can be distinguished from both of these species based on host, biogeography, association with a conifer bark beetle, and odour in culture as well as various morphological characteristics noted previously.

A comparison of DNA sequences for three gene regions provided strong support for our view that *C. bhutanensis prov. nom.* represents a previously undescribed taxon. Sequence data for the ITS regions alone did not provide convincing separation between the new species, *C. moniliformis* and *C. moniliformopsis*. However, addition of β-tubulin and EF1-α sequences provided clear resolution to the clades in which these three species reside. Phylogenetically, *C. bhutanensis prov. nom.* grouped within the larger *C. coerulescens* clade (Witthuhn *et al.* 1998) together with *C. moniliformis* and *C. moniliformopsis* as its closest relatives. This clade is separate from the *C. fimbriata* clade, in which the other *Ceratocystis* spp. with hat-shaped ascospores reside (Witthuhn *et al.* 1999, Barnes *et al.* 2003a). This study also provides the first DNA sequence data for *C. moniliformopsis* and supports the view that this is a distinct species, even though it is morphologically very similar to *C. moniliformis* (Yuan & Mohammed 2002).

Besides *C. polonica*, *C. laricicola* and *C. rufipenni*, *C. bhutanensis prov. nom.* is the fourth *Ceratocystis* sp. known to be associated with a conifer bark beetle. The new *Ceratocystis* sp. from Bhutan is, however, very different to the other three species, morphologically, phenotypically and phylogenically. *Ceratocystis polonica*, *C. laricicola* and *C. rufipenni* are closely related to each other and form part of the *C. coerulescens* spp. complex on conifers (Harrington & Wingfield 1998, Witthuhn *et al.* 1998). In contrast, *C. bhutanensis prov. nom.* is more distantly related to species of the *C. coerulescens* complex on conifers, but phylogenetically groups closely with *C. moniliformis* and *C. moniliformopsis* that occur on hardwoods in tropical, sub-tropical and temperate areas of the world.

With its fast growth, its rapid degeneration under standard laboratory conditions and its intensive aroma *C. bhutanensis prov. nom.* phenotypically also resembles *C. moniliformis* and *C. moniliformopsis* and differs from other *Ceratocystis* spp. that are associated with conifer bark beetles. The intensive odour of *C. bhutanensis prov. nom.* is of special interest, since this is a general characteristic of *Ceratocystis* spp. that are not intimately and specifically associated with insects. The intensive aroma of these species is viewed as an adaptation to attract various insects that are unspecifically involved in dissemination of these fungi (Kile 1993, Harrington & Wingfield 1998). In contrast to *C. bhutanensis prov. nom.*, cultures of *C. polonica*, *C. laricicola* and *C. rufipenni* lack an intensive aroma,

which is considered as a modification to the consistent association with bark beetles (Yamaoka *et al.* 1997, Harrington & Wingfield 1998).

Ips schmutzenhoferi is an insect that is biologically very similar to *I. typographus* and *I. cembrae* (Postner 1974, Christiansen & Bakke 1988, Schmutzenhofer 1988). Both of the latter insects carry a wide range of *Ophiostoma* spp. and their anamorphs, and they are particularly interesting in that they are also consistently associated with a pathogenic *Ceratocystis* sp. (Solheim 1986, Redfern *et al.* 1987, Solheim 1992, Yamaoka *et al.* 1997, Yamaoka *et al.* 1998, Kirisits 2001). In this respect, we might have expected to encounter a *Ceratocystis* sp. associated with *I. schmutzenhoferi* in Bhutan. However, the fact that *C. bhutanensis prov. nom.* was isolated only from adult insects at one locality and not from beetles obtained from other sites, from galleries or symptomatic sapwood tissue is intriguing and raises the question about the intimacy of the relationship between this fungus and *I. schmutzenhoferi*.

Ceratocystis bhutanensis prov. nom. may be a rare associate of *I. schmutzenhoferi* or may show a restricted geographical distribution. Variation in the assemblages of fungi associated with bark beetles between different study sites has also been well documented and this might explain the isolation results in the present study. For example, *C. polonica* has been reported as a frequent or even as the most dominate associate of *I. typographus* in some parts of Europe, while it was not recorded or occurred rarely in studies conducted in other parts of the continent (Solheim 1986, 1992, 1993, Kirisits *et al.* 2000, Kirisits 2001). It has also been suggested that the population dynamics of *I. typographus* has a strong influence on the incidence and frequency of *C. polonica*, the fungus occurring less frequently during endemic periods, but becoming more frequent during outbreaks of the insect (Solheim 1993). Similar phenomena may also occur in the *I. schmutzenhoferi* – *C. bhutanensis prov. nom.* system. Phoretic mites of bark beetles are also known to carry ascospores of *Ceratocystis* and *Ophiostoma* spp. (Moser, Perry & Solheim 1989, Moser, Perry & Furuta 1997) and it might be possible that *C. bhutanensis prov. nom.* was in fact isolated from mites attached to the beetles and not directly from *I. schmutzenhoferi*.

An alternative view is that the isolation of *C. bhutanensis prov. nom.* from *I. schmutzenhoferi* was only accidental and that this fungus is only casually associated with this conifer bark beetle. Its unusual features for a *Ceratocystis* sp. associated with conifer bark beetles, especially its intensive aroma, and its close phylogenetic relationship to two

Ceratocystis spp. from hardwoods might support this suggestion. At present the ecology of *C. bhutanensis prov. nom.* remains enigmatic. Further investigations, especially isolations from various niches at the type locality of the fungus and within the entire distribution range of *I. schmutzenhoferi* in Western and Central Bhutan are desirable, in order to understand the incidence and ecology of *C. bhutanensis prov. nom.* and its relationships with *I. schmutzenhoferi* more thoroughly.

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

Species	Isolate no.[d]	Alternative numbers[e]	GenBank accession nr.	Year of isolation	Host	Geographical origin	Associated insect	Collector(s)
C. moniliformis	CMW 8240[a]	None	AY528989[e] AY529000[f] AY529010[g]	2001	*Cassia fistula*	Punakha, Bhutan	None	M. J. Wingfield, T. Kirisits & D. B. Chhetri
"	CMW 8238[c]	"	N/A	"	"	"	"	"
"	CMW 9590[a, c]	"	AY528985[e] AY528996[f] AY529006[g]	2002	*Eucalyptus grandis*	Mpumalanga, South Africa	"	J. Roux
"	CMW 4114[a]	"	AY528986[e] AY528997[f] AY529007[g]	1997	*Schizolobium parahybum*	Ecuador, South America	"	M. J. Wingfield
"	CMW 10134[c]	"	N/A	2002	*Eucalyptus grandis*	Mpumalanga, South Africa	"	M. van Wyk
C. moniliformopsis	CMW 9986[a, c]	CBS 109441	AY528987[e] AY528998[f] AY529008[g]	1999	*Eucalyptus obliqua*	Tazmania, Australia	"	Z. Q. Yuan
"	CMW 10214[a, c]	None	AY528988[e] AY528999[f] AY529009[g]	1989	*Eucalyptus sieberi*	Victoria, Australia	"	M. J. Dudzinski
"	CMW 10215[c]	"	N/A	1990	"	"	"	"
C. bhutanensis prov. nom.	CMW 8215[a]	PREM 57805	AY528953[e] AY528958[f] AY528963[g]	2001	*Picea spinulosa*	Jelekha, Bhutan	*Ips schmutzenhoferi*	M. J. Wingfield , T. Kirisits & D. B. Chhetri
"	CMW 8242[a, b]	CBS 112907 PREM 57809	AY528951[e] AY528956[f] AY528961[g]	"	"	"	"	"
"	CMW 8217[a, b, c]	PREM 57807	AY528952[e] AY528957[f] AY528962[g]	"	"	"	"	"

Table 1. (Continued) Isolates of *Ceratocystis* used in this study.

Species	Isolate no.[d]	Alternative numbers[e]	GenBank accession nr.	Year of isolation	Host	Geographical origin	Associated insect	Collector(s)
C. bhutanensis prov. nom.	CMW 8241[a, b, c]	PREM 57808	N/A	2001	*Picea spinulosa*	Jelekha, Bhutan	*Ips schmutzenhoferi*	M. J. Wingfield, T. Kirisits & D. B. Chhetri
"	CMW 8396[a]	BH 8/5 PREM 57812	N/A	"	"	"	"	"
"	CMW 8399[a]	BH 8/8	AY528954[e] AY528959[f] AY528964[g]	"	"	"	"	"
"	CMW 8243[a, b]	CBS 112908 PREM 57810	N/A	"	"	"	"	"
"	CMW 8108[a, b]	CBS 112905	N/A	"	"	"	"	"
"	CMW 8244[a, b, c]	PREM 57811	N/A	"	"	"	"	"
C. virescens	CMW 3276[a]	None	AY528984[e] AY528990[f] AY528991[g]	1963	*Quercus* sp.	Warrenber, N.Y., USA	None	T. Hinds

N/A refers to accession numbers not available at present.
[a, b, c, d, e, f, g] Isolates marked with [a] were sequenced, those marked with [b] were used for morphological descriptions and those marked with [c] were 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, BH to the culture collection of the Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Universität für Bodenkultur Wien (BOKU), Vienna, Austria and PREM to the National Fungal Herbarium (PREM), Pretoria, South Africa. GenBank accession numbers that are marked with [e] represent the ITS sequences, those marked with an [f] represent the β-tubulin sequences and those marked with a [g] represent the elongation factor sequences.

Table 2. Comparison of *C. bhutanensis prov. nom.* with morphologically similar species, *C. moniliformis* and *C. moniliformopsis*.

Characteristic	*C. bhutanensis prov. nom.*	*C. moniliformis* (Hedgcock 1906)	*C. moniliformopsis* (Yuan & Mohammed 2002)
ASCOMATA			
Base			
Colour	Dark brown to black	Brown to black	Dark brown to black
Diameter	138 - 178 µm	90 - 180 µm	200 - 300 µm
Ornamentation	Conical spines and hyphal hairs	Conical spines (sparse)	Hyphal hairs & conical spines
Form	Globose	Globose	Ovoid
Neck			
Colour	Dark brown to black becoming light brown towards apex	Light brown becoming transparent at the apex [a]	Dark brown to black
Disc-form at base	Yes	Yes [a]	Yes
Length	453 - 519 µm	730 - 896 µm [a]	470 - 780 µm
Width (Tip)	12 - 14 µm	14 µm [a]	18 - 22 µm
Width (Base)	34 - 42 µm	39.2 - 51.8 µm [a]	40 - 50 µm
Ostiolar hyphae			
Shape	Divergent	Divergent	Convergent
Measurement	18 - 26 µm	12 - 18 x 2 µm	25 - 45 x 1.5 - 2 µm
Ascus	Not seen	Fugacious	Not Seen
Ascospores			
Colour	Hyaline	Hyaline	Hyaline
Shape (Side view)	Hat-shaped	Oval, one side flat	Hat-shaped
Measurements	4 - 6 x 2 - 5 µm	4 - 5 x 3 - 4 µm	4 - 5 x 2 - 2.5 µm
Texture	Mucilaginous	Slimy grey mass	Gelatinous sheath

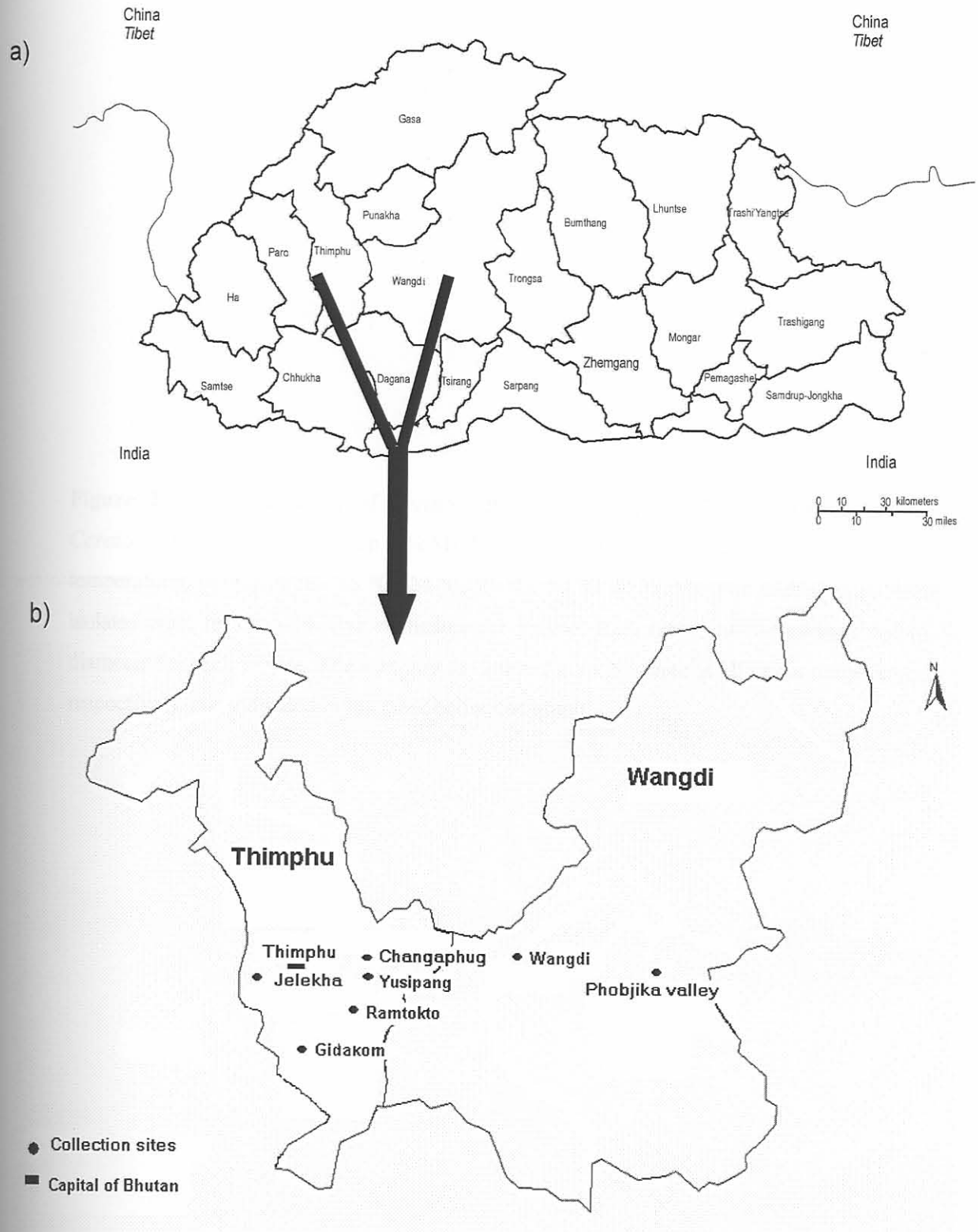
[a] Description by Bakshi (1951).

Table 2 (continued). Comparison of *C. bhutanensis prov. nom.* with morphologically similar species, *C. moniliformis* and *C. moniliformopsis.*

Characteristic	***C. bhutanensis prov. nom.***	***C. moniliformis*** (Hedgcock 1906)	***C. moniliformopsis*** (Yuan & Mohammed 2002)
CONIDIOMATA			
Conidiophores			
Measurements	23 - 39 x 4 - 6 µm	(1) 3 - 13.7 x 3.5 - 8.9 µm [a] (2) 7.3 - 13.7 x 4.5 µm [a]	5 - 32.5 x 4 - 5.3 µm
Shape	Phialides	Phialides (2 types)	Phialides (2 types)
Conidia			
Shape	(1) Cylindrical (2) Barrel-shaped	(1) Oval or cylindrical [a] (2) Cylindrical [a]	(1) Cylindrical (2) Oblong or ellipsoidal
Measurements	(1) 7 - 9 x 1 - 3 µm (2) 3 - 5 x 2 - 3 µm	(1) 7.3 - 13.7 x 3.5 - 8.9 µm [a] (2) 4.3 - 15.5 x 1 - 2.5 µm [a]	(1) 13 - 21 x 2 - 3 µm (2) 12 - 17.5 x 5 - 7.5 µm
CULTURE			
Growth rate	20 mm per day at 25 °C in the dark	60 mm in 10 days at 22 °C in the dark [a]	6.3 - 7.5 mm per day at 22 °C in the dark
Colour	Cream-buff to dark olive to black	Hyaline to grey to black	Colourless to white grey, centre becoming greenish brown
Odour	Fermenting odour	Pear drops [a]	None
Mycelia	Smooth and granulated	Coarsely granular	Smooth

[a] Description by Bakshi (1951).

Figure 1. a) A map of Bhutan with all the administrative districts (dzongkhags) of the country and Bhutan´s capital Thimphu, b) A map of the dzongkhags Thimphu and Wangdue Phodrang (Wangdi) showing the localities where samples for fungal isolation were collected from *Picea spinulosa* and *Pinus wallichiana*. *Ceratocystis bhutanensis prov. nom.* was only isolated from individuals of *Ips schmutzenhoferi* obtained from Jelekha.


a)
China
Tibet
China
Tibet
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Punakha
Thimphu
Paro
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Wangdi
Bumthang
Lhuntse
Trashi Yangtse
Trongsa
Trashigang
Mongar
Zhemgang
Samtse
Chhukha
Dagana
Tsirang
Serpang
Pemagashel
Samdrup-Jongkha
India
India
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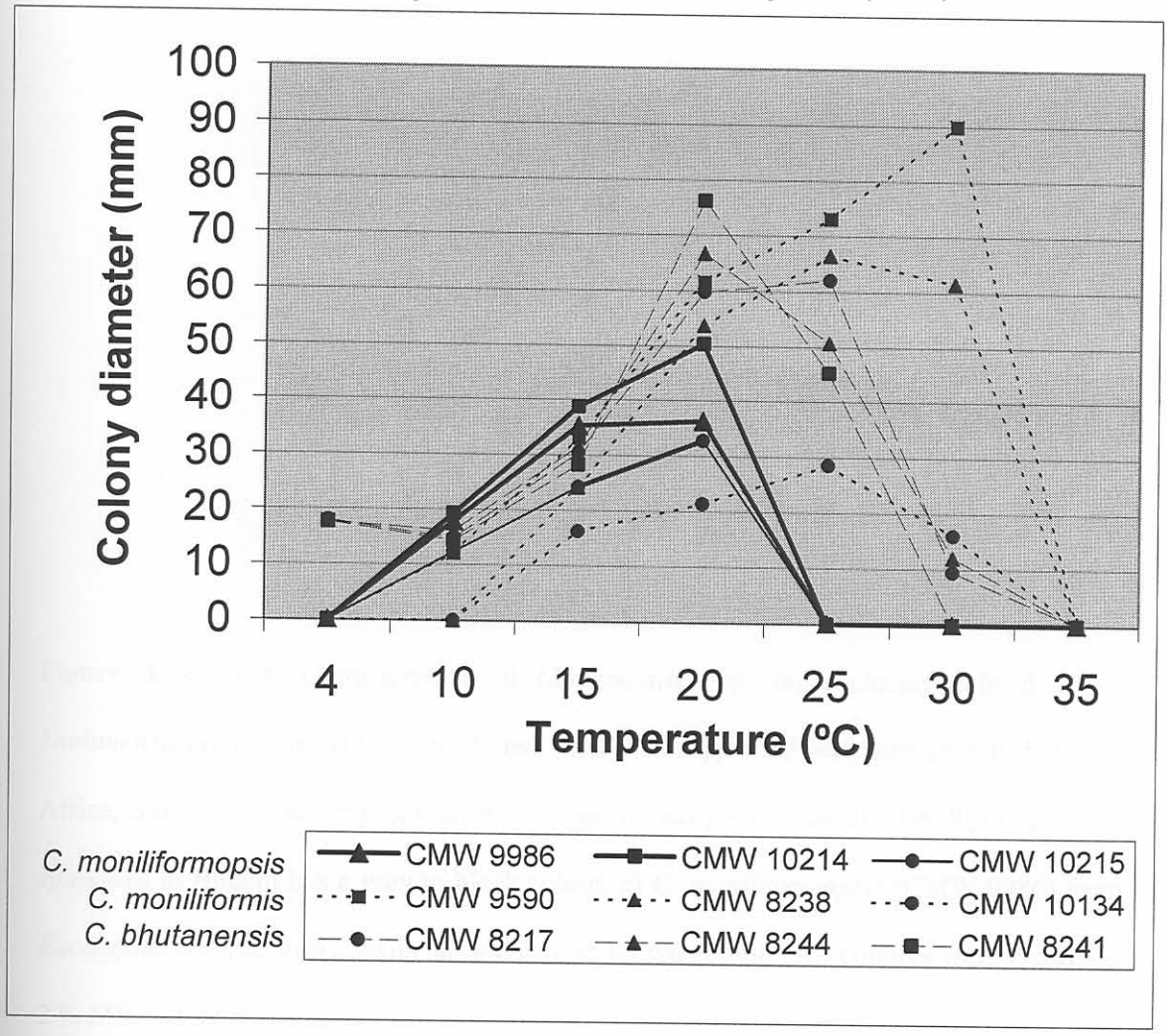
0 10 30 miles
b)
N
Wangdi
Thimphu
Thimphu
Changaphug
Wangdi
Jelekha
Yusipang
Phobjika valley
Ramtokto
Gidakom
Collection sites
Capital of Bhutan

Figure 2. Colony diameter of *Ceratocystis moniliformopsis*, *C. moniliformis* and the *Ceratocystis* sp. from Bhutan on 2 % MEA after three days of incubation at eight different temperatures (4 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C & 35 °C). For each species three isolates were tested, with five replicates per isolate. Bars represent the average colony diameter for each isolate. The standard deviation for each isolate at all seven temperatures respectively, are indicated in the table below the graph.



Temperature/ Isolate	4 °C	10 °C	15 °C	20 °C	25 °C	30 °C	35 °C
CMW 9986	0.0	0.2	2.1	1.3	0.0	0.0	0.0
CMW 10214	0.0	4.7	0.9	2.8	0.0	0.0	0.0
CMW 10215	0.0	1.0	2.0	5.2	0.0	0.0	0.0
CMW 9590	0.0	3.0	0.8	0.89	1.2	0.0	0.0
CMW 8238	0.0	0.0	2.6	0.3	1.5	1.0	0.0
CMW 10134	0.0	0.0	1.9	5.5	15.5	0.6	0.0
CMW 8217	1.6	1.5	1.0	1.2	2.7	0.4	0.0
CMW 8244	2.1	0.8	2.5	3.4	2.9	0.9	0.0
CMW 8241	1.2	0.5	2.7	5.9	3.6	0.0	0.0

Figure 3. Cultural characteristics of *Ceratocystis* spp. most closely related to *C. bhutanensis prov. nom.* a) *C. moniliformis* (CMW 9590) *from Eucalyptus grandis* in South Africa, has a white to grey colour, b) *C. bhutanensis prov. nom.* (CMW 8217) from *P. spinulosa* in Bhutan has a grey to black colour, c) *C. moniliformopsis* (CMW 9986) from *Eucalyptus obliqua* in Australia has a cream to brown colour. All cultures were grown on 2 % MEA at 20 °C for approximately 10 days.

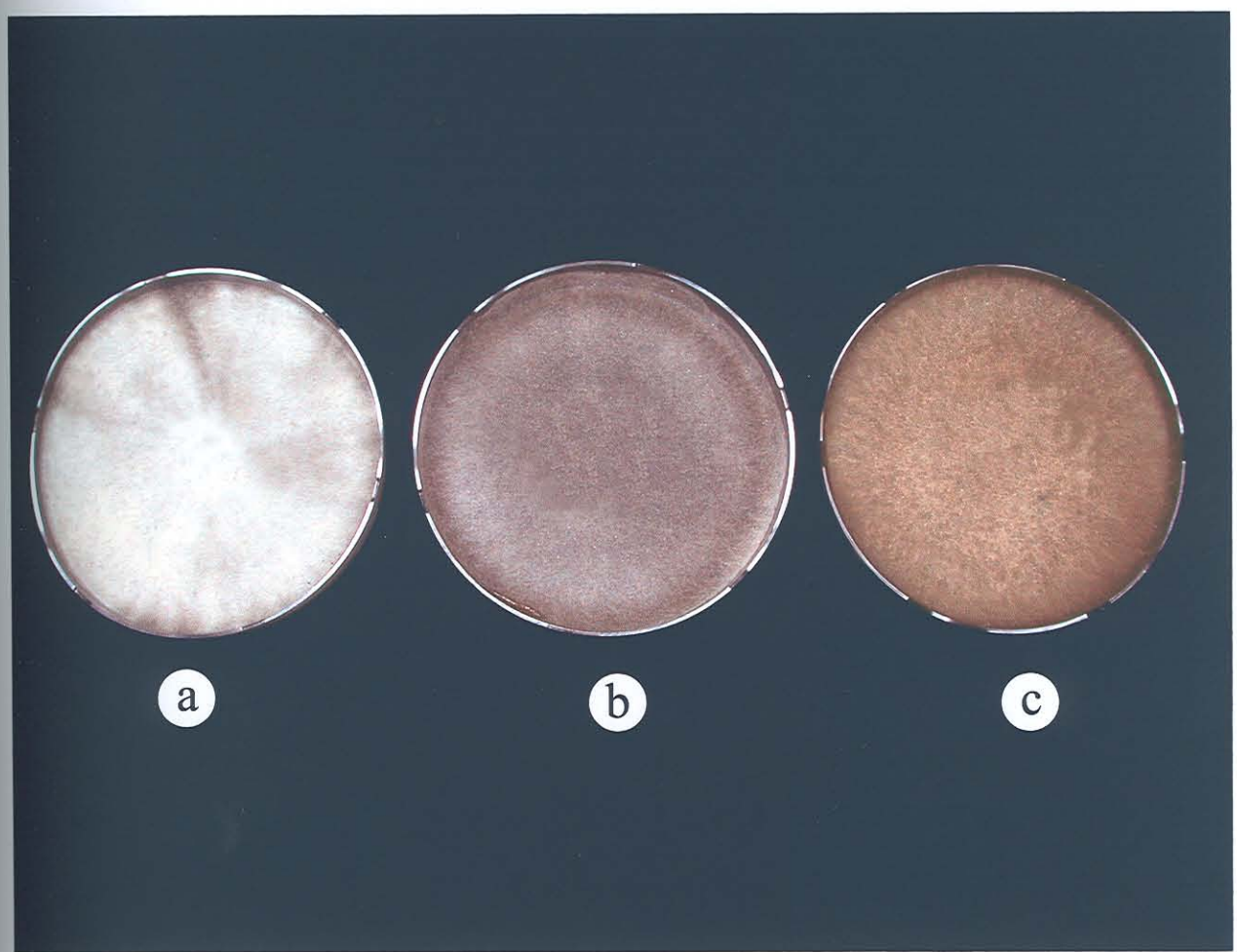


Figure 4. Morphological characteristics of *C. bhutanensis prov. nom.* (PREM 57807, CMW 8217), a) Globose ascomata with long neck (scalebar = 40 µm), b) Ascomatal base with short, conical spines and hyphal ornamentation (scalebar = 40 µm), c) Divergent ostiolar hyphae on the top of the ascomatal neck (scalebar = 10 µm), d) Hat-shaped ascospore in side view (scalebar = 5 µm), e) Hyphae with smooth edges (scalebar = 5 µm), f) Hyphae with rough edges (scalebar = 5 µm), g) Cylindrical conidia forming a chain (scalebar = 5 µm), h) Barrel-shaped conidia (scalebar = 5 µm), i) Phialidic conidiophore with emerging cylindrical conidium (scalebar = 10 µm)

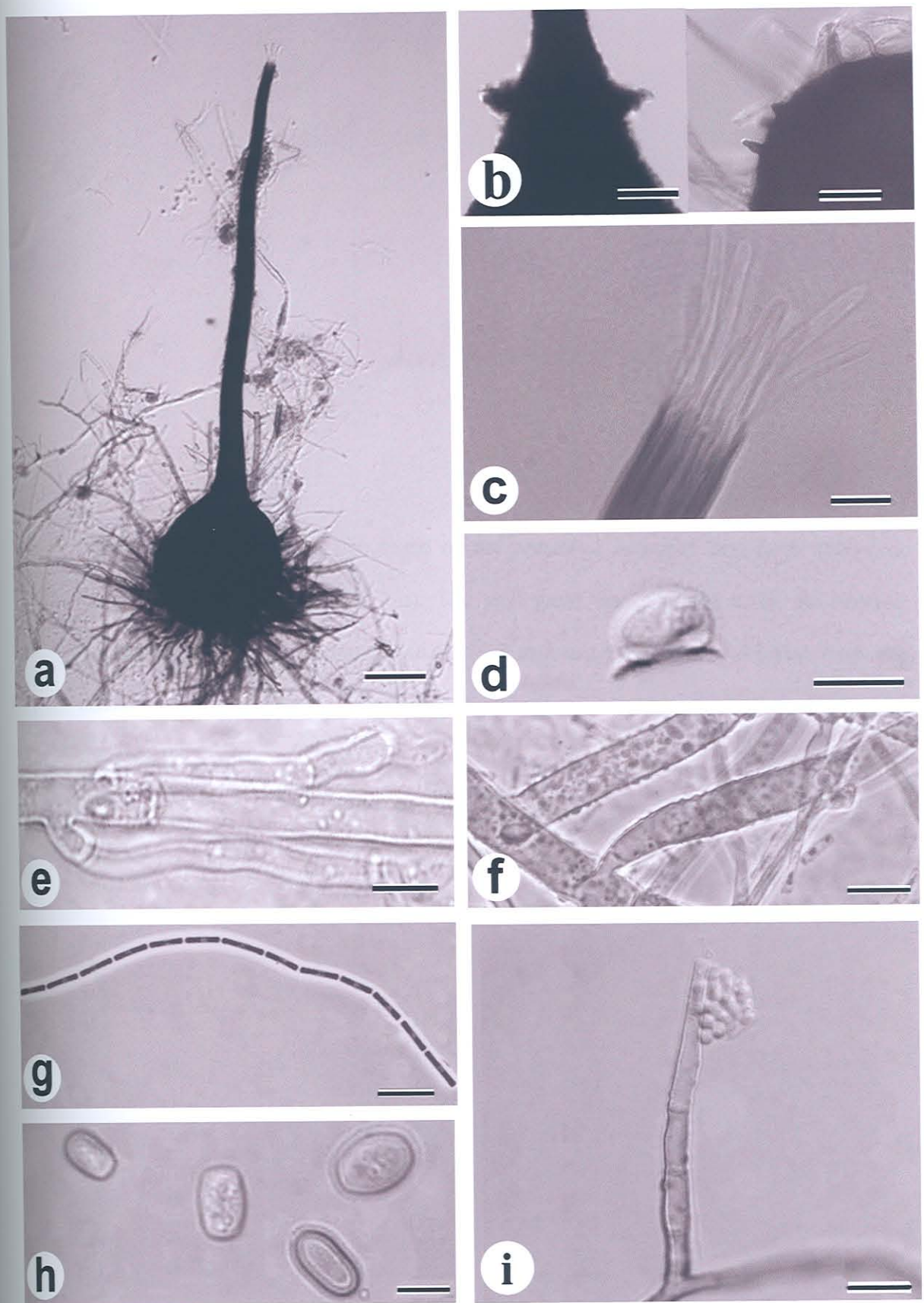
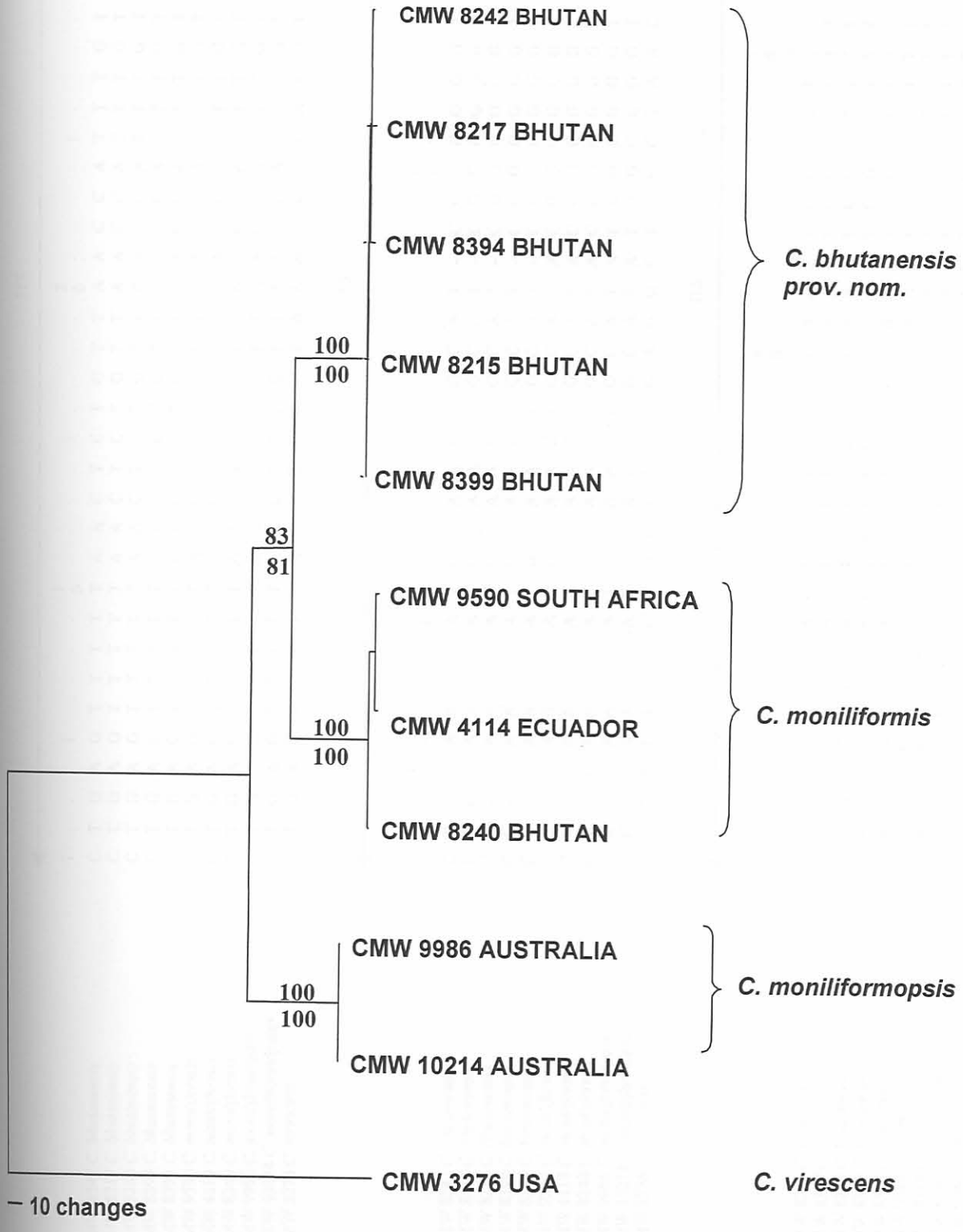

a
b
c
d
e
f
g
h
i

Figure 5. A phylogenetic tree based on the combined sequence data from three gene regions (ITS, β-tubulin and EF1-α). The phylogram was obtained using the heuristic search option based on parsimony and *C. virescens* was treated as the out-group. Bootstrap values are indicated above of the branches while Bayesian values are indicated below the branches.



ITS

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CMW 8242 *C. bhutanensis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 8217 *C. bhutanensis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 8215 *C. bhutanensis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 8399 *C. bhutanensis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 8394 *C. bhutanensis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 9590 *C. moniliformis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 4114 *C. moniliformis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 8240 *C. moniliformis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 9986 *C. moniliformopsis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 10214 *C. moniliformopsis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 3276 *C. virescens*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	-	T	A	A	A	C	C	A	T	A	T	G	T	G	A	A	C	A	T	A

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CMW 8242 *C. bhutanensis*	C	C	-	-	A	A	A	C	A	T	G	-	A	A	C	T	G	C	A	T	-	**C**	G	G	C	G	G	G	T	C	T	C	C	C	G	C
CMW 8217 *C. bhutanensis*	C	C	-	-	A	A	A	C	A	T	G	-	A	A	C	T	G	C	A	T	-	**C**	G	G	C	G	G	G	T	C	T	C	C	C	G	C
CMW 8215 *C. bhutanensis*	C	C	-	-	A	A	A	C	A	T	G	-	A	A	C	T	G	C	A	T	-	**C**	G	G	C	G	G	G	T	C	T	C	C	C	G	C
CMW 8399 *C. bhutanensis*	C	C	-	-	A	A	A	C	A	T	G	-	A	A	C	T	G	C	A	T	-	**C**	G	G	C	G	G	G	T	C	T	C	C	C	G	C
CMW 8394 *C. bhutanensis*	C	C	-	-	A	A	A	C	A	T	G	-	A	A	C	T	G	C	A	T	-	**C**	G	G	C	G	G	G	T	C	T	C	C	C	G	C
CMW 9590 *C. moniliformis*	C	C	-	-	A	A	A	C	A	T	G	-	A	A	C	T	G	C	A	T	**A**	**C**	G	G	C	G	G	G	T	C	T	C	C	C	G	C
CMW 4114 *C. moniliformis*	C	C	-	-	A	A	A	C	A	T	G	-	A	A	C	T	G	C	A	T	**A**	**C**	G	G	C	G	G	G	T	C	T	C	C	C	G	C
CMW 8240 *C. moniliformis*	C	C	-	-	A	A	A	C	A	T	G	-	A	A	C	T	G	C	A	T	**A**	**C**	G	G	C	G	G	G	T	C	T	C	C	C	G	C
CMW 9986 *C. moniliformopsis*	C	C	-	-	A	A	A	C	A	T	G	-	A	A	C	T	G	C	A	T	**A**	**T**	G	G	C	G	G	G	T	C	T	C	C	C	G	C
CMW 10214 *C. moniliformopsis*	C	C	-	-	A	A	A	C	A	T	G	-	A	A	C	T	G	C	A	T	**A**	**T**	G	G	C	G	G	G	T	C	T	C	C	C	G	C
CMW 3276 *C. virescens*	C	C	T	A	T	T	A	G	C	T	G	C	T	T	T	G	G	A	G	G	**C**	**T**	T	G	G	T	A	A	C	A	C	A	A	A	G	T

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CMW 8242 *C. bhutanensis*	C	A	G	-	C	A	G	T	A	C	T	C	T	T	G	-	-	-	A	A	C	T	C	G	T	T	T	T	T	-	-	A	T	A	T	A
CMW 8217 *C. bhutanensis*	C	A	G	-	C	A	G	T	A	C	T	C	T	T	G	-	-	-	A	A	C	T	C	G	T	T	T	T	T	-	-	A	T	A	T	A
CMW 8215 *C. bhutanensis*	C	A	G	-	C	A	G	T	A	C	T	C	T	T	G	-	-	-	A	A	C	T	C	G	T	T	T	T	T	-	-	A	T	A	T	A
CMW 8399 *C. bhutanensis*	C	A	G	-	C	A	G	T	A	C	T	C	T	T	G	-	-	-	A	A	C	T	C	G	T	T	T	T	T	-	-	A	T	A	T	A
CMW 8394 *C. bhutanensis*	C	A	G	-	C	A	G	T	A	C	T	C	T	T	G	-	-	-	A	A	C	T	C	G	T	T	T	T	T	-	-	A	T	A	T	A
CMW 9590 *C. moniliformis*	C	A	G	-	C	A	G	T	A	C	T	C	T	T	G	-	-	-	A	A	C	T	C	G	T	T	T	T	T	-	-	A	T	A	T	A
CMW 4114 *C. moniliformis*	C	A	G	-	C	A	G	T	A	C	T	C	T	T	G	-	-	-	A	A	C	T	C	G	T	T	T	T	T	-	-	A	T	A	T	A
CMW 8240 *C. moniliformis*	C	A	G	-	C	A	G	T	A	C	T	C	T	T	G	-	-	-	A	A	C	T	C	G	T	T	T	T	T	-	-	A	T	A	T	A
CMW 9986 *C. moniliformopsis*	C	A	G	-	C	A	G	T	A	C	T	C	T	T	G	-	-	-	A	A	C	T	C	G	T	T	T	T	T	-	-	A	T	A	T	A
CMW 10214 *C. moniliformopsis*	C	A	G	-	C	A	G	T	A	C	T	C	T	T	G	-	-	-	A	A	C	T	C	G	T	T	T	T	T	-	-	A	T	A	T	A
CMW 3276 *C. virescens*	C	T	G	C	C	G	G	T	A	G	T	A	T	T	T	T	A	A	A	A	C	T	C	T	T	T	T	T	T	T	T	A	T	T	C	T

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CMW 8242 *C. bhutanensis*	A	A	G	A	A	T	T	T	A	T	T	C	A	T	T	G	C	T	G	A	**G**	T	A	G	C	A	T	T	T	T	A	T	A	A	A	T
CMW 8217 *C. bhutanensis*	A	A	G	A	A	T	T	T	A	T	T	C	A	T	T	G	C	T	G	A	**G**	T	A	G	C	A	T	T	T	T	A	T	A	A	A	T
CMW 8215 *C. bhutanensis*	A	A	G	A	A	T	T	T	A	T	T	C	A	T	T	G	C	T	G	A	**G**	T	A	G	C	A	T	T	T	T	A	T	A	A	A	T
CMW 8399 *C. bhutanensis*	A	A	G	A	A	T	T	T	A	T	T	C	A	T	T	G	C	T	G	A	**G**	T	A	G	C	A	T	T	T	T	A	T	A	A	A	T
CMW 8394 *C. bhutanensis*	A	A	G	A	A	T	T	T	A	T	T	C	A	T	T	G	C	T	G	A	**T**	T	A	G	C	A	T	T	T	T	A	T	A	A	A	T
CMW 9590 *C. moniliformis*	A	A	G	A	A	T	T	T	A	T	T	C	A	T	T	G	C	T	G	A	**G**	T	A	G	C	A	T	T	T	T	A	T	A	A	A	T
CMW 4114 *C. moniliformis*	A	A	G	A	A	T	T	T	A	T	T	C	A	T	T	G	C	T	G	A	**G**	T	A	G	C	A	T	T	T	T	A	T	A	A	A	T
CMW 8240 *C. moniliformis*	A	A	G	A	A	T	T	T	A	T	T	C	A	T	T	G	C	T	G	A	**G**	T	A	G	C	A	T	T	T	T	A	T	A	A	A	T
CMW 9986 *C. moniliformopsis*	A	A	G	A	A	T	T	T	A	T	T	C	A	T	T	G	C	T	G	A	**G**	T	A	G	C	A	T	T	T	T	A	T	A	A	A	T
CMW 10214 *C. moniliformopsis*	A	A	G	A	A	T	T	T	A	T	T	C	A	T	T	G	C	T	G	A	**G**	T	A	G	C	A	T	T	T	T	A	T	A	A	A	T
CMW 3276 *C. virescens*	A	A	A	G	A	A	T	T	A	T	T	C	A	T	T	G	C	T	G	A	**G**	T	G	G	C	A	T	T	A	A	C	A	T	A	A	T

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CMW 8242 *C. bhutanensis*	G	T	A	T	T	A	A	A	A	C	T	T	T	C	A	A	C	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A
CMW 8217 *C. bhutanensis*	G	T	A	T	T	A	A	A	A	C	T	T	T	C	A	A	C	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A
CMW 8215 *C. bhutanensis*	G	T	A	T	T	A	A	A	A	C	T	T	T	C	A	A	C	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A
CMW 8399 *C. bhutanensis*	G	T	A	T	T	A	A	A	A	C	T	T	T	C	A	A	C	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A
CMW 8394 *C. bhutanensis*	G	T	A	T	T	A	A	A	A	C	T	T	T	C	A	A	C	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A
CMW 9590 *C. moniliformis*	G	T	A	T	T	A	A	A	A	C	T	T	T	C	A	A	C	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A
CMW 4114 *C. moniliformis*	G	T	A	T	T	A	A	A	A	C	T	T	T	C	A	A	C	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A
CMW 8240 *C. moniliformis*	G	T	A	T	T	A	A	A	A	C	T	T	T	C	A	A	C	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A
CMW 9986 *C. moniliformopsis*	G	T	A	T	T	A	A	A	A	C	T	T	T	C	A	A	C	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A
CMW 10214 *C. moniliformopsis*	G	T	A	T	T	A	A	A	A	C	T	T	T	C	A	A	C	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A
CMW 3276 *C. virescens*	A	A	G	T	T	A	A	A	A	C	T	T	T	C	A	A	C	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A

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CMW 8242 *C. bhutanensis*	G	C	A	T	C	G	A	T	G	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A
CMW 8217 *C. bhutanensis*	G	C	A	T	C	G	A	T	G	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A
CMW 8215 *C. bhutanensis*	G	C	A	T	C	G	A	T	G	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A
CMW 8399 *C. bhutanensis*	G	C	A	T	C	G	A	T	G	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A
CMW 8394 *C. bhutanensis*	G	C	A	T	C	G	A	T	G	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A
CMW 9590 *C. moniliformis*	G	C	A	T	C	G	A	T	G	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A
CMW 4114 *C. moniliformis*	G	C	A	T	C	G	A	T	G	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A
CMW 8240 *C. moniliformis*	G	C	A	T	C	G	A	T	G	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A
CMW 9986 *C. moniliformopsis*	G	C	A	T	C	G	A	T	G	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A
CMW 10214 *C. moniliformopsis*	G	C	A	T	C	G	A	T	G	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A
CMW 3276 *C. virescens*	G	C	A	T	C	G	A	T	G	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	C	G	T	A	A

ITS

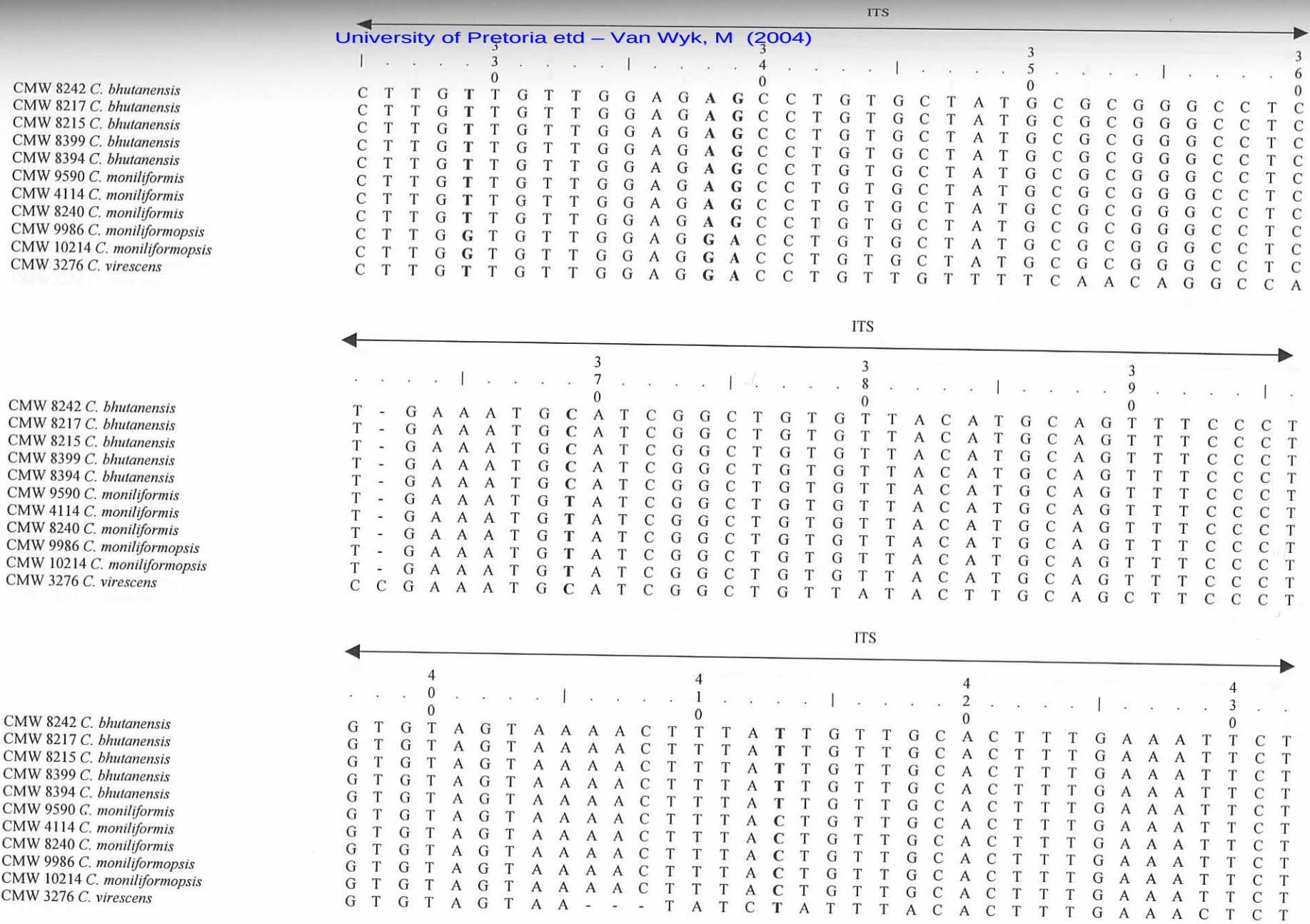
	210	220	230	240	250
CMW 8242 *C. bhutanensis*	T G T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T
CMW 8217 *C. bhutanensis*	T G T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T
CMW 8215 *C. bhutanensis*	T G T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T
CMW 8399 *C. bhutanensis*	T G T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T
CMW 8394 *C. bhutanensis*	T G T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T
CMW 9590 *C. moniliformis*	T G T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T
CMW 4114 *C. moniliformis*	T G T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T
CMW 8240 *C. moniliformis*	T G T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T
CMW 9986 *C. moniliformopsis*	T G T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T
CMW 10214 *C. moniliformopsis*	T G T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T
CMW 3276 *C. virescens*	T G T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T

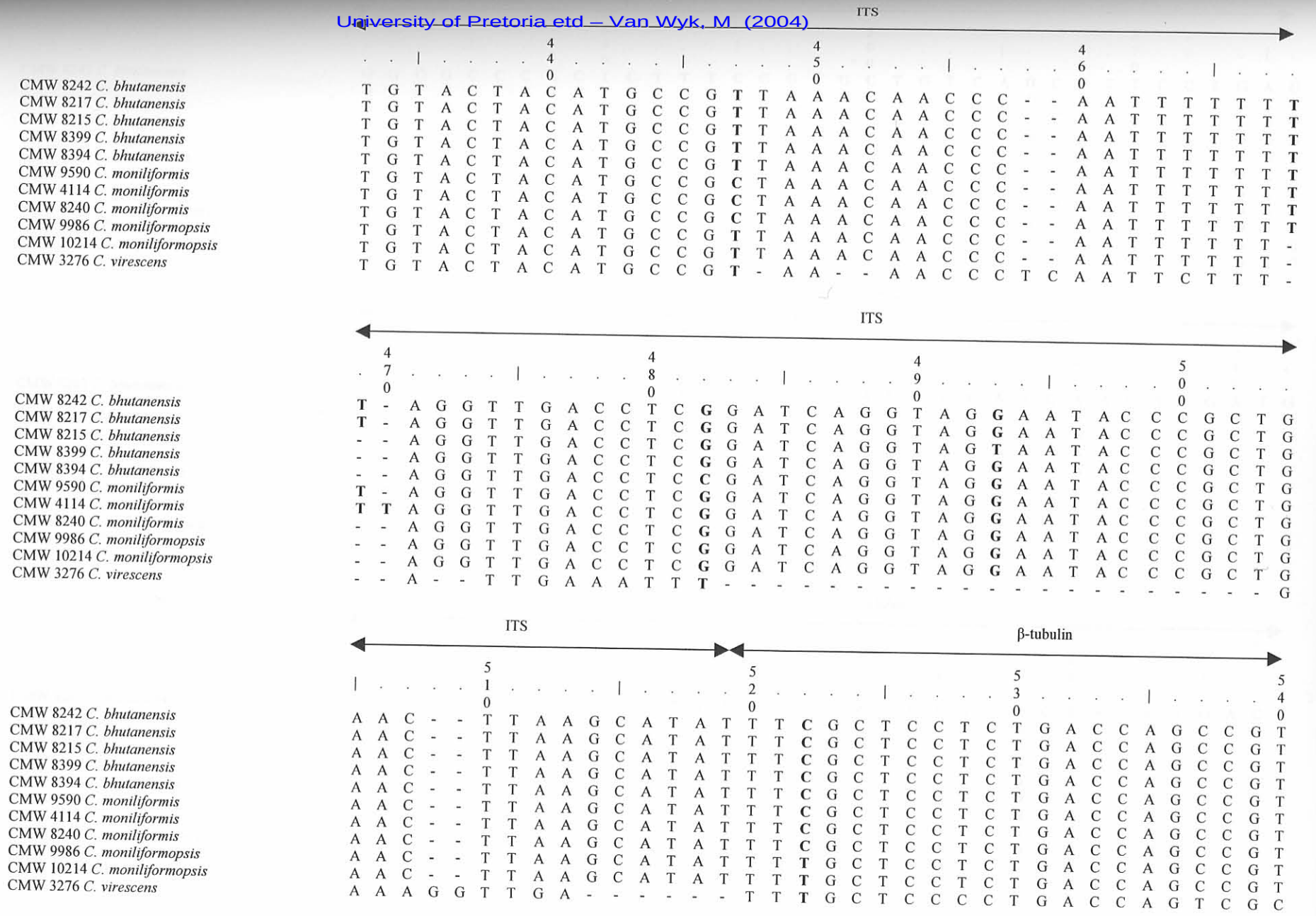
ITS

	260	270	280
CMW 8242 *C. bhutanensis*	G A A C G C A C A T T G C G C C C A G C A G T A C T C T G C T G G G C A
CMW 8217 *C. bhutanensis*	G A A C G C A C A T T G C G C C C A G C A G T A C T C T G C T G G G C A
CMW 8215 *C. bhutanensis*	G A A C G C A C A T T G C G C C C A G C A G T A C T C T G C T G G G C A
CMW 8399 *C. bhutanensis*	G A A C G C A C A T T G C G C C C A G C A G T A C T C T G C T G G G C A
CMW 8394 *C. bhutanensis*	G A A C G C A C A T T G C G C C C A G C A G T A C T C T G C T G G G C A
CMW 9590 *C. moniliformis*	G A A C G C A C A T T G C G C C C A G C A G T A C T C T G C T G G G C A
CMW 4114 *C. moniliformis*	G A A C G C A C A T T G C G C C C A G C A G T A C T C T G C T G G G C A
CMW 8240 *C. moniliformis*	G A A C G C A C A T T G C G C C C A G C A G T A C T C T G C T G G G C A
CMW 9986 *C. moniliformopsis*	G A A C G C A C A T T G C G C C C A G C A G T A C T C T G C T G G G C A
CMW 10214 *C. moniliformopsis*	G A A C G C A C A T T G C G C C C A G C A G T A C T C T G C T G G G C A
CMW 3276 *C. virescens*	G A A C G C A C A T T G C G C C T G G C A G T A T T C A G C C A G G C A

ITS

	290	300	310	320
CMW 8242 *C. bhutanensis*	T G C C T G T C C G A G C G T C A T T T C A C C A C T C A A G C T C T G
CMW 8217 *C. bhutanensis*	T G C C T G T C C G A G C G T C A T T T C A C C A C T C A A G C T C T G
CMW 8215 *C. bhutanensis*	T G C C T G T C C G A G C G T C A T T T C A C C A C T C A A G C T C T G
CMW 8399 *C. bhutanensis*	T G C C T G T C C G A G C G T C A T T T C A C C A C T C A A G C T C T G
CMW 8394 *C. bhutanensis*	T G C C T G T C C G A G C G T C A T T T C A C C A C T C A A G C T C T G
CMW 9590 *C. moniliformis*	T G C C T G T C C G A G C G T C A T T T C A C C A C T C A A G C T C T G
CMW 4114 *C. moniliformis*	T G C C T G T C C G A G C G T C A T T T C A C C A C T C A A G C T C T G
CMW 8240 *C. moniliformis*	T G C C T G T C C G A G C G T C A T T T C A C C A C T C A A G C T C T G
CMW 9986 *C. moniliformopsis*	T G C C T G T C C G A G C G T C A T T T C A C C A C T C A A G C T A T G
CMW 10214 *C. moniliformopsis*	T G C C T G T C C G A G C G T C A T T T C A C C A C T C A A G C T A T G
CMW 3276 *C. virescens*	T G C C T G T C C G A G C G T C A T T T C A C C A C T C A A G C T C C G





β-tubulin

Isolate										550										560										570						
CMW 8242 *C. bhutanensis*	G	G	C	G	C	**C**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A	G	C	G	T	T	C	C	T	G	A	G
CMW 8217 *C. bhutanensis*	G	G	C	G	C	**C**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A	G	C	G	T	T	C	C	T	G	A	G
CMW 8215 *C. bhutanensis*	G	G	C	G	C	**C**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A	G	C	G	T	T	C	C	T	G	A	G
CMW 8399 *C. bhutanensis*	G	G	C	G	C	**C**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A	G	C	G	T	T	C	C	T	G	A	G
CMW 8394 *C. bhutanensis*	G	G	C	G	C	**C**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A	G	C	G	T	T	C	C	T	G	A	G
CMW 9590 *C. moniliformis*	G	G	C	G	C	**T**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A	G	C	G	T	T	C	C	T	G	A	G
CMW 4114 *C. moniliformis*	G	G	C	G	C	**T**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A	G	C	G	T	T	C	C	T	G	A	G
CMW 8240 *C. moniliformis*	G	G	C	G	C	**T**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A	G	C	G	T	T	C	C	T	G	A	G
CMW 9986 *C. moniliformopsis*	G	G	C	G	C	**C**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A	G	C	G	T	T	C	C	T	G	A	G
CMW 10214 *C. moniliformopsis*	G	G	C	G	C	**C**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A	G	C	G	T	T	C	C	T	G	A	G
CMW 3276 *C. virescens*	G	G	T	G	C	**T**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	C	G	T	C	A	G	C	G	T	G	C	C	C	G	A	G

β-tubulin

Isolate				580										590										600										610		
CMW 8242 *C. bhutanensis*	C	T	C	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	**C**	A	A	G	A	A	C	A	T	G	A	T	G
CMW 8217 *C. bhutanensis*	C	T	C	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	**C**	A	A	G	A	A	C	A	T	G	A	T	G
CMW 8215 *C. bhutanensis*	C	T	C	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	**C**	A	A	G	A	A	C	A	T	G	A	T	G
CMW 8399 *C. bhutanensis*	C	T	C	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	**C**	A	A	G	A	A	C	A	T	G	A	T	G
CMW 8394 *C. bhutanensis*	C	T	C	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	**C**	A	A	G	A	A	C	A	T	G	A	T	G
CMW 9590 *C. moniliformis*	C	T	C	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	**T**	A	A	G	A	A	C	A	T	G	A	T	G
CMW 4114 *C. moniliformis*	C	T	C	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	**T**	A	A	G	A	A	C	A	T	G	A	T	G
CMW 8240 *C. moniliformis*	C	T	C	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	**T**	A	A	G	A	A	C	A	T	G	A	T	G
CMW 9986 *C. moniliformopsis*	C	T	C	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	**C**	A	A	G	A	A	C	A	T	G	A	T	G
CMW 10214 *C. moniliformopsis*	C	T	C	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	**C**	A	A	G	A	A	C	A	T	G	A	T	G
CMW 3276 *C. virescens*	C	T	C	A	C	C	A	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	**C**	A	A	G	A	A	C	A	T	G	A	T	G

β-tubulin

Isolate								620										630										640								
CMW 8242 *C. bhutanensis*	G	C	T	G	C	T	T	C	C	G	A	T	T	T	C	C	G	C	A	A	C	G	G	T	C	G	C	T	A	C	C	T	G	A	C	C
CMW 8217 *C. bhutanensis*	G	C	T	G	C	T	T	C	C	G	A	T	T	T	C	C	G	C	A	A	C	G	G	T	C	G	C	T	A	C	C	T	G	A	C	C
CMW 8215 *C. bhutanensis*	G	C	T	G	C	T	T	C	C	G	A	T	T	T	C	C	G	C	A	A	C	G	G	T	C	G	C	T	A	C	C	T	G	A	C	C
CMW 8399 *C. bhutanensis*	G	C	T	G	C	T	T	C	C	G	A	T	T	T	C	C	G	C	A	A	C	G	G	T	C	G	C	T	A	C	C	T	G	A	C	C
CMW 8394 *C. bhutanensis*	G	C	T	G	C	T	T	C	C	G	A	T	T	T	C	C	G	C	A	A	C	G	G	T	C	G	C	T	A	C	C	T	G	A	C	C
CMW 9590 *C. moniliformis*	G	C	T	G	C	T	T	C	C	G	A	T	T	T	C	C	G	C	A	A	C	G	G	T	C	G	C	T	A	C	C	T	G	A	C	C
CMW 4114 *C. moniliformis*	G	C	T	G	C	T	T	C	C	G	A	T	T	T	C	C	G	C	A	A	C	G	G	T	C	G	C	T	A	C	C	T	G	A	C	C
CMW 8240 *C. moniliformis*	G	C	T	G	C	T	T	C	C	G	A	T	T	T	C	C	G	C	A	A	C	G	G	T	C	G	C	T	A	C	C	T	G	A	C	C
CMW 9986 *C. moniliformopsis*	G	C	T	G	C	T	T	C	C	G	A	T	T	T	C	C	G	C	A	A	C	G	G	T	C	G	C	T	A	C	C	T	G	A	C	C
CMW 10214 *C. moniliformopsis*	G	C	T	G	C	T	T	C	C	G	A	T	T	T	C	C	G	C	A	A	C	G	G	T	C	G	C	T	A	C	C	T	G	A	C	C
CMW 3276 *C. virescens*	G	C	T	G	C	T	T	C	C	G	A	T	T	T	C	C	G	C	A	A	C	G	G	T	C	G	C	T	A	C	C	T	G	A	C	T

β-tubulin

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CMW 8242 *C. bhutanensis*	T	G	C	T	C	T	G	C	C	A	T	T	T	T	G	T	A	A	G	T	T	-	-	-	-	-	-	-	-	-	C	C	T	T	C	T
CMW 8217 *C. bhutanensis*	T	G	C	T	C	T	G	C	C	A	T	T	T	T	G	T	A	A	G	T	T	-	-	-	-	-	-	-	-	-	C	C	T	T	C	T
CMW 8215 *C. bhutanensis*	T	G	C	T	C	T	G	C	C	A	T	T	T	T	G	T	A	A	G	T	T	-	-	-	-	-	-	-	-	-	C	C	T	T	C	T
CMW 8399 *C. bhutanensis*	T	G	C	T	C	T	G	C	C	A	T	T	T	T	G	T	A	A	G	T	T	-	-	-	-	-	-	-	-	-	C	C	T	T	C	T
CMW 8394 *C. bhutanensis*	T	G	C	T	C	T	G	C	C	A	T	T	T	T	G	T	A	A	G	T	T	-	-	-	-	-	-	-	-	-	C	C	T	T	C	T
CMW 9590 *C. moniliformis*	T	G	C	T	C	T	G	C	C	A	T	C	T	T	G	T	A	A	G	T	T	A	T	-	-	-	-	-	-	-	-	-	-	-	C	T
CMW 4114 *C. moniliformis*	T	G	C	T	C	T	G	C	C	A	T	C	T	T	G	T	A	A	G	T	T	A	T	-	-	-	-	-	-	-	-	-	-	-	C	T
CMW 8240 *C. moniliformis*	T	G	C	T	C	T	G	C	C	A	T	C	T	T	G	T	A	A	G	T	T	A	T	-	-	-	-	-	-	-	-	-	-	-	C	T
CMW 9986 *C. moniliformopsis*	T	G	T	T	C	T	G	C	C	A	T	C	T	T	G	T	A	A	G	T	T	A	T	T	T	T	T	G	G	A	G	G	A	A	C	T
CMW 10214 *C. moniliformopsis*	T	G	T	T	C	T	G	C	C	A	T	C	T	T	G	T	A	A	G	T	T	A	T	T	T	T	T	G	G	A	G	G	A	A	C	T
CMW 3276 *C. virescens*	T	G	C	T	C	T	G	C	T	A	T	C	T	T	G	T	A	T	G	T	T	-	T	T	T	-	-	-	C	A	C	C	A	A	C	T

β-tubulin

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CMW 8242 *C. bhutanensis*	C	C	C	C	T	G	C	A	T	C	-	A	-	T	A	C	T	T	G	G	A	G	T	A	T	C	A	T	T	C	A	C	T	A	A	C
CMW 8217 *C. bhutanensis*	C	C	C	C	T	G	C	A	T	C	-	A	-	T	A	C	T	T	G	G	A	G	T	A	T	C	A	T	T	C	A	C	T	A	A	C
CMW 8215 *C. bhutanensis*	C	C	C	C	T	G	C	A	T	C	-	A	-	T	A	C	T	T	G	G	A	G	T	A	T	C	A	T	T	C	A	C	T	A	A	C
CMW 8399 *C. bhutanensis*	C	C	C	C	T	G	C	A	T	C	-	A	-	T	A	C	T	T	G	G	A	G	T	A	T	C	A	T	T	C	A	C	T	A	A	C
CMW 8394 *C. bhutanensis*	C	C	C	C	T	G	C	A	T	C	-	A	-	T	A	C	T	T	G	G	A	G	T	A	T	C	A	T	T	C	A	C	T	A	A	C
CMW 9590 *C. moniliformis*	T	T	T	T	C		T	T	T	G	T	A	G	T	A	T	T	T	G	G	A	G	C	A	T	G	A	T	T	C	A	C	T	A	A	C
CMW 4114 *C. moniliformis*	T	T	T	T	C	-	T	T	T	G	T	A	G	T	A	T	T	T	G	G	A	G	C	A	T	G	A	T	T	C	A	C	T	A	A	C
CMW 8240 *C. moniliformis*	T	T	T	T	C	-	T	T	T	G	T	A	G	T	A	T	T	T	G	G	A	G	C	A	T	G	A	T	T	C	A	C	T	A	A	C
CMW 9986 *C. moniliformopsis*	T	T	T	T	C	G	T	T	T	C	-	A	-	T	A	T	T	T	G	G	A	G	C	A	-	C	A	T	T	G	A	C	T	A	A	C
CMW 10214 *C. moniliformopsis*	T	T	T	T	C	G	T	T	T	C	-	A	-	T	A	T	T	T	G	G	A	G	C	A	-	C	A	T	T	G	A	C	T	A	A	C
CMW 3276 *C. virescens*	-	-	-	-	-	-	-	-	-	-	-	A	-	T	A	T	T	T	G	-	T	C	T	A	T	C	A	T	T	-	A	G	T	-	-	-

β-tubulin

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CMW 8242 *C. bhutanensis*	A	T	T	C	A	T	T	T	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	C	T
CMW 8217 *C. bhutanensis*	A	T	T	C	A	T	T	T	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	C	T
CMW 8215 *C. bhutanensis*	A	T	T	C	A	T	T	T	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	C	T
CMW 8399 *C. bhutanensis*	A	T	T	C	A	T	T	T	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	C	T
CMW 8394 *C. bhutanensis*	A	T	T	C	A	T	T	T	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	C	T
CMW 9590 *C. moniliformis*	A	T	-	-	A	T	T	T	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	C	T
CMW 4114 *C. moniliformis*	A	T	-	-	A	T	T	T	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	C	T
CMW 8240 *C. moniliformis*	A	T	-	-	A	T	T	T	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	C	T
CMW 9986 *C. moniliformopsis*	A	T	A	T	A	T	T	T	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	C	T
CMW 10214 *C. moniliformopsis*	A	T	A	T	A	T	T	T	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	C	T
CMW 3276 *C. virescens*	T	T	A	T	A	T	T	T	G	C	A	T	G	G	A	T	C	T	T	T	G	C	T	A	A	C	A	C	C	T	C	T	T	T	C	T

β-tubulin

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CMW 8242 *C. bhutanensis*	-	-	-	-	-	-	-	-	-	A	T	A	G	C	C	G	T	G	G	T	A	A	G	G	T	C	G	C	C	A	T	G	A	A	G	G
CMW 8217 *C. bhutanensis*	-	-	-	-	-	-	-	-	-	A	T	A	G	C	C	G	T	G	G	T	A	A	G	G	T	C	G	C	C	A	T	G	A	A	G	G
CMW 8215 *C. bhutanensis*	-	-	-	-	-	-	-	-	-	A	T	A	G	C	C	G	T	G	G	T	A	A	G	G	T	C	G	C	C	A	T	G	A	A	G	G
CMW 8399 *C. bhutanensis*	-	-	-	-	-	-	-	-	-	A	T	A	G	C	C	G	T	G	G	T	A	A	G	G	T	C	G	C	C	A	T	G	A	A	G	G
CMW 8394 *C. bhutanensis*	-	-	-	-	-	-	-	-	-	A	T	A	G	C	C	G	T	G	G	T	A	A	G	G	T	C	G	C	C	A	T	G	A	A	G	G
CMW 9590 *C. moniliformis*	-	-	-	-	-	**C**	**T**	-	-	A	T	A	G	C	C	G	T	G	G	T	A	A	G	G	T	C	G	C	C	A	T	G	A	A	G	G
CMW 4114 *C. moniliformis*	-	-	-	-	-	**C**	**T**	-	-	A	T	A	G	C	C	G	T	G	G	T	A	A	G	G	T	C	G	C	C	A	T	G	A	A	G	G
CMW 8240 *C. moniliformis*	-	-	-	-	-	**C**	**T**	-	-	A	T	A	G	C	C	G	T	G	G	T	A	A	G	G	T	C	G	C	C	A	T	G	A	A	G	G
CMW 9986 *C. moniliformopsis*	-	-	-	-	-	-	-	-	-	A	T	A	G	C	C	G	T	G	G	T	A	A	G	G	T	C	G	C	C	A	T	G	A	A	G	G
CMW 10214 *C. moniliformopsis*	-	-	-	-	-	-	-	-	-	A	T	A	G	C	C	G	T	G	G	T	A	A	G	G	T	C	G	C	C	A	T	G	A	A	G	G
CMW 3276 *C. virescens*	T	T	C	G	T	**C**	**T**	T	T	A	T	A	G	C	C	G	T	G	G	T	A	A	G	G	T	C	G	C	C	A	T	G	A	A	G	G

β-tubulin

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CMW 8242 *C. bhutanensis*	A	G	G	T	T	G	A	G	G	A	C	C	A	G	A	T	G	C	G	C	A	A	C	G	T	C	C	A	G	A	A	C	A	A	G	A
CMW 8217 *C. bhutanensis*	A	G	G	T	T	G	A	G	G	A	C	C	A	G	A	T	G	C	G	C	A	A	C	G	T	C	C	A	G	A	A	C	A	A	G	A
CMW 8215 *C. bhutanensis*	A	G	G	T	T	G	A	G	G	A	C	C	A	G	A	T	G	C	G	C	A	A	C	G	T	C	C	A	G	A	A	C	A	A	G	A
CMW 8399 *C. bhutanensis*	A	G	G	T	T	G	A	G	G	A	C	C	A	G	A	T	G	C	G	C	A	A	C	G	T	C	C	A	G	A	A	C	A	A	G	A
CMW 8394 *C. bhutanensis*	A	G	G	T	T	G	A	G	G	A	C	C	A	G	A	T	G	C	G	C	A	A	C	G	T	C	C	A	G	A	A	C	A	A	G	A
CMW 9590 *C. moniliformis*	A	G	G	T	T	G	A	G	G	A	C	C	A	G	A	T	G	C	G	C	A	A	C	G	T	C	C	A	G	A	A	C	A	A	G	A
CMW 4114 *C. moniliformis*	A	G	G	T	T	G	A	G	G	A	C	C	A	G	A	T	G	C	G	C	A	A	C	G	T	C	C	A	G	A	A	C	A	A	G	A
CMW 8240 *C. moniliformis*	A	G	G	T	T	G	A	G	G	A	C	C	A	G	A	T	G	C	G	C	A	A	C	G	T	C	C	A	G	A	A	C	A	A	G	A
CMW 9986 *C. moniliformopsis*	A	G	G	T	T	G	A	G	G	A	C	C	A	G	A	T	G	C	G	C	A	A	C	G	T	C	C	A	G	A	A	C	A	A	G	A
CMW 10214 *C. moniliformopsis*	A	G	G	T	T	G	A	G	G	A	C	C	A	G	A	T	G	C	G	C	A	A	C	G	T	C	C	A	G	A	A	C	A	A	G	A
CMW 3276 *C. virescens*	A	G	G	T	T	G	A	G	G	A	C	C	A	G	A	T	G	C	G	C	A	A	C	G	T	C	C	A	G	A	A	C	A	A	G	A

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CMW 8242 *C. bhutanensis*	A	C	T	C	C	T	C	T	T	A	C	T	T	C	G	T	T	G	A	G	T	G	G	A	T	**C**	C	C	C	A	A	C	A	A	C	G
CMW 8217 *C. bhutanensis*	A	C	T	C	C	T	C	T	T	A	C	T	T	C	G	T	T	G	A	G	T	G	G	A	T	**C**	C	C	C	A	A	C	A	A	C	G
CMW 8215 *C. bhutanensis*	A	C	T	C	C	T	C	T	T	A	C	T	T	C	G	T	T	G	A	G	T	G	G	A	T	**C**	C	C	C	A	A	C	A	A	C	G
CMW 8399 *C. bhutanensis*	A	C	T	C	C	T	C	T	T	A	C	T	T	C	G	T	T	G	A	G	T	G	G	A	T	**C**	C	C	C	A	A	C	A	A	C	G
CMW 8394 *C. bhutanensis*	A	C	T	C	C	T	C	T	T	A	C	T	T	C	G	T	T	G	A	G	T	G	G	A	T	**T**	C	C	C	A	A	C	A	A	C	G
CMW 9590 *C. moniliformis*	A	C	T	C	C	T	C	T	T	A	C	T	T	C	G	T	T	G	A	G	T	G	G	A	T	**C**	C	C	C	A	A	C	A	A	C	G
CMW 4114 *C. moniliformis*	A	C	T	C	C	T	C	T	T	A	C	T	T	C	G	T	T	G	A	G	T	G	G	A	T	**T**	C	C	C	A	A	C	A	A	C	G
CMW 8240 *C. moniliformis*	A	C	T	C	C	T	C	T	T	A	C	T	T	C	G	T	T	G	A	G	T	G	G	A	T	**T**	C	C	C	A	A	C	A	A	C	G
CMW 9986 *C. moniliformopsis*	A	C	T	C	C	T	C	T	T	A	C	T	T	C	G	T	T	G	A	G	T	G	G	A	T	**C**	C	C	C	A	A	C	A	A	C	G
CMW 10214 *C. moniliformopsis*	A	C	T	C	C	T	C	T	T	A	C	T	T	C	G	T	T	G	A	G	T	G	G	A	T	**C**	C	C	C	A	A	C	A	A	C	G
CMW 3276 *C. virescens*	A	C	T	C	C	T	C	T	T	A	C	T	T	T	G	T	T	G	A	G	T	G	G	A	T	**C**	C	C	C	A	A	C	A	A	C	G

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	|	.	.	.	.	870	.	.	.	.	|	.	.	.	.	880	.	.	.	.	|	.	.	.	.	890	.	.	.	.	|	.	.	.	.	900
CMW 8242 *C. bhutanensis*	T	C	C	A	G	A	C	C	G	C	**C**	C	T	**G**	T	G	C	T	C	C	A	T	T	C	C	T	C	C	C	C	G	T	G	G	C	C
CMW 8217 *C. bhutanensis*	T	C	C	A	G	A	C	C	G	C	**C**	C	T	**G**	T	G	C	T	C	C	A	T	T	C	C	T	C	C	C	C	G	T	G	G	C	C
CMW 8215 *C. bhutanensis*	T	C	C	A	G	A	C	C	G	C	**C**	C	T	**G**	T	G	C	T	C	C	A	T	T	C	C	T	C	C	C	C	G	T	G	G	C	C
CMW 8399 *C. bhutanensis*	T	C	C	A	G	A	C	C	G	C	**C**	C	T	**G**	T	G	C	T	C	C	A	T	T	C	C	T	C	C	C	C	G	T	G	G	C	C
CMW 8394 *C. bhutanensis*	T	C	C	A	G	A	C	C	G	C	**C**	C	T	**G**	T	G	C	T	C	C	A	T	T	C	C	T	C	C	C	C	G	T	G	G	C	C
CMW 9590 *C. moniliformis*	T	C	C	A	G	A	C	C	G	C	**T**	C	T	**T**	T	G	C	T	C	C	A	T	T	C	C	T	C	C	C	C	G	T	G	G	C	C
CMW 4114 *C. moniliformis*	T	C	C	A	G	A	C	C	G	C	**T**	C	T	**T**	T	G	C	T	C	C	A	T	T	C	C	T	C	C	C	C	G	T	G	G	C	C
CMW 8240 *C. moniliformis*	T	C	C	A	G	A	C	C	G	C	**T**	C	T	**T**	T	G	C	T	C	C	A	T	T	C	C	T	C	C	C	C	G	T	G	G	C	C
CMW 9986 *C. moniliformopsis*	T	C	C	A	G	A	C	C	G	C	**T**	C	T	**C**	T	G	C	T	C	C	A	T	T	C	C	T	C	C	C	C	G	T	G	G	C	C
CMW 10214 *C. moniliformopsis*	T	C	C	A	G	A	C	C	G	C	**T**	C	T	**C**	T	G	C	T	C	C	A	T	T	C	C	T	C	C	C	C	G	T	G	G	C	C
CMW 3276 *C. virescens*	T	C	C	A	G	A	C	T	G	C	**C**	C	T	**G**	T	G	C	T	C	C	A	T	T	C	C	T	C	C	C	C	G	T	G	G	C	C

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CMW 8242 *C. bhutanensis*	T	C	A	A	G	A	T	G	T	C	T	T	C	**G**	A	C	**C**	T	T	C	G	T	**T**	G	G	T	A	A	C	T	C	T	A	C	T	G
CMW 8217 *C. bhutanensis*	T	C	A	A	G	A	T	G	T	C	T	T	C	**G**	A	C	**A**	T	T	C	G	T	**T**	G	G	T	A	A	C	T	C	T	A	C	T	G
CMW 8215 *C. bhutanensis*	T	C	A	A	G	A	T	G	T	C	T	T	C	**G**	A	C	**C**	T	T	C	G	T	**T**	G	G	T	A	A	C	T	C	T	A	C	T	G
CMW 8399 *C. bhutanensis*	T	C	A	A	G	A	T	G	T	C	T	T	C	**G**	A	C	**C**	T	T	C	G	T	**T**	G	G	T	A	A	C	T	C	T	A	C	T	G
CMW 8394 *C. bhutanensis*	T	C	A	A	G	A	T	G	T	C	T	T	C	**G**	A	C	**C**	T	T	C	G	T	**T**	G	G	T	A	A	C	T	C	T	A	C	T	G
CMW 9590 *C. moniliformis*	T	C	A	A	G	A	T	G	T	C	T	T	C	**G**	A	C	**C**	T	T	C	G	T	**C**	G	G	T	A	A	C	T	C	T	A	C	T	G
CMW 4114 *C. moniliformis*	T	C	A	A	G	A	T	G	T	C	T	T	C	**G**	A	C	**C**	T	T	C	G	T	**C**	G	G	T	A	A	C	T	C	T	A	C	T	G
CMW 8240 *C. moniliformis*	T	C	A	A	G	A	T	G	T	C	T	T	C	**G**	A	C	**C**	T	T	C	G	T	**C**	G	G	T	A	A	C	T	C	T	A	C	T	G
CMW 9986 *C. moniliformopsis*	T	C	A	A	G	A	T	G	T	C	T	T	C	**C**	A	C	**C**	T	T	C	G	T	**C**	G	G	T	A	A	C	T	C	T	A	C	T	G
CMW 10214 *C. moniliformopsis*	T	C	A	A	G	A	T	G	T	C	T	T	C	**C**	A	C	**C**	T	T	C	G	T	**C**	G	G	T	A	A	C	T	C	T	A	C	T	G
CMW 3276 *C. virescens*	T	C	A	A	G	A	T	G	T	C	G	T	C	**T**	A	C	**C**	T	T	C	G	T	**C**	G	G	T	A	A	C	T	C	G	A	C	T	G

β-tubulin

	.	.	.	940	.	.	.	.	|	.	.	.	.	950	.	.	.	.	|	.	.	.	.	960	.	.	.	.	|	.	.	.	.	970	.	.
CMW 8242 *C. bhutanensis*	C	**T**	A	T	C	C	A	G	G	A	G	C	T	**G**	T	T	C	A	A	G	C	G	**C**	A	T	T	G	G	**T**	G	A	G	C	A	G	T
CMW 8217 *C. bhutanensis*	C	**T**	A	T	C	C	A	G	G	A	G	C	T	**G**	T	T	C	A	A	G	C	G	**C**	A	T	T	G	G	**T**	G	A	G	C	A	G	T
CMW 8215 *C. bhutanensis*	C	**T**	A	T	C	C	A	G	G	A	G	C	T	**G**	T	T	C	A	A	G	C	G	**C**	A	T	T	G	G	**T**	G	A	G	C	A	G	T
CMW 8399 *C. bhutanensis*	C	**C**	A	T	C	C	A	G	G	A	G	C	T	**G**	T	T	C	A	A	G	C	G	**C**	A	T	T	G	G	**T**	G	A	G	C	A	G	T
CMW 8394 *C. bhutanensis*	C	**C**	A	T	C	C	A	G	G	A	G	C	T	**G**	T	T	C	A	A	G	C	G	**C**	A	T	T	G	G	**T**	G	A	G	C	A	G	T
CMW 9590 *C. moniliformis*	C	**C**	A	T	C	C	A	G	G	A	G	C	T	**C**	T	T	C	A	A	G	C	G	**T**	A	T	T	G	G	**C**	G	A	G	C	A	G	T
CMW 4114 *C. moniliformis*	C	**C**	A	T	C	C	A	G	G	A	G	C	T	**C**	T	T	C	A	A	G	C	G	**T**	A	T	T	G	G	**C**	G	A	G	C	A	G	T
CMW 8240 *C. moniliformis*	C	**C**	A	T	C	C	A	G	G	A	G	C	T	**C**	T	T	C	A	A	G	C	G	**T**	A	T	T	G	G	**C**	G	A	G	C	A	G	T
CMW 9986 *C. moniliformopsis*	C	**T**	A	T	C	C	A	G	G	A	G	C	T	**C**	T	T	C	A	A	G	C	G	**C**	A	T	T	G	G	**T**	G	A	G	C	A	G	T
CMW 10214 *C. moniliformopsis*	C	**T**	A	T	C	C	A	G	G	A	G	C	T	**C**	T	T	C	A	A	G	C	G	**C**	A	T	T	G	G	**T**	G	A	G	C	A	G	T
CMW 3276 *C. virescens*	C	**C**	A	T	C	C	A	G	G	A	G	C	T	**G**	T	T	C	A	A	G	C	G	**T**	A	T	T	G	G	**C**	G	A	G	C	A	G	T

β-tubulin

								980										990										1000								
CMW 8242 *C. bhutanensis*	T	C	A	C	T	G	C	C	A	T	G	T	T	C	C	G	T	C	G	C	A	A	G	G	C	T	T	T	C	T	T	G	C	A	T	T
CMW 8217 *C. bhutanensis*	T	C	A	C	T	G	C	C	A	T	G	T	T	C	C	G	T	C	G	C	A	A	G	G	C	T	T	T	C	T	T	G	C	A	T	T
CMW 8215 *C. bhutanensis*	T	C	A	C	T	G	C	C	A	T	G	T	T	C	C	G	T	C	G	C	A	A	G	G	C	T	T	T	C	T	T	G	C	A	T	T
CMW 8399 *C. bhutanensis*	T	C	A	C	T	G	C	C	A	T	G	T	T	C	C	G	T	C	G	C	A	A	G	G	C	T	T	T	C	T	T	G	C	A	T	T
CMW 8394 *C. bhutanensis*	T	C	A	C	T	G	C	C	A	T	G	T	T	C	C	G	T	C	G	C	A	A	G	G	C	T	T	T	C	T	T	G	C	A	T	T
CMW 9590 *C. moniliformis*	T	C	A	C	T	G	C	C	A	T	G	T	T	C	C	G	T	C	G	C	A	A	G	G	C	T	T	T	C	T	T	G	C	A	T	T
CMW 4114 *C. moniliformis*	T	C	A	C	T	G	C	C	A	T	G	T	T	C	C	G	T	C	G	C	A	A	G	G	C	T	T	T	C	T	T	G	C	A	T	T
CMW 8240 *C. moniliformis*	T	C	A	C	T	G	C	C	A	T	G	T	T	C	C	G	T	C	G	C	A	A	G	G	C	T	T	T	C	T	T	G	C	A	T	T
CMW 9986 *C. moniliformopsis*	T	C	A	C	T	G	C	C	A	T	G	T	T	C	C	G	T	C	G	C	A	A	G	G	C	T	T	T	C	T	T	G	C	A	T	T
CMW 10214 *C. moniliformopsis*	T	C	A	C	T	G	C	C	A	T	G	T	T	C	C	G	T	C	G	C	A	A	G	G	C	T	T	T	C	T	T	G	C	A	T	T
CMW 3276 *C. virescens*	T	C	A	C	T	G	C	T	A	T	G	T	T	C	C	G	T	C	G	C	A	A	G	G	C	T	T	T	C	T	T	G	C	A	T	T

β-tubulin

		1010										1020										1030										1040				
CMW 8242 *C. bhutanensis*	G	G	T	A	C	A	C	T	A	A	C	G	A	G	G	G	T	A	T	G	G	A	C	G	A	G	A	T	G	G	A	G	T	T	C	A
CMW 8217 *C. bhutanensis*	G	G	T	A	C	A	C	T	A	A	C	G	A	G	G	G	T	A	T	G	G	A	C	G	A	G	A	T	G	G	A	G	T	T	C	A
CMW 8215 *C. bhutanensis*	G	G	T	A	C	A	C	T	A	A	C	G	A	G	G	G	T	A	T	G	G	A	C	G	A	G	A	T	G	G	A	G	T	T	C	A
CMW 8399 *C. bhutanensis*	G	G	T	A	C	A	C	T	A	A	C	G	A	G	G	G	T	A	T	G	G	A	C	G	A	G	A	T	G	G	A	G	T	T	C	A
CMW 8394 *C. bhutanensis*	G	G	T	A	C	A	C	T	A	A	C	G	A	G	G	G	T	A	T	G	G	A	C	G	A	G	A	T	G	G	A	G	T	T	C	A
CMW 9590 *C. moniliformis*	G	G	T	A	C	A	C	T	A	A	C	G	A	G	G	G	T	A	T	G	G	A	C	G	A	G	A	T	G	G	A	G	T	T	C	A
CMW 4114 *C. moniliformis*	G	G	T	A	C	A	C	T	A	A	C	G	A	G	G	G	T	A	T	G	G	A	C	G	A	G	A	T	G	G	A	G	T	T	C	A
CMW 8240 *C. moniliformis*	G	G	T	A	C	A	C	T	A	A	C	G	A	G	G	G	T	A	T	G	G	A	C	G	A	G	A	T	G	G	A	G	T	T	C	A
CMW 9986 *C. moniliformopsis*	G	G	T	A	C	A	C	T	A	A	C	G	A	G	G	G	T	A	T	G	G	A	C	G	A	G	A	T	G	G	A	G	T	T	C	A
CMW 10214 *C. moniliformopsis*	G	G	T	A	C	A	C	T	A	A	C	G	A	G	G	G	T	A	T	G	G	A	C	G	A	G	A	T	G	G	A	G	T	T	C	A
CMW 3276 *C. virescens*	G	G	T	A	C	A	C	T	G	G	T	G	A	G	G	G	T	A	T	G	G	A	C	G	A	G	A	T	G	G	A	G	T	T	C	A

β-tubulin

EF1-α

						1050										1060										1070										1080
CMW 8242 *C. bhutanensis*	C	T	G	A	G	G	C	G	A	G	A	A	G	T	T	C	G	A	G	A	A	T	A	A	G	T	C	T	-	C	C	C	C	A	A	A
CMW 8217 *C. bhutanensis*	C	T	G	A	G	G	C	G	A	G	A	A	G	T	T	C	G	A	G	A	A	T	A	A	G	T	C	T	-	C	C	C	C	A	A	A
CMW 8215 *C. bhutanensis*	C	T	G	A	G	G	C	G	A	G	A	A	G	T	T	C	G	A	G	A	A	T	A	A	G	T	C	T	-	C	C	C	C	A	A	A
CMW 8399 *C. bhutanensis*	C	T	G	A	G	G	C	G	A	G	A	A	G	T	T	C	G	A	G	A	A	T	A	A	G	T	C	T	-	C	C	C	C	A	A	A
CMW 8394 *C. bhutanensis*	C	T	G	A	G	G	C	G	A	G	A	A	G	T	T	C	G	A	G	A	A	T	A	A	G	T	C	T	-	C	C	C	C	A	A	A
CMW 9590 *C. moniliformis*	C	T	G	A	G	G	C	G	A	G	A	A	G	T	T	C	G	A	G	A	A	T	A	A	G	T	C	T	**A**	C	C	C	C	A	A	A
CMW 4114 *C. moniliformis*	C	T	G	A	G	G	C	G	A	G	A	A	G	T	T	C	G	A	G	A	A	T	A	A	G	T	C	T	**A**	C	C	C	C	A	A	A
CMW 8240 *C. moniliformis*	C	T	G	A	G	G	C	G	A	G	A	A	G	T	T	C	G	A	G	A	A	T	A	A	G	T	C	T	**A**	C	C	C	C	A	A	A
CMW 9986 *C. moniliformopsis*	C	T	G	A	G	G	C	G	A	G	A	A	G	T	T	C	G	A	G	A	A	T	A	A	G	T	C	T	-	C	C	C	C	A	A	A
CMW 10214 *C. moniliformopsis*	C	T	G	A	G	G	C	G	A	G	A	A	G	T	T	C	G	A	G	A	A	T	A	A	G	T	C	T	-	C	C	C	C	A	A	A
CMW 3276 *C. virescens*	C	T	G	A	G	G	C	G	A	G	A	A	G	T	T	C	G	A	G	A	A	T	A	A	G	T	C	T	**C**	C	C	C	C	A	T	C

EF1-α

									1090										1100										1110						
CMW 8242 *C. bhutanensis*	A	C	C	-	A	A	T	C	C	C	G	C	G	A	C	A	T	C	A	A	T	C	G	A	A	C	C	G	A	A	C	A	A	G	A	T
CMW 8217 *C. bhutanensis*	A	C	C	-	A	A	T	C	C	C	G	C	G	A	C	A	T	C	A	A	T	C	G	A	A	C	C	G	A	A	C	A	A	G	A	T
CMW 8215 *C. bhutanensis*	A	C	C	-	A	A	T	C	C	C	G	C	G	A	C	A	T	C	A	A	T	C	G	A	A	C	C	G	A	A	C	A	A	G	A	T
CMW 8399 *C. bhutanensis*	A	C	C	-	A	A	T	C	C	C	G	C	G	A	C	A	T	C	A	A	T	C	G	A	A	C	C	G	A	A	C	A	A	G	A	T
CMW 8394 *C. bhutanensis*	A	C	C	-	A	A	T	C	C	C	G	C	G	A	C	A	T	C	A	A	T	C	G	A	A	C	C	G	A	A	C	A	A	G	A	T
CMW 9590 *C. moniliformis*	A	C	C	-	A	A	A	C	C	C	G	C	G	A	T	A	C	C	A	G	C	C	G	A	A	C	T	G	A	A	C	A	A	G	A	T
CMW 4114 *C. moniliformis*	A	C	C	-	A	A	A	C	C	C	G	C	G	A	T	A	C	C	A	G	C	C	G	A	A	C	T	G	A	A	C	A	A	G	A	T
CMW 8240 *C. moniliformis*	A	C	C	-	A	A	A	C	C	C	G	C	G	A	T	A	C	C	A	G	C	C	G	A	A	C	C	G	A	A	C	A	A	G	A	T
CMW 9986 *C. moniliformopsis*	A	A	C	-	A	A	C	A	C	C	G	C	G	A	C	A	C	C	A	A	T	C	G	A	A	T	C	A	A	C	C	A	A	G	A	T
CMW 10214 *C. moniliformopsis*	A	A	C	-	A	A	C	A	C	C	G	C	G	A	C	A	C	C	A	A	T	C	G	A	A	T	C	A	A	C	C	A	A	G	A	T
CMW 3276 *C. virescens*	C	A	G	T	C	T	T	A	C	A	T	A	T	T	A	T	T	G	A	T	A	T	C	A	T	A	T	C	C	C	T	C	C	G	T	T

EF1-α

				1120										1130										1140										1150		
CMW 8242 *C. bhutanensis*	A	C	C	G	A	T	A	G	G	A	T	G	T	G	T	T	G	C	T	A	G	T	G	A	T	G	C	G	T	T	T	T	C	T	A	C
CMW 8217 *C. bhutanensis*	A	C	C	G	A	T	A	G	G	A	T	G	T	G	T	T	G	C	T	A	G	T	G	A	T	G	C	G	T	T	T	T	C	T	A	C
CMW 8215 *C. bhutanensis*	A	C	C	G	A	T	A	G	G	A	T	G	T	G	T	T	G	C	T	A	G	T	G	A	T	G	C	G	T	T	T	T	C	T	A	C
CMW 8399 *C. bhutanensis*	A	C	C	G	A	T	A	G	G	A	T	G	T	G	T	T	G	C	T	A	G	T	G	A	T	G	C	G	T	T	T	T	C	T	A	C
CMW 8394 *C. bhutanensis*	A	C	C	G	A	T	A	G	G	A	T	G	T	G	T	T	G	C	T	A	G	T	G	A	T	G	C	G	T	T	T	T	C	T	A	C
CMW 9590 *C. moniliformis*	A	C	C	G	A	G	A	G	G	A	T	A	A	G	T	T	G	C	T	A	G	C	G	G	T	G	T	G	T	T	T	T	C	-	A	C
CMW 4114 *C. moniliformis*	A	C	C	G	A	G	A	G	G	A	T	A	A	G	T	T	G	C	T	A	G	C	G	G	T	G	T	G	T	T	T	T	C	-	A	C
CMW 8240 *C. moniliformis*	A	C	C	G	A	G	A	G	G	A	T	A	A	G	T	T	G	C	T	A	G	C	G	G	T	G	T	G	T	T	T	T	C	-	A	C
CMW 9986 *C. moniliformopsis*	A	C	A	G	A	T	C	G	G	A	T	G	A	G	T	T	G	C	T	G	G	T	C	A	T	G	C	G	T	T	T	T	C	C	A	C
CMW 10214 *C. moniliformopsis*	A	C	A	G	A	T	C	G	G	A	T	G	A	G	T	T	G	C	T	G	G	T	C	A	T	G	C	G	T	T	T	T	C	C	A	C
CMW 3276 *C. virescens*	A	C	G	A	G	C	T	T	C	A	A	T	C	A	A	T	A	A	T	G	A	A	A	A	C	T	T	G	A	C	T	G	T	T	C	A

EF1-α

								1160										1170										1180								
CMW 8242 *C. bhutanensis*	G	C	A	G	C	G	T	T	C	A	G	T	C	T	T	G	T	A	T	A	A	A	G	G	T	T	G	A	G	T	T	G	A	T	T	T
CMW 8217 *C. bhutanensis*	G	C	A	G	C	G	T	T	C	A	G	T	C	T	T	G	T	A	T	A	A	A	G	G	T	T	G	A	G	T	T	G	A	T	T	T
CMW 8215 *C. bhutanensis*	G	C	A	G	C	G	T	T	C	A	G	T	C	T	T	G	T	A	T	A	A	A	G	G	T	T	G	A	G	T	T	G	A	T	T	T
CMW 8399 *C. bhutanensis*	G	C	A	G	C	G	T	T	C	A	G	T	C	T	T	G	T	A	T	A	A	A	G	G	T	T	G	A	G	T	T	G	A	T	T	T
CMW 8394 *C. bhutanensis*	G	C	A	G	C	G	T	T	C	A	G	T	C	T	T	G	T	A	T	A	A	A	G	G	T	T	G	A	G	T	T	G	A	T	T	T
CMW 9590 *C. moniliformis*	G	C	A	G	C	G	T	T	C	A	G	T	C	T	T	G	T	A	T	G	G	A	G	G	T	T	G	A	G	T	T	G	T	T	T	T
CMW 4114 *C. moniliformis*	G	C	A	G	C	G	T	T	C	A	G	T	C	T	T	G	T	A	T	G	G	A	G	G	T	T	G	A	G	T	T	G	T	T	T	T
CMW 8240 *C. moniliformis*	G	C	A	G	C	G	T	T	C	A	G	T	C	T	T	G	T	A	T	A	G	A	G	G	T	T	G	A	G	T	T	G	T	T	T	T
CMW 9986 *C. moniliformopsis*	G	C	A	G	C	G	T	T	T	A	G	C	C	T	T	G	C	A	A	A	G	A	G	G	T	C	G	A	G	T	T	G	T	T	T	T
CMW 10214 *C. moniliformopsis*	G	C	A	G	C	G	T	T	T	A	G	C	C	T	T	G	C	A	A	A	G	A	G	G	T	C	G	A	G	T	T	G	T	T	T	T
CMW 3276 *C. virescens*	C	C	G	G	T	T	T	C	G	A	A	T	T	C	G	T	T	G	T	T	G	G	A	T	C	T	G	A	G	T	C	T	T	G	T	G

EF1-α

Isolate		1190									1200										1210										1220				
CMW 8242 *C. bhutanensis*	T	**C**	A	T	G	A	G	G	C	A	**G**	**C**	**C**	C	T	**A**	C	C	C	-	C	G	C	C	-	T	G	G	C	G	G	G	G	T	A	G
CMW 8217 *C. bhutanensis*	T	**C**	A	T	G	A	G	G	C	A	**G**	**C**	**C**	C	T	**A**	C	C	C	-	C	G	C	C	-	T	G	G	C	G	G	G	G	T	A	G
CMW 8215 *C. bhutanensis*	T	**C**	A	T	G	A	G	G	C	A	**G**	**C**	**C**	C	T	**A**	C	C	C	-	C	G	C	C	-	T	G	G	C	G	G	G	G	T	A	G
CMW 8399 *C. bhutanensis*	T	**C**	A	T	G	A	G	G	C	A	**G**	**C**	**C**	C	T	**A**	C	C	C	-	C	G	C	C	-	T	G	G	C	G	G	G	G	T	A	G
CMW 8394 *C. bhutanensis*	T	**C**	A	T	G	A	G	G	C	A	**G**	**C**	**C**	C	T	**A**	C	C	C	-	C	G	C	C	-	T	G	G	C	G	G	G	G	T	A	G
CMW 9590 *C. moniliformis*	T	**G**	A	T	G	A	G	G	C	A	**A**	**T**	**A**	C	T	**A**	C	C	C	**A**	C	G	C	C	-	T	G	G	C	G	G	G	G	T	A	G
CMW 4114 *C. moniliformis*	T	**G**	A	T	G	A	G	G	C	A	**A**	**T**	**A**	C	T	**A**	C	C	C	**A**	C	G	C	C	-	T	G	G	C	G	G	G	G	T	A	G
CMW 8240 *C. moniliformis*	T	**G**	A	T	G	A	G	G	C	A	**A**	**T**	**A**	C	T	**A**	C	C	C	**A**	C	G	C	C	-	T	G	G	C	G	G	G	G	T	A	G
CMW 9986 *C. moniliformopsis*	T	**C**	A	T	G	A	G	G	C	A	**A**	**C**	**A**	C	T	**G**	C	C	C	-	C	G	C	C	-	T	G	G	C	G	G	G	G	T	A	G
CMW 10214 *C. moniliformopsis*	T	**C**	A	T	G	A	G	G	C	A	**A**	**C**	**A**	C	T	**G**	C	C	C	-	C	G	C	C	-	T	G	G	C	G	G	G	G	T	A	G
CMW 3276 *C. virescens*	T	**C**	C	C	T	A	C	C	C	A	**C**	**C**	**T**	G	A	**A**	C	A	G	**C**	C	A	A	T	C	T	C	A	A	T	A	A	A	A	T	C

EF1-α

Isolate						1230										1240										1250										1260
CMW 8242 *C. bhutanensis*	T	**G**	T	T	G	T	G	T	T	**G**	T	G	**A**	A	A	A	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**A**
CMW 8217 *C. bhutanensis*	T	**G**	T	T	G	T	G	T	T	**G**	T	G	**A**	A	A	A	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**A**
CMW 8215 *C. bhutanensis*	T	**G**	T	T	G	T	G	T	T	**G**	T	G	**A**	A	A	A	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**A**
CMW 8399 *C. bhutanensis*	T	**G**	T	T	G	T	G	T	T	**G**	T	G	**A**	A	A	A	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**A**
CMW 8394 *C. bhutanensis*	T	**G**	T	T	G	T	G	T	T	**G**	T	G	**A**	A	A	A	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**A**
CMW 9590 *C. moniliformis*	T	**T**	T	T	G	T	G	T	T	**A**	T	G	**A**	A	A	A	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**A**
CMW 4114 *C. moniliformis*	T	**T**	T	T	G	T	G	T	T	**A**	T	G	**A**	A	A	A	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**
CMW 8240 *C. moniliformis*	T	**T**	T	T	G	T	G	T	T	**A**	T	G	**A**	A	A	A	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**
CMW 9986 *C. moniliformopsis*	T	**T**	T	T	G	T	G	T	T	**G**	T	G	**G**	A	A	A	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**
CMW 10214 *C. moniliformopsis*	T	**T**	T	T	G	T	G	T	T	**G**	T	G	**G**	A	A	A	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**
CMW 3276 *C. virescens*	C	**C**	A	T	G	C	T	T	G	**T**	T	C	**G**	A	A	C	C	C	C	A	C	T	A	G	C	C	T	A	G	G	A	T	T	G	A	**A**

EF1-α

Isolate										1270										1280										1290						
CMW 8242 *C. bhutanensis*	G	G	A	A	G	**A**	**C**	A	T	A	C	A	A	G	G	G	T	**A**	**T**	**A**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**C**	T	T
CMW 8217 *C. bhutanensis*	G	G	A	A	G	**A**	**C**	A	T	A	C	A	A	G	G	G	T	**A**	**T**	**A**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**C**	T	T
CMW 8215 *C. bhutanensis*	G	G	A	A	G	**A**	**C**	A	T	A	C	A	A	G	G	G	T	**A**	**T**	**A**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**T**	T	T
CMW 8399 *C. bhutanensis*	G	G	A	A	G	**A**	**C**	A	T	A	C	A	A	G	G	G	T	**A**	**T**	**A**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**T**	T	T
CMW 8394 *C. bhutanensis*	G	G	A	A	G	**A**	**C**	A	T	A	C	A	A	G	G	G	T	**A**	**T**	**A**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**C**	T	T
CMW 9590 *C. moniliformis*	G	G	A	A	G	**T**	**C**	A	T	A	C	A	A	G	G	G	T	**A**	**C**	**A**	A	T	T	A	C	C	C	C	G	C	T	**A**	T	**T**	T	T
CMW 4114 *C. moniliformis*	G	G	A	A	G	**T**	**C**	A	T	A	C	A	A	G	G	G	T	**A**	**C**	**A**	A	T	T	A	C	C	C	C	G	C	T	**A**	T	**T**	T	T
CMW 8240 *C. moniliformis*	G	G	A	A	G	**T**	**C**	A	T	A	C	A	A	G	G	G	T	**A**	**C**	**A**	A	T	T	A	C	C	C	C	G	C	T	**A**	T	**T**	T	T
CMW 9986 *C. moniliformopsis*	G	G	A	A	G	**C**	**T**	A	T	A	C	A	A	G	G	G	T	**G**	**T**	**T**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**T**	T	T
CMW 10214 *C. moniliformopsis*	G	G	A	A	G	**C**	**T**	A	T	A	C	A	A	G	G	G	T	**G**	**T**	**T**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**T**	T	T
CMW 3276 *C. virescens*	C	C	C	C	T	**G**	G	C	A	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

EF1-α

				1300										1310										1320										1330		
CMW 8242 *C. bhutanensis*	G	T	A	C	C	C	T	G	T	A	C	A	A	G	G	A	C	C	C	G	C	T	G	T	G	T	G	G	G	A	A	G	G	T	A	C
CMW 8217 *C. bhutanensis*	G	T	A	C	C	C	T	G	T	A	C	A	A	G	G	A	C	C	C	G	C	T	G	T	G	T	G	G	G	A	A	G	G	T	A	C
CMW 8215 *C. bhutanensis*	G	T	A	C	C	C	T	G	T	A	C	A	A	G	G	A	C	C	C	G	C	T	G	T	G	T	G	G	G	A	A	G	G	T	A	C
CMW 8399 *C. bhutanensis*	G	T	A	C	C	C	T	G	T	A	C	A	A	G	G	A	C	C	C	G	C	T	G	T	G	T	G	G	G	A	A	G	G	T	A	C
CMW 8394 *C. bhutanensis*	G	T	A	C	C	C	T	G	T	A	C	A	A	G	G	A	C	C	C	G	C	T	G	T	G	T	G	G	G	A	A	G	G	T	A	C
CMW 9590 *C. moniliformis*	G	C	C	C	C	C	T	G	T	A	C	A	A	A	G	A	C	C	C	G	T	T	T	T	G	T	G	G	G	A	A	G	G	A	A	C
CMW 4114 *C. moniliformis*	G	C	C	C	C	C	T	G	T	A	C	A	A	A	G	A	C	C	C	G	T	T	T	T	G	T	G	G	G	A	A	G	G	A	A	C
CMW 8240 *C. moniliformis*	G	C	C	C	C	C	T	G	T	A	C	A	A	A	G	A	C	C	C	G	T	T	T	T	G	T	G	G	G	A	A	G	G	A	A	C
CMW 9986 *C. moniliformopsis*	G	T	C	C	C	C	T	G	T	A	C	A	A	G	G	A	C	C	C	G	C	T	G	T	G	T	G	A	A	A	G	G	G	C	A	C
CMW 10214 *C. moniliformopsis*	G	T	C	C	C	C	T	G	T	A	C	A	A	G	G	A	C	C	C	G	C	T	G	T	G	T	G	A	A	A	G	G	G	C	A	C
CMW 3276 *C. virescens*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	C	C	T	C	T	T	A	C	A	C	A	A	C	A	A	A	C	C

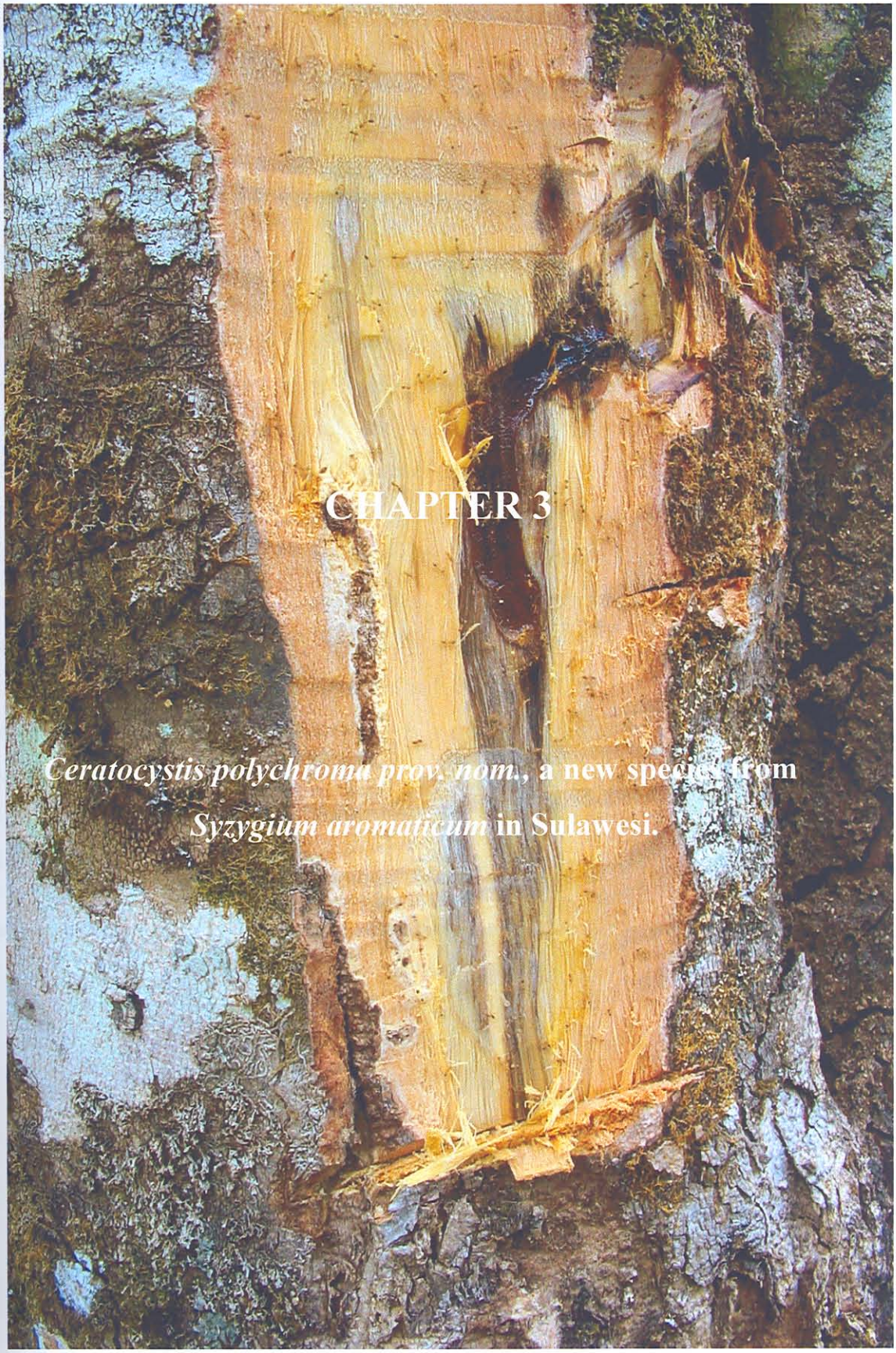
EF1-α

								1340										1350										1360								
CMW 8242 *C. bhutanensis*	C	C	T	G	G	T	C	A	C	A	C	A	C	A	T	C	C	A	G	T		-		C	A	T	G	C	A	T	G	C	T	C	T	T
CMW 8217 *C. bhutanensis*	C	C	T	G	G	T	C	A	C	A	C	A	C	A	T	C	C	A	G	T		-		C	A	T	G	C	A	T	G	C	T	C	T	T
CMW 8215 *C. bhutanensis*	C	C	T	G	G	T	C	A	C	A	C	A	C	A	T	C	C	A	G	T		-		C	A	T	G	C	A	T	G	C	T	C	T	T
CMW 8399 *C. bhutanensis*	C	C	T	G	G	T	C	A	C	A	C	A	C	A	T	C	C	A	G	T		-		C	A	T	G	C	A	T	G	C	T	C	T	T
CMW 8394 *C. bhutanensis*	C	C	T	G	G	T	C	A	C	A	C	A	C	A	T	C	C	A	G	T		-		C	A	T	G	C	A	T	G	C	T	C	T	T
CMW 9590 *C. moniliformis*	C	C	T	G	G	T	T	A	C	G	C	C	C	C	T	C	T	A	A	T		-		C	A	T	G	C	A	T	G	C	T	C	T	T
CMW 4114 *C. moniliformis*	C	C	T	G	G	T	T	A	C	G	C	C	C	C	T	C	T	A	A	T		-		C	A	T	G	C	A	T	G	C	T	C	T	T
CMW 8240 *C. moniliformis*	C	C	T	G	G	T	T	A	C	G	C	C	C	C	T	C	T	A	A	T		-		C	A	T	G	C	A	T	G	C	T	C	T	T
CMW 9986 *C. moniliformopsis*	C	C	T	G	G	T	C	A	T	C	T	C	C	A	T	C	C	C	A	T	C	T	A	C	A	T	G	C	A	A	G	G	T	C	T	C
CMW 10214 *C. moniliformopsis*	C	C	T	G	G	T	C	A	T	C	T	C	C	A	T	C	C	C	A	T	C	T	A	C	A	T	G	C	A	A	G	G	T	C	T	C
CMW 3276 *C. virescens*	T	T	G	C	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	A	T	G	C	A	T	T	G	T	A	T	C

EF1-α

		1370										1380										1390										1400				
CMW 8242 *C. bhutanensis*	-	C	G	G	C	A	T	G	A	T	A	G	T	G	T	C	T	T	G	T	T	T	G	G	G	T	G	T	T	G	G	T	T	C	G	C
CMW 8217 *C. bhutanensis*	-	C	G	G	C	A	T	G	A	T	A	G	T	G	T	C	T	T	G	T	T	T	G	G	G	T	A	T	T	G	G	T	T	C	G	C
CMW 8215 *C. bhutanensis*	-	C	G	G	C	A	T	G	A	T	A	G	T	G	T	C	T	T	G	T	T	T	G	G	G	T	A	T	T	G	G	T	T	C	G	C
CMW 8399 *C. bhutanensis*	-	C	G	G	C	A	T	G	A	T	G	G	T	G	T	C	T	T	G	T	T	T	G	G	G	T	A	T	T	G	G	T	T	C	G	C
CMW 8394 *C. bhutanensis*	-	C	G	G	C	A	T	G	A	T	A	G	T	G	T	C	T	T	G	T	T	T	G	G	G	T	G	T	T	G	G	T	T	C	G	C
CMW 9590 *C. moniliformis*	A	C	G	-	T	A	T	G	G	T	G	G	G	G	T	C	T	T	G	T	T	T	G	G	G	T	A	T	T	G	G	T	T	A	G	C
CMW 4114 *C. moniliformis*	A	C	G	-	T	A	T	G	G	T	G	G	G	G	T	C	T	T	G	T	T	T	G	G	T	T	G	T	T	G	G	T	T	A	G	C
CMW 8240 *C. moniliformis*	A	C	G	-	T	A	T	G	G	T	G	G	G	G	T	C	T	T	G	T	T	T	G	G	T	T	G	T	T	G	G	T	T	A	G	C
CMW 9986 *C. moniliformopsis*	T	T	G	C	A	C	G	A	C	-	-	-	-	G	T	C	T	T	G	T	T	T	G	A	T	T	A	T	T	G	G	T	T	C	G	C
CMW 10214 *C. moniliformopsis*	T	T	G	C	A	C	G	A	C	-	-	-	-	G	T	C	T	T	G	T	T	T	G	A	T	T	A	T	T	G	G	T	T	C	G	C
CMW 3276 *C. virescens*	-	-	-	-	T	A	T	G	C	-	-	-	-	A	T	G	C	C	A	T	T	T	A	C	A	A	T	A	A	-	-	-	-	-	-	-

	1410														1420
CMW 8242 *C. bhutanensis*	A	**C**	T	T	**C**	**A**	T	C	**T**	A	G	**C**	A	T	C	A
CMW 8217 *C. bhutanensis*	A	**C**	T	T	**C**	**A**	T	C	**T**	A	G	**C**	A	T	C	A
CMW 8215 *C. bhutanensis*	A	**C**	T	T	**C**	**A**	T	C	**T**	A	G	**C**	A	T	C	A
CMW 8399 *C. bhutanensis*	A	**C**	T	T	**C**	**A**	T	C	**T**	A	G	**C**	A	T	C	A
CMW 8394 *C. bhutanensis*	A	**C**	T	T	**C**	**A**	T	C	**T**	A	G	**C**	A	T	C	A
CMW 9590 *C. moniliformis*	A	**G**	T	T	**A**	**A**	T	C	**C**	A	G	**C**	A	T	C	A
CMW 4114 *C. moniliformis*	A	**G**	T	T	**A**	**A**	T	C	**C**	A	G	**C**	A	T	C	A
CMW 8240 *C. moniliformis*	A	**G**	T	T	**A**	**A**	T	C	**C**	A	G	**C**	A	T	C	A
CMW 9986 *C. moniliformopsis*	A	**C**	T	T	**A**	**G**	T	C	**T**	A	G	**G**	A	T	C	A
CMW 10214 *C. moniliformopsis*	A	**C**	T	T	**A**	**G**	T	C	**T**	A	G	**G**	A	T	C	A
CMW 3276 *C. virescens*	-	**C**	T	T	A	A	**A**	**C**	C	A	**A**	T	C	T	C	A



CHAPTER 3

Ceratocystis polychroma prov. nom., a new species from *Syzygium aromaticum* in Sulawesi.

ABSTRACT

Clove decline is the most serious disease affecting *Syzygium aromaticum* in Northern Sulawesi, Indonesia. The aetiology of this disease has never been established. Diseased *S. aromaticum* trees show symptoms of wilt and defoliation, and die in large numbers. Clove decline was found to affect 20-80 % of trees at eighteen sites investigated during a recent survey of the disease. Dying trees are typically infested with the woodborer *Hexamitodera semivelutina*. Larval tunnels are associated with extensive discolouration of the xylem vessels, which has a streaked appearance. Isolations from discoloured wood and larval galleries consistently yielded a *Ceratocystis* spp. very similar to *C. fimbriata.* Comparisons of DNA sequence data for the Internal Transcribed Spacer (ITS) regions, β-tubulin and the Transcription Elongation Factor 1-α (EF1- α) region showed clearly that this *Ceratocystis* sp. resides in a clade distinct from *C. fimbriata* or any of the other known *Ceratocystis* spp. 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 to be known as *C. polychroma prov. nom.*

INTRODUCTION

Clove represents a commonly used spice worldwide. It is produced from the unopened flower buds of the evergreen tree, *Syzygium aromaticum* L. Merr. & Perry (Myrtaceae) (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* Roberts, Eden-Green, Jones and 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 dieback of clove trees.

Although *H. semivelutina* is closely associated with clove dieback 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 tissue, especially that 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 *Ceratocystis fimbriata* Ell. & Halst. (Liew *et al.* 2003) based only on morphological characteristics. The aim of the present study was to identify the *Ceratocystis* sp. from dying clove trees more comprehensively, based on morphological and DNA sequence comparisons.

MATERIALS AND METHODS

Fungal isolates

Diseased clove trees at eighteen different locations in northern Sulawesi (Fig. 1) 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 Kembesr (Chapter 4, Fig. 1). Trees were examined for signs of insect infestation and fungal infection (Fig. 1). Larval tunnels were inspected on site, with the aid of a 10-x 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* were stored in ethanol and are maintained in the collection of the School of Land, Water & Crop Sciences, University of Sydney, Australia.

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 the pieces of wood placed between carrots as described by Moller & De Vay (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 vol^{-1}, SIGMA, Steinheim, Germany) and Thiamine (0.001 g vol^{-1}, 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), Baarn, Netherlands. Holotype material of the new *Ceratocystis* sp. from Sulawesi, consisting of dried cultures on MEA is kept at the National Fungal Herbarium (PREM), Pretoria, South Africa (Table 1).

DNA extraction

Mycelium from actively growing cultures on 2 % MEA plates, for each representative isolate chosen (Table 1) was scraped into Eppendorf tubes and lyophilised for 2 days. 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).

PCR amplification

Both ITS regions (ITS1 and ITS2) including the 5.8S gene of the 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 °C. Part of the β-tubulin gene was amplified using primers βt1a and βt1b (Glass & Donaldson 1995) at an annealing temperature of 56 °C. The EF1-α gene was amplified with the primers EF1-728F and EF1-986R (Carbone & Kohn 1999) at an annealing temperature of 58 °C.

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 x 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 °C for 2 min, followed by 10 cycles at 94 °C for 20 s, x °C (x = the annealing temperature specified for each set of primers as noted above) for 40 s and 72 °C 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 °C 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).

DNA Sequencing and analyses

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 as used for the PCR reactions. Sequence reactions were run on an ABI PRISM™ 3100 Autosequencer (Applied BioSystems, Foster City, California,

U.S.A) 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 are available in GenBank (Table 1). Sequences were aligned manually and analysed using PAUP version 4.0b10* [Phylogenetic Analysis Using Parsimony (and other methods)] (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* (Davids.) Moreau was used as the out-group. 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 100^{th} tree and printing every 10^{th} tree. To avoid including trees that might have been sampled before convergence of the Markov chain, a number of 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 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. Prior to the growth studies, the isolates were grown for two weeks at 20 °C. 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 °C as well as at temperatures ranging from 10 °C to 35 °C, at five-degree intervals. Two measurements of colony diameter at right angles to each other were made every second day, for 16 days and averages were computed. The entire experiment was repeated once.

Morphological characteristics were described from 14-day-old cultures, on 2 % MEA supplemented with Streptomycin Sulphate (0.001 g.vol^{-1}, Sigma, Steinheim, Germany). 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, CMW11443, CMW 11449). Ranges, averages, and standard deviations of the corresponding measurements were calculated. 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 discolouration 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 (Fig. 1). Others originated from carrot baiting of the discoloured wood.

PCR amplification

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 EF1- α resulted in amplicons of ~300 bp.

DNA Sequencing and analysis

Partition homogeneity tests for the sequence data sets of all three genes resulted in a P-value of 0.05. The dataset had a value equal to the required value and the three genes could thus be combined. The combined sequences of the ITS and β-tubulin and EF1- α genes resulted in a dataset of 1383 characters (Appendix). Of these characters, 804 were constant while 292 characters were parsimony-uninformative and 287 were parsimony-informative. Analysis of this dataset resulted in three most parsimonious trees (Fig. 2.), with a tree 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 datasets, 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 & Wingfield, *C. fimbriata* and *C. albofundus* Wingfield, De Beer & 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. 2).

Species of *Ceratocystis* included in the analyses resided in four well-resolved clades (Fig. 2). The *Ceratocystis* sp. from clove in Sulawesi did not group with any known *Ceratocystis* spp. It formed a single, well-supported sub-clade with a bootstrap support of 98 %. The other sub-clades represented isolates of *C. fimbriata*, *C. albofundus* and *C. pirilliformis* respectively (Fig. 2). This result was confirmed using the Bayesian analysis, with a branch node possibility of 100 % (Fig. 2). DNA-based comparisons thus show that the *Ceratocystis* sp. from Sulawesi represents a previously undescribed species of *Ceratocystis*.

Morphology and cultural characteristics

Three different culture morphologies were observed for the *Ceratocystis* sp. from dying *S. aromaticum* trees (Chapter 4). For the purpose of this chapter, only one culture group was chosen to represent this species. The isolates reached 90 mm in 16 days at the optimum temperature for growth of 25 °C. Growth at 10 °C was slow with cultures reaching only an average 10 mm in 16 days, and at 30 °C growth was slow reaching an average of 45 mm in 16 days. Colony colour differed at different temperatures. At 15 °C cultures had a distinct dark, olive green (23m) colour (Fig. 3a) while at 20 °C it had a white to buff brown colour (17'"I) (Fig. 3b). Cultures incubated at 25 °C had a honey colour (19 "k) (Fig. 3c.) and cultures at 30 °C had a hazel colour (11'k) (Fig. 3d). Cultures produced a fruity odour, similar to that produced by *C. fimbriata*.

Morphological characteristics of the *Ceratocystis* sp. from clove were most similar to those of *C. fimbriata.* The ascomatal bases of the teleomorph were black and globose and ornamented

with hyphal hairs (Fig. 4a). The ostiolar hyphae were divergent (Fig. 4b), exuding sticky masses of hat-shaped ascospores (Fig. 4c). The anamorph of the *Ceratocystis* sp. is a typical *Thielaviopsis* sp., with phialidic conidiogenous cells (Fig. 4d). Typical cylindrical and barrel-shaped conidia were observed (Fig. 4e & g). Cultures produced chlamydospores, either singly or in chains (Fig. 4f). This fungus could easily be distinguished from *C. albofundus* (Wingfield *et al.* 1996) and *C. pirilliformis* (Barnes *et al.* 2003), the two other *Ceratocystis* spp. with hat-shaped ascospores in the *C. fimbriata* complex, based on ascomatal colour and shape.

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

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 describing it as a new taxon, *C. polychroma prov. nom.*

Ceratocystis polychroma, Van Wyk, Liew & Wingfield

Etymology: *polychroma* reflecting the different colours of cultures at different temperatures.

Stat.conid.: *Thielaviopsis*

(Fig. 3-4)

Coloniae ad 15 °C olivaceo-virides, infra olivaceae, ad 20 °C albae vel fulvo-brunneae, inframellinae, ad 25 °C supra infraque mellinae, et ad 30 °C supra infraque avellinae. *Mycelium* plerumque in medio immersum; mycelium aerium album adest. *Crescit* optime ad 25 °C, nullo incremento supra 35 °C, deminuto ad 10 °C. *Hyphae* leves, in septis non constrictae, 3–5 µm. *Bases ascomatum* atrobrunneae vel nigrae, globosae, hyphis ornatae, bases (208-) 217-261 (-269) µm diametro. *Colla ascomatae* basi atrobrunnea vel nigra, apicem versus laetescentes, (837-) 849-1071 (-1187) µm longa, basi (42-) 44-54 (-57) µm lata, apice (15-) 16-18 (-20) µm lata. *Hyphae ostiolares* divergentes, hyalinae, (31-) 33-43 (-46) µm longae. *Asci* non visi. *Ascosporae* lateraliter visae cucullatae, aseptatae, hyalinae, in vagina investitae, cum vagina 5-7 x 3-4 µm, sine vagina 4-5 x 3-4 µm. Ascosporae in massis mucilagineis fulvo-luteis in apicibus collorum ascomatum cumulantes. *Anamorpha Thielaviopsis*: conidiophora in mycelia singula, hyalina, basibus tumidis, apicem versus angustata, 53-81 (-103) µm longa, basi 4-6 µm lata, apicibus 3-4 µm lata. Evolutio *conidii* phialidici per parietes annulares faciendas, *conidia* duarum formarum: conidia primaria hyalina, aseptata, cylindrica, (13-) 16-24 (-26) x 3-5 µm; conidia secondaria hyalina, aseptata, doliiformia, 9-11 x 6-8 µm, in catenis portata. *Chlamydospora* elliptica, parietibus crassis, levia, "argus" brunnea, 11-14 x 8-14 µm, in agaro inclusa, singula vel in catenis facta.

Typus: **Sulawesi**: Toliangoki, isolated from larval tunnel of *Hexamitoderma semivelutina* (Coleoptera: Cerambycidae) on *Syzigium aromaticum*, December 2002, *E. C. Y. Liew*, (PREM 57818 – holotypus, living culture: CMW 11424).

Colonies olive green (23m), reverse olive (21"m) at 15 °C (Fig. 3a), at 20 °C colonies white to buff brown (17"'I), reverse honey (19"k) (Fig. 3b), at 25 °C colonies honey (19"k), reverse honey (19"k) (Fig. 3c), and at 30 °C colonies are hazel (11'k), reverse hazel (11'k) (Fig. 3d) in colour. *Mycelium* mostly submerged in medium, sparse white aerial mycelium present. *Optimal temperature* for growth 25 °C, no growth above 35 °C, diminished growth at 10 °C. *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 in diameter. *Ascomatal necks* dark brown to black at 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 sheath, 5-7 x 3-4 µm with sheath, 4-5 x 3-4 µm without sheath. Ascospores accumulated in buff-yellow (19d) mucilaginous masses on the apices of ascomatal necks. *Thielaviopsis anamorph*: conidiophores occurring singly on mycelia, hyaline, 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 *conidium* development through ring wall building, *conidia* of two types: primary conidia hyaline, aseptate, cylindrical (13-) 16–24 (-26) x 3-5 µm, secondary conidia hyaline, aseptate, barrel-shaped 9-11 x 6-8 µm, borne in chains. *Chlamydospores* oval, thick walled, smooth, argus brown (13m), 11-14 x 8-14 µm, embedded in agar, formed singly or in chains, terminally.

Additional specimens examined: **Sulawesi**: Kiaea, isolated from larval tunnels of *H. semivelutina* on *S. aromaticum*, December 2002, *E. C. Y. Liew*, (culture CMW 11443, PREM 57820); same collecting data (culture CMW 11419, PREM 57817); Rumoong, isolated from larvae tunnel of *H. semivelutina* on *S. aromaticum*, December 2002, *E. C. Y. Liew*, (culture CMW 11449, PREM 57821).

DISCUSSION

Ceratocystis polychroma prov. nom. 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 extensive red streaked discolouration of the living wood that is found associated with the borer. Morphologically, *C. polychroma prov. nom.* most closely resembles *C. fimbriata*. This explains why Liew *et al.* (2003) tentatively identified the fungus as *C. fimbriata*. 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 prov. nom.* can, however, be distinguished from *C. fimbriata* and all other *Ceratocystis* spp. based on morphology, growth in culture and DNA-based comparisons.

Ceratocystis polychroma prov. nom. produces colonies that are white to green in colour whereas isolates of *C. fimbriata* are typically olivacious green in culture. *Ceratocystis*

fimbriata cultures tend to produce obvious aerial mycelium, which is different to *C. polychroma prov. nom.* that produces a sparse white mat of mycelium on the surface of cultures. The bases of the ascomatal necks are much wider in *C. polychroma prov. nom.* than in *C. fimbriata* and the barrel-shaped conidia are also much wider in the former than the latter species.

Together with *C. polychroma prov. nom.*, there are seven *Ceratocystis* spp. with hat shaped ascospores. Other species include *C. fimbriata* (Halstead 1890, Upadhyay 1981), *C. moniliformis* Hedgc. (Davidson 1935, Hunt 1956), *C. albofundus* (Morris, Wingfield & De Beer 1993, Wingfield *et al.* 1996), *C. moniliformopsis* Yuan & Mohamm. (Yuan & Mohammed 2002), *C. pirilliformis* (Barnes *et al.* 2003) and *C. acericola* Griffin (Grylls & Siefert 1993). Of these fungi, only *C. fimbriata* (Upadhyay 1981), *C. pirilliformis* (Barnes *et al.* 2003) and *C. polychroma prov. nom.* 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 prov. nom.* 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 prov. nom. and *C. fimbriata* are clearly similar and they are also phylogenetically closely related. Sequence data for the ITS regions of the ribosomal DNA operon alone showed that *C. polychroma prov. nom.* is different to *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 prov. nom.* represents a unique group, although it resides in the clade including *C. fimbriata*, *C. pirilliformis* and *C. albofundus*. This clade is strongly separated from the *C. coerulescens* clade (Witthuhn *et al.* 1998), which also includes species with hat-shaped ascospores.

Ceratocystis polychroma prov. nom. is closely associated with damage to clove trees by the cerambycid beetle, *H. semivelutina*. Association with an insect is not unusual amongst *Ceratocystis* spp., that require wounds for infection and are known to be vectored by insects (Iton 1966, Seifert, Wingfield & Kendrick 1993). Numerous species of *Ceratocystis,* such as *C. fimbriata*, produce fruity aromatics and are thus attractive to casual insects such as picnic

beetles (Coleoptera: Nitidulidae) and flies (Diptera) that transport them to freshly made wounds on trees (Moller & De Vay 1968, Hinds 1972). This is very different to species such as *C. polonica* Siemaszko, *C. laricicola* Redfern & Minter and *C. rufipenni* Wingfield, Harrington & 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 prov. nom.* 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 prov. nom.* 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 *Bursaphelechus 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 prov. nom. resides in a genus of very well known pathogens of woody plants (Kile 1993). Its association with dramatic dieback of cloves and the very characteristic discolouration 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 dieback disease of clove trees in Sulawesi.

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

Species	Isolate no.	Alternative numbers	GenBank accession nr.	Date of isolation	Host	Geographical origin	Associated insect	Collector(s)
C. fimbriata	CMW 2218[a]	None	AF395680[d] N/A[e] AY528974[f]	1991	*Platanus* sp.	France	None	Grosclaude, C.
"	CMW 2219[a]	"	AF395679[d] N/A[e] AY528975[f]	"	"	"	"	"
C. albofundus	CMW 5329[a]	"	AF388947[d] N/A[e] N/A[f]	1999	*Acacia mearnsii*	Uganda	"	Roux, J.
"	CMW 5943[a]	"	N/A[d] N/A[e] N/A[f]	2000	"	"	"	"
C. pirilliformis	CMW 6569[a]	"	N/A[d] N/A[e] AY528982[f]	"	*Eucalyptus nitens*	Australia	"	Wingfield, M.J.
"	CMW 6579[a]	"	N/A[d] N/A[e] AY528983[f]	"	"	"	"	"
C. polychroma prov. nom	CMW 11424[a, b, c]	PREM 57818 CBS N/A	AY528966[d] AY528970[e] AY528978[f]	2002	*Syzygium aromaticum*	Sulawesi, Indonesia	*Hexamitodera semivelutina*	Liew, E. C. Y. & Wingfield, M. J.
"	CMW 11436[a]	PREM 57819 CBS N/A	AY528967[d] AY528971[e] AY528979[f]	"	"	"	"	"
"	CMW 11443[a, b, c]	PREM 57820 CBS N/A	N/A[d] N/A[e] N/A[f]	"	"	"	"	"
"	CMW 11449[a, b, c]	PREM 57821 CBS N/A	AY528968[d] AY528972[e] AY528980[f]	"	"	"	"	"
"	CMW 11455[a]	PREM 57822 CBS N/A	AY528969[d] AY528973[e] AY528981[f]	"	"	"	"	"
C. virescens	CMW 3276[a]	None	AY528984[d] AY528990[e] AY528991[f]	1963	*Quercus* sp.	U. S. A.	None	Hinds, T.

N/A refers to accession numbers not available at present.

[a, b, c, d, e, f] Isolates marked with [a] CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, those marked with [b] were sequenced, those marked with [c] represents isolates that were used for morphological descriptions, GenBank accession numbers that are marked with [d] reprenesnt the ITS sequences, those marked with [e] represent the β-tubulin sequences and those marked with [f] represent the Elongation Factor sequences.

Figure 1. Symptoms of the disease caused by *C. polychroma prov. nom.* in Sulawesi, Indonesia, a) diseased and dead *Syzygium aromaticum* trees, b) internal symptoms showing sap stain in the wood, c-d) tunnels in the wood caused by *Hexamitodera semivelutina* larvae along with sap stain damage, e) the *H. semivelutina* larvae isolated inside the *S. aromaticum* trees.


a

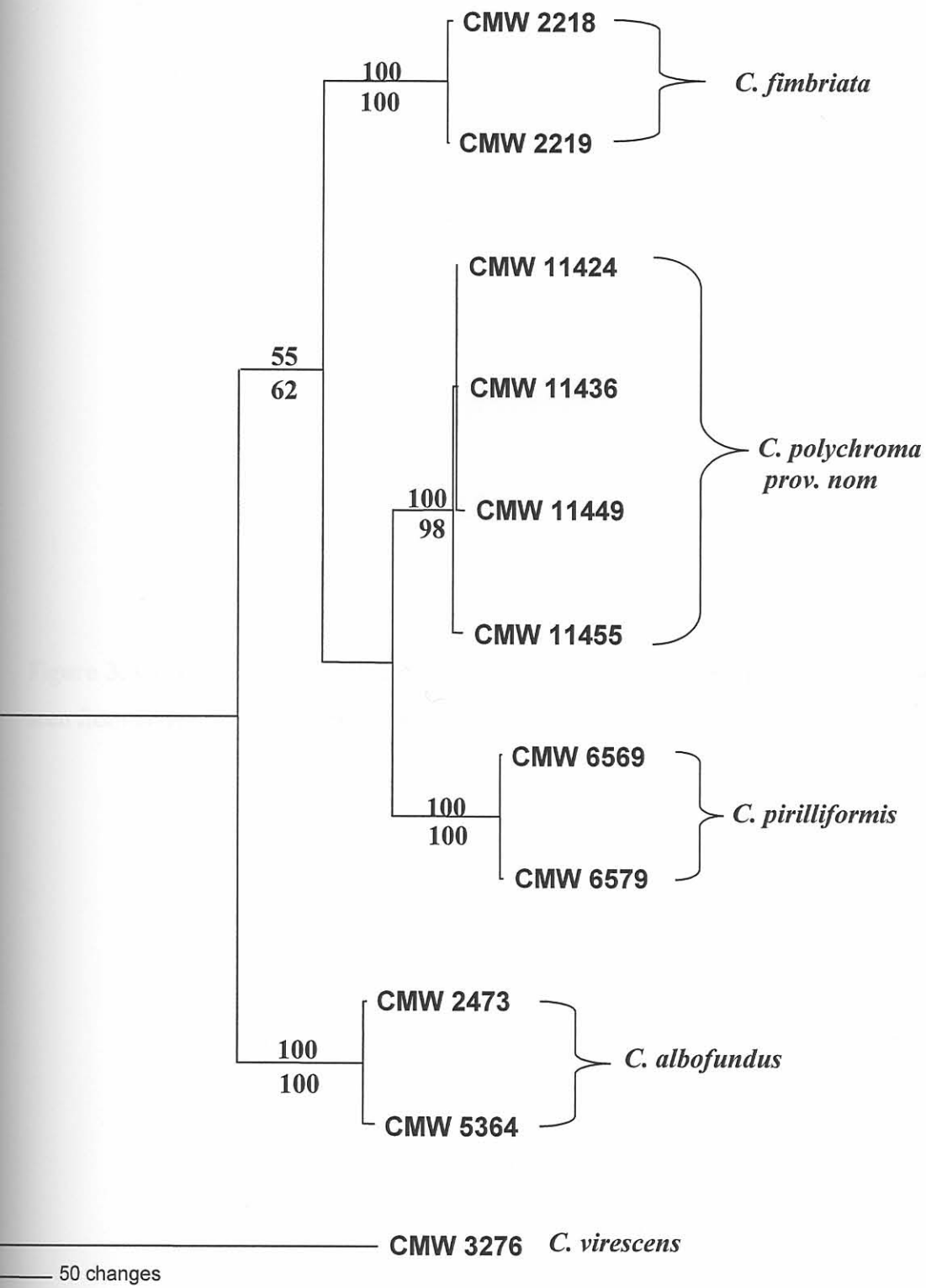

b


c


d


e

Figure 2. A phylogenetic tree based on the combined sequence data for three gene regions; ITS, β-tubulin and Ef1-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* was treated as the out-group.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100
CMW 2219
C. fimbriata
CMW 11424
55
62
CMW 11436
C. polychroma
prov. nom
100
98
CMW 11449
CMW 11455
CMW 6569
100
100
C. pirilliformis
CMW 6579
CMW 2473
100
100
C. albofundus
CMW 5364
CMW 3276
C. virescens
50 changes

Figure 3. Growth and morphology of *C. polychroma prov. nom.* at different temperatures as seen from above and below. a) 15 °C, b) 20 °C, c) 25 °C, d) 30 °C.

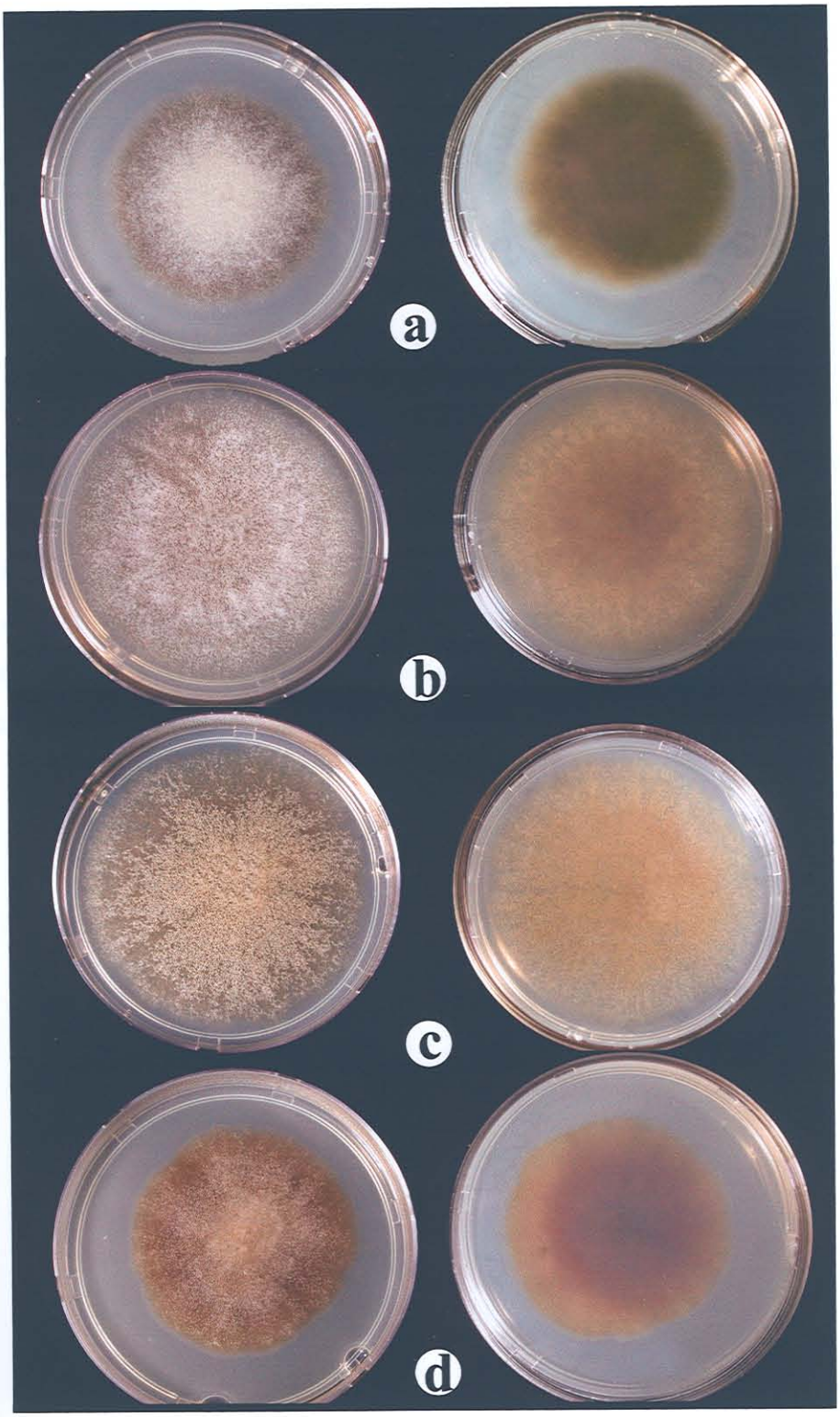
a
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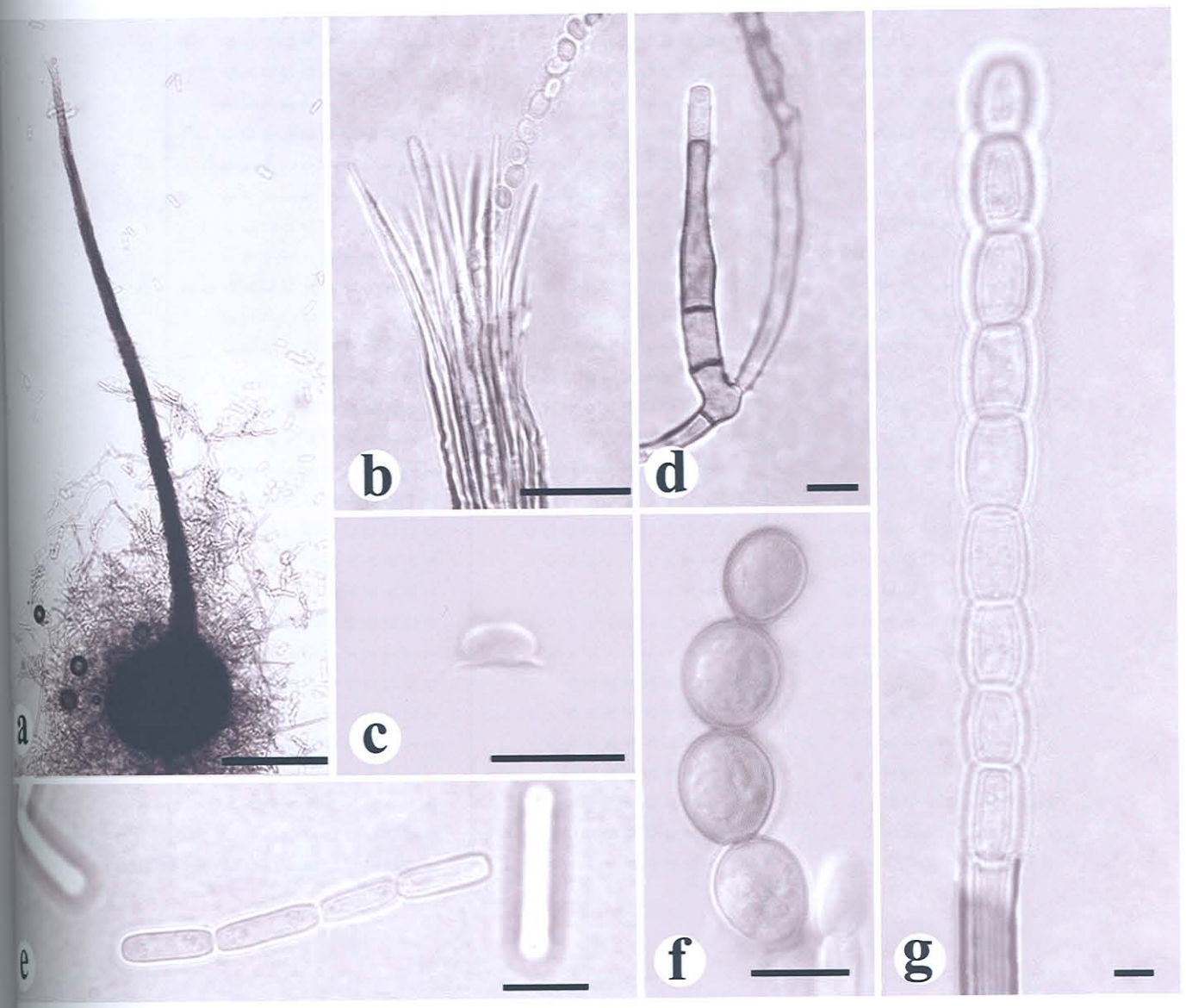
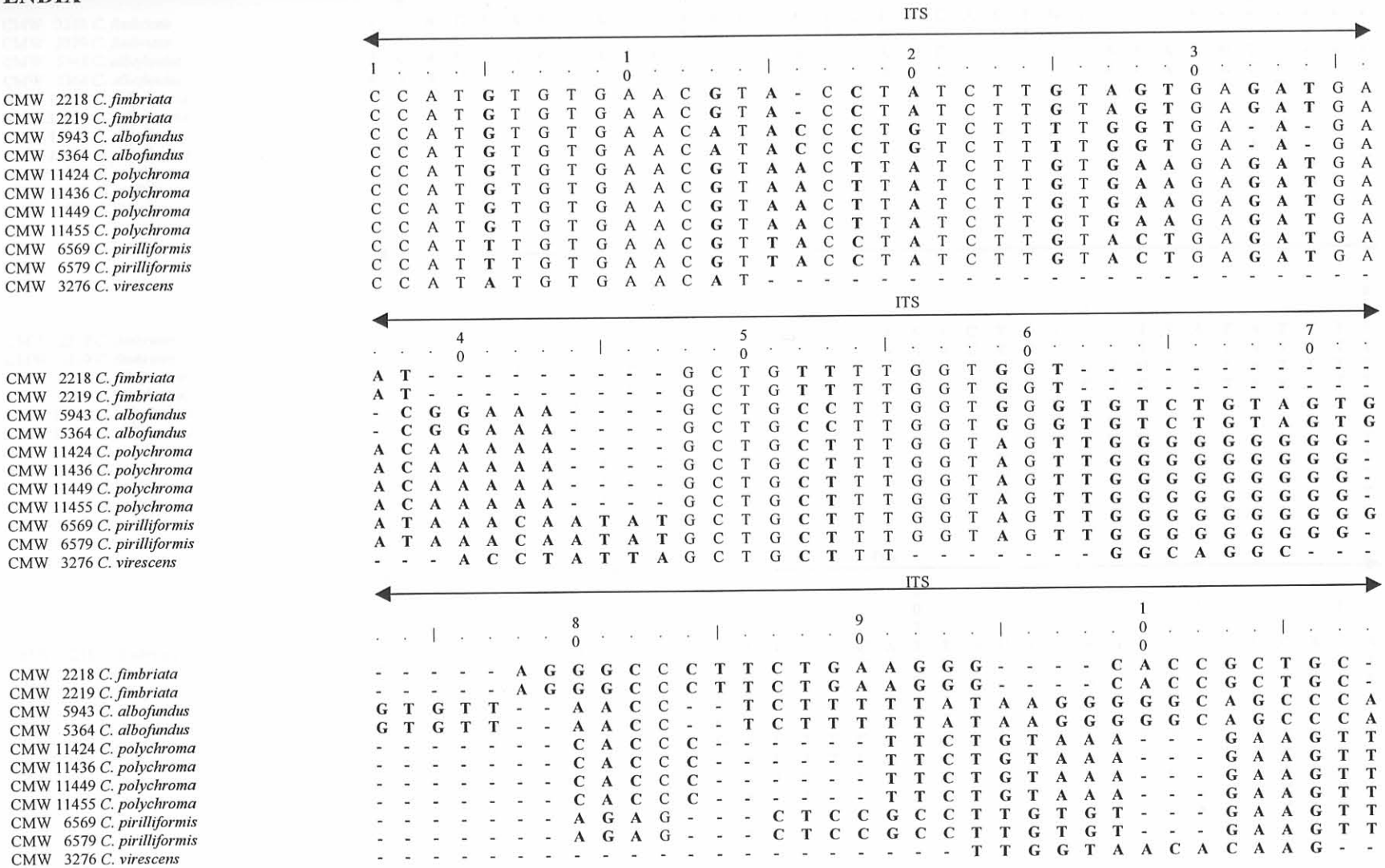
Figure 4. Morphological characteristics of *C. polychroma prov. nom.* (CMW 11424). a) Globose ascomatal base (scale bar = 200 µm), b) Divergent ostiolar hyphae (scale bar = 20 µm), c) Hat-shaped ascospore in side view (scale bar = 10 µm), d) Phialidic conidiogenous cell with emerging cylindrical conidium (scale bar = 5 µm), e) Cylindrical conidia in a chain (scale bar = 10 µm), f) Chain of chlamydospores (scale bar = 10 µm), g) Barrel-shaped conidia in a chain (scale bar = 5 µm).



APPENDIX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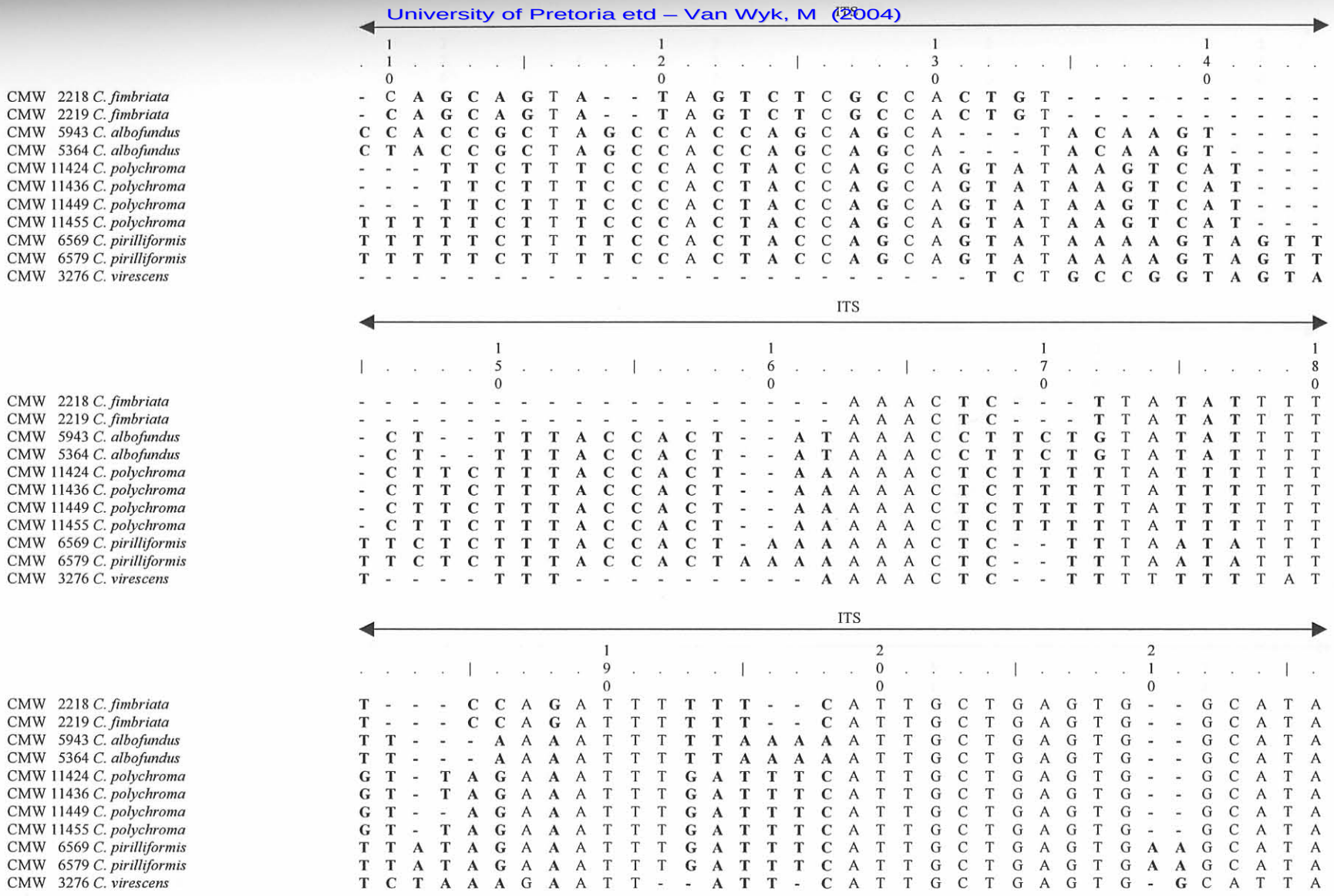
CMW 2219 C. fimbriata
CMW 5943 C. albofundus
CMW 5364 C. albofundus
CMW 11424 C. polychroma
CMW 11436 C. polychroma
CMW 11449 C. polychroma
CMW 11455 C. polychroma
CMW 6569 C. pirilliformis
CMW 6579 C. pirilliformis
CMW 3276 C. virescens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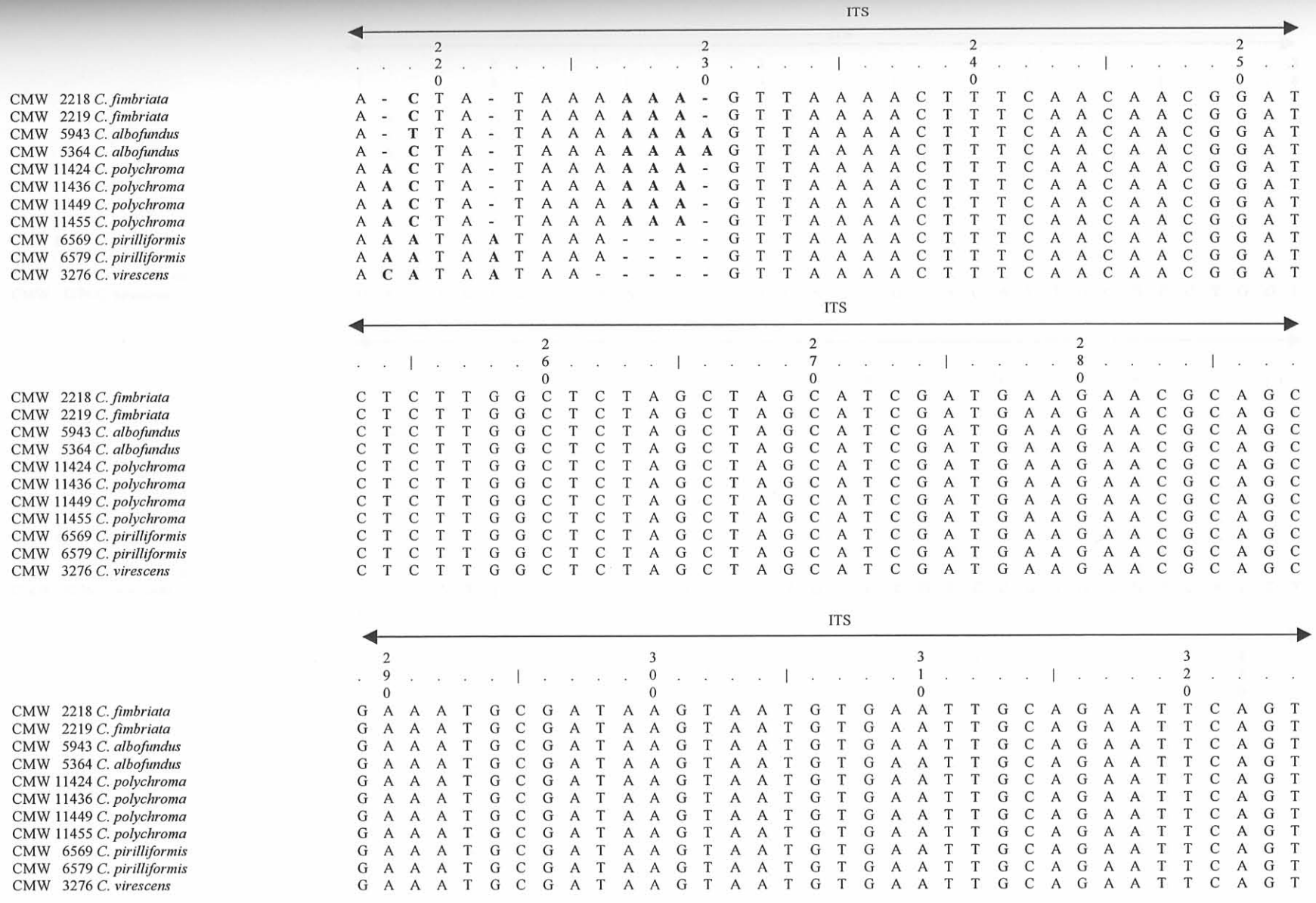
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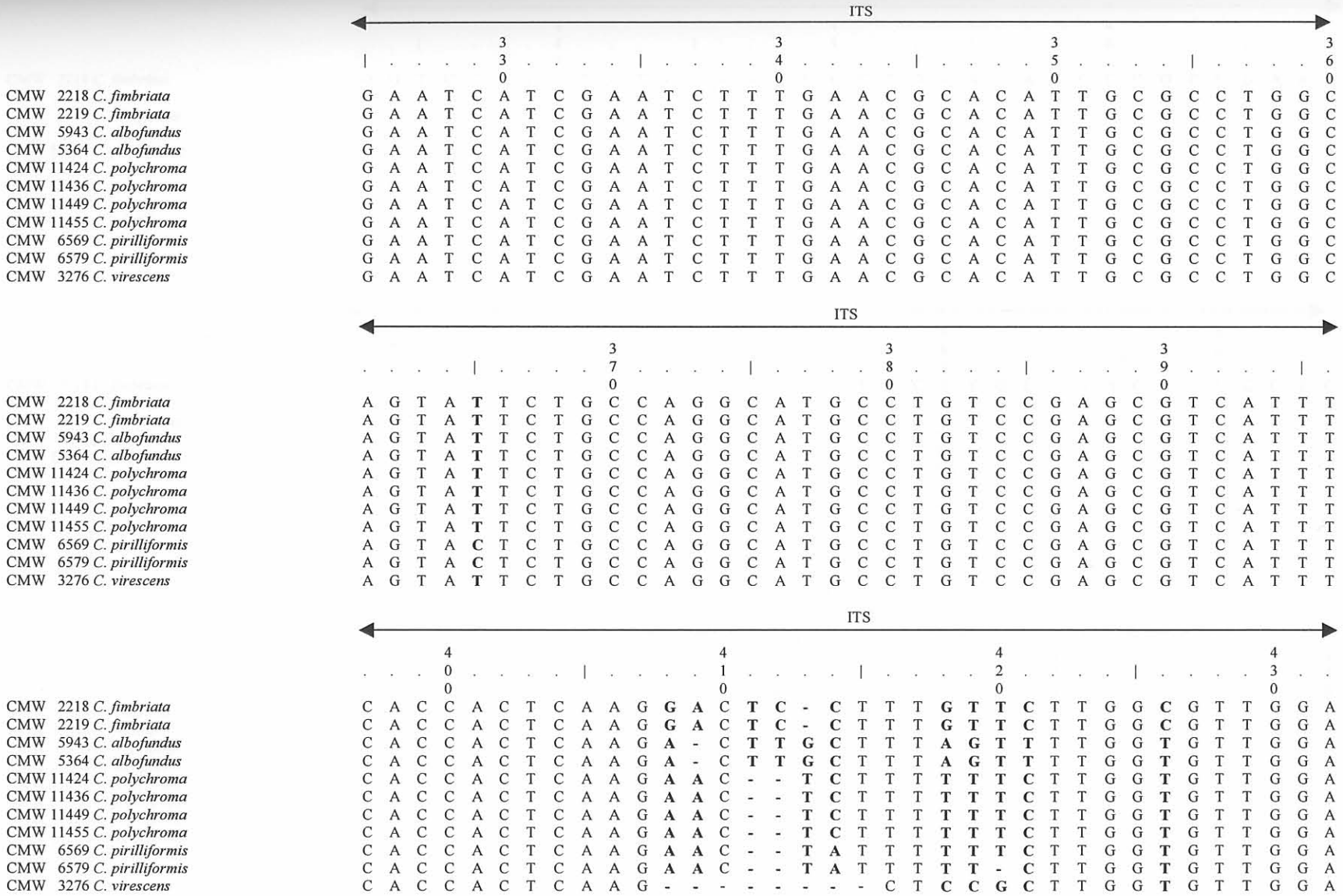
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CMW 11436 C. polychroma
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CMW 6579 C. pirilliformis
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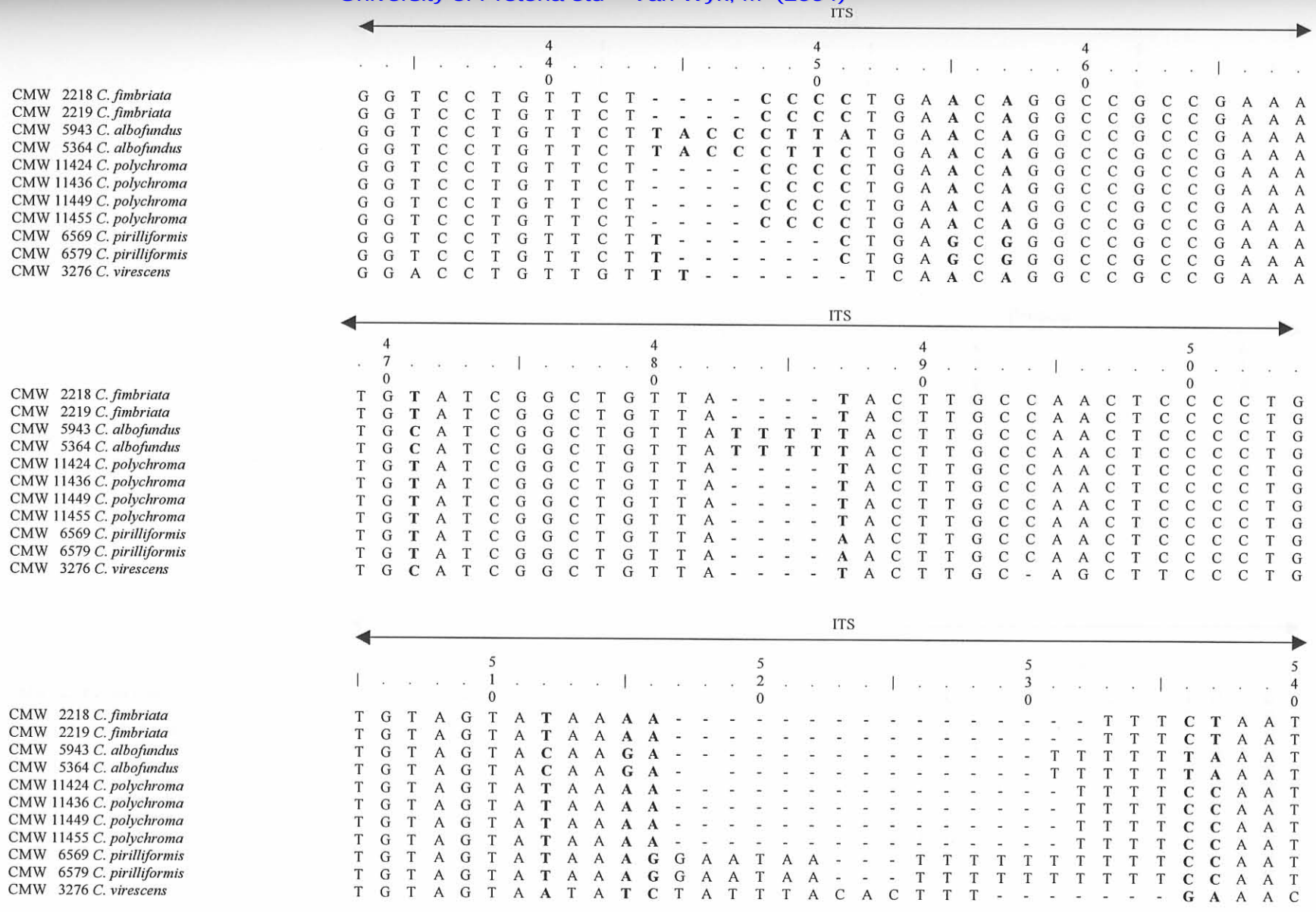
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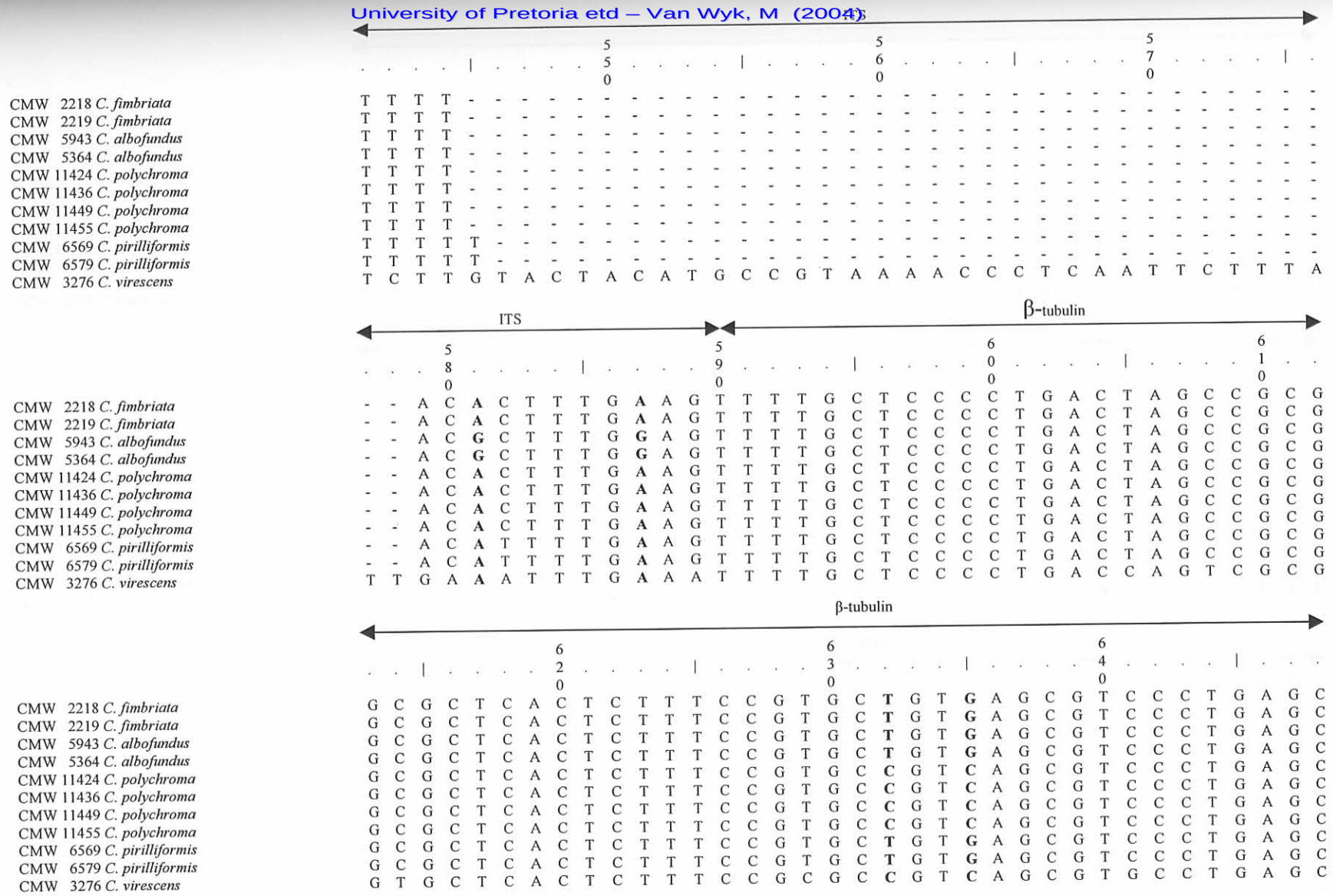


β-tubulin

							650										660										670									
CMW 2218 *C. fimbriata*	T	T	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	C	A	A	G	A	A	C	A	T	G	A	T	G	G
CMW 2219 *C. fimbriata*	T	T	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	C	A	A	G	A	A	C	A	T	G	A	T	G	G
CMW 5943 *C. albofundus*	T	T	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	C	A	A	G	A	A	C	A	T	G	A	T	G	G
CMW 5364 *C. albofundus*	T	T	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	C	A	A	G	A	A	C	A	T	G	A	T	G	G
CMW 11424 *C. polychroma*	T	T	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	C	A	A	G	A	A	C	A	T	G	A	T	G	G
CMW 11436 *C. polychroma*	T	T	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	C	A	A	G	A	A	C	A	T	G	A	T	G	G
CMW 11449 *C. polychroma*	T	T	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	C	A	A	G	A	A	C	A	T	G	A	T	G	G
CMW 11455 *C. polychroma*	T	T	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	C	A	A	G	A	A	C	A	T	G	A	T	G	G
CMW 6569 *C. pirilliformis*	T	T	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	C	A	A	G	A	A	C	A	T	G	A	T	G	G
CMW 6579 *C. pirilliformis*	T	T	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	C	A	A	G	A	A	C	A	T	G	A	T	G	G
CMW 3276 *C. virescens*	T	G	A	C	C	C	A	G	C	A	G	A	T	G	T	T	C	G	A	C	C	C	C	A	A	G	A	A	C	A	T	G	A	T	G	G

β-tubulin

	680										690										700										710					
CMW 2218 *C. fimbriata*	C	T	G	C	T	T	C	C	G	A	C	T	T	C	C	G	C	A	A	C	G	G	C	C	G	T	T	A	C	C	T	G	A	C	**T**	T
CMW 2219 *C. fimbriata*	C	T	G	C	T	T	C	C	G	A	C	T	T	C	C	G	C	A	A	C	G	G	C	C	G	T	T	A	C	C	T	G	A	C	**T**	T
CMW 5943 *C. albofundus*	C	T	G	C	T	T	C	C	G	A	C	T	T	C	C	G	C	A	A	C	G	G	C	C	G	T	T	A	C	C	T	G	A	C	**T**	T
CMW 5364 *C. albofundus*	C	T	G	C	T	T	C	C	G	A	C	T	T	C	C	G	C	A	A	C	G	G	C	C	G	T	T	A	C	C	T	G	A	C	**T**	T
CMW 11424 *C. polychroma*	C	T	G	C	T	T	C	C	G	A	C	T	T	C	C	G	C	A	A	C	G	G	C	C	G	T	T	A	C	C	T	G	A	C	**C**	T
CMW 11436 *C. polychroma*	C	T	G	C	T	T	C	C	G	A	C	T	T	C	C	G	C	A	A	C	G	G	C	C	G	T	T	A	C	C	T	G	A	C	**C**	T
CMW 11449 *C. polychroma*	C	T	G	C	T	T	C	C	G	A	C	T	T	C	C	G	C	A	A	C	G	G	C	C	G	T	T	A	C	C	T	G	A	C	**C**	T
CMW 11455 *C. polychroma*	C	T	G	C	T	T	C	C	G	A	C	T	T	C	C	G	C	A	A	C	G	G	C	C	G	T	T	A	C	C	T	G	A	C	**C**	T
CMW 6569 *C. pirilliformis*	C	T	G	C	T	T	C	C	G	A	C	T	T	C	C	G	C	A	A	C	G	G	C	C	G	T	T	A	C	C	T	G	A	C	**T**	T
CMW 6579 *C. pirilliformis*	C	T	G	C	T	T	C	C	G	A	C	T	T	C	C	G	C	A	A	C	G	G	C	C	G	T	T	A	C	C	T	G	A	C	**T**	T
CMW 3276 *C. virescens*	C	T	G	C	T	T	C	C	G	A	T	T	T	C	C	G	C	A	A	C	G	G	T	C	G	C	T	A	C	C	T	G	A	C	**T**	T

β-tubulin

					720										730										740										750	
CMW 2218 *C. fimbriata*	G	C	T	C	T	G	C	C	A	T	C	T	T	G	T	A	**C**	G	T	A	T	A	C	C	C	**C**	-	T	T	**G**	**A**	A	A	A	G	A
CMW 2219 *C. fimbriata*	G	C	T	C	T	G	C	C	A	T	C	T	T	G	T	A	**C**	G	T	A	T	A	C	C	C	**C**	-	T	T	**G**	**A**	A	A	A	G	A
CMW 5943 *C. albofundus*	G	C	T	C	T	G	C	C	A	T	C	T	T	G	T	A	**C**	G	T	A	T	A	C	C	C	-	**T**	T	T	**G**	**A**	A	A	A	G	A
CMW 5364 *C. albofundus*	G	C	T	C	T	G	C	C	A	T	C	T	T	G	T	A	**C**	G	T	A	T	A	C	C	C	-	**T**	T	T	**G**	**A**	A	A	A	G	A
CMW 11424 *C. polychroma*	G	C	T	C	T	G	C	C	A	T	C	T	T	G	T	A	**T**	G	T	A	T	A	C	C	C	-	**T**	T	T	**C**	**G**	A	A	A	G	A
CMW 11436 *C. polychroma*	G	C	T	C	T	G	C	C	A	T	C	T	T	G	T	A	**T**	G	T	A	T	A	C	C	C	-	**T**	T	T	**C**	**G**	A	A	A	G	A
CMW 11449 *C. polychroma*	G	C	T	C	T	G	C	C	A	T	C	T	T	G	T	A	**T**	G	T	A	T	A	C	C	C	-	**T**	T	T	**C**	**G**	A	A	A	G	A
CMW 11455 *C. polychroma*	G	C	T	C	T	G	C	C	A	T	C	T	T	G	T	A	**T**	G	T	A	T	A	C	C	C	-	**T**	T	T	**C**	**G**	A	A	A	G	A
CMW 6569 *C. pirilliformis*	G	C	T	C	T	G	C	C	A	T	C	T	T	G	T	A	**C**	G	T	A	T	A	C	C	C	**T**	**T**	T	T	**G**	**A**	A	A	-	G	A
CMW 6579 *C. pirilliformis*	G	C	T	C	T	G	C	C	A	T	C	T	T	G	T	A	**C**	G	T	A	T	A	C	C	C	**T**	**T**	T	T	**G**	**A**	A	A	-	G	A
CMW 3276 *C. virescens*	G	C	T	C	T	G	C	C	A	T	C	T	T	G	T	A	**T**	G	T	-	-	-	-	-	-	**T**	**T**	T	T	**C**	**A**	-	-	-	-	-

β-tubulin

Isolate	Sequence
	. . . | . . . . 760 . . . . | . . . . 770 . . . . | . . . . 780 . . . . | . .
CMW 2218 *C. fimbriata*	T T A G C C C A T T G C T G T T T T C T T C G T A C A T G T G C C T C C
CMW 2219 *C. fimbriata*	T T A G C C C A T T G C T G T T T T C T T C G T A C A T G T G C C T C C
CMW 5943 *C. albofundus*	C T A G C C C A T G C T T G T T T T C T T T G T A C A T G T A C - T - C
CMW 5364 *C. albofundus*	C T A G C C C A T G C T T G T T T T C T T T G T A C A T G T A C - T - A
CMW 11424 *C. polychroma*	T T A G C C C A T T G C T G T T T T C T T C G T A C A T G T A C C C C C
CMW 11436 *C. polychroma*	T T A G C C C A T T G C T G T T T T C T T C G T A C A T G T A C C C C C
CMW 11449 *C. polychroma*	T T A G C C C A T T G C T G T T T T C T T C G T A C A T G T A C C C C C
CMW 11455 *C. polychroma*	T T A G C C C A T T G C T G T T T T C T T C G T A C A T G T A C C C C C
CMW 6569 *C. pirilliformis*	T T A G C C C A A T G C T G T T T T C T T C C T A C A T G T A C C C C C
CMW 6579 *C. pirilliformis*	T T A G C C C A A T G C T G T T T T C T T C C T A C A T G T A C C C C C
CMW 3276 *C. virescens*	- - - - - C C A A - - - - - - - - - - - - - C T A T A T T T G T C T A C

β-tubulin

Isolate	Sequence
	. . | . . . . 790 . . . . | . . . . 800 . . . . | . . . . 810 . . . . | . . . .
CMW 2218 *C. fimbriata*	T C T G T T G C T C A T G C A A C T A T G C T T T C T A T G A C C A T T
CMW 2219 *C. fimbriata*	T C T G T T G C T C A T G C A A C T A T G C T T T C T A T G A C C A T T
CMW 5943 *C. albofundus*	A C T G T T G C T G A T G C A A C T G T G A T T T C T A T G A C T A T T
CMW 5364 *C. albofundus*	A C T G T T G C T G A T G C A A C T G T G A T T T C T A T G A C T A T T
CMW 11424 *C. polychroma*	T C T G C T G C T C A T G C A A C T G T G C T T T C C A T G A C C A T T
CMW 11436 *C. polychroma*	T C T G C T G C T C A T G C A A C T G T G C T T T C C A T G A C C A T T
CMW 11449 *C. polychroma*	T C T G C T G C T C A T G C A A C T G T G C T T T C C A T G A C C A T T
CMW 11455 *C. polychroma*	T C T G C T G C T C A T G C A A C T G T G C T T T C C A T G A C C A T T
CMW 6569 *C. pirilliformis*	T C T G C T G C C C A T G C A A C T G T G C T T T C C A T G A C C A T T
CMW 6579 *C. pirilliformis*	T C T G C T G C C C A T G C A A C T G T G C T T T C C A T G A C C A T T
CMW 3276 *C. virescens*	A T T A - - G T T C A T G - - - - - - - - - T T T G C A T G G A T C T T

β-tubulin

Isolate	Sequence
	. 820 . . . . | . . . . 830 . . . . | . . . . 840 . . . . | . . . . 850 . . . .
CMW 2218 *C. fimbriata*	T G C T A A C C C T T T T T T C T T C C C C T C T C T A C T T T A C A G
CMW 2219 *C. fimbriata*	T G C T A A C C C T T T T T T C T T C C C C T C T C T A C T T T A C A G
CMW 5943 *C. albofundus*	T G C T A A C C C C A T T T T C T T C T C - - - T C T A C T T T A C A G
CMW 5364 *C. albofundus*	C G C T A A C C C C A T T T T C T T C T C - - - T C T A C T T T A C A G
CMW 11424 *C. polychroma*	T G C T A A C T A T T T T T T C T T C C C T C C T C T A C T T T A C A G
CMW 11436 *C. polychroma*	T G C T A A C T A T T T T T T C T T C C C T C C T C T A C T T T A C A G
CMW 11449 *C. polychroma*	T G C T A A C T A T T T T T T C T T C C C T C C T C T A C T T T A C A G
CMW 11455 *C. polychroma*	T G C T A A C T A T T T T T T C T T C C C T C C T C T A C T T T A C A G
CMW 6569 *C. pirilliformis*	T G C T A A C C C T T T T T T C T T C T C T T C T C T A C T T T A C A G
CMW 6579 *C. pirilliformis*	T G C T A A C C C T T T T T T C T T C T C T T C T C T A C T T T A C A G
CMW 3276 *C. virescens*	T G C T A A C A C C T C T T T C T T T C G T C - - - - - - T T T A T A G

β-tubulin

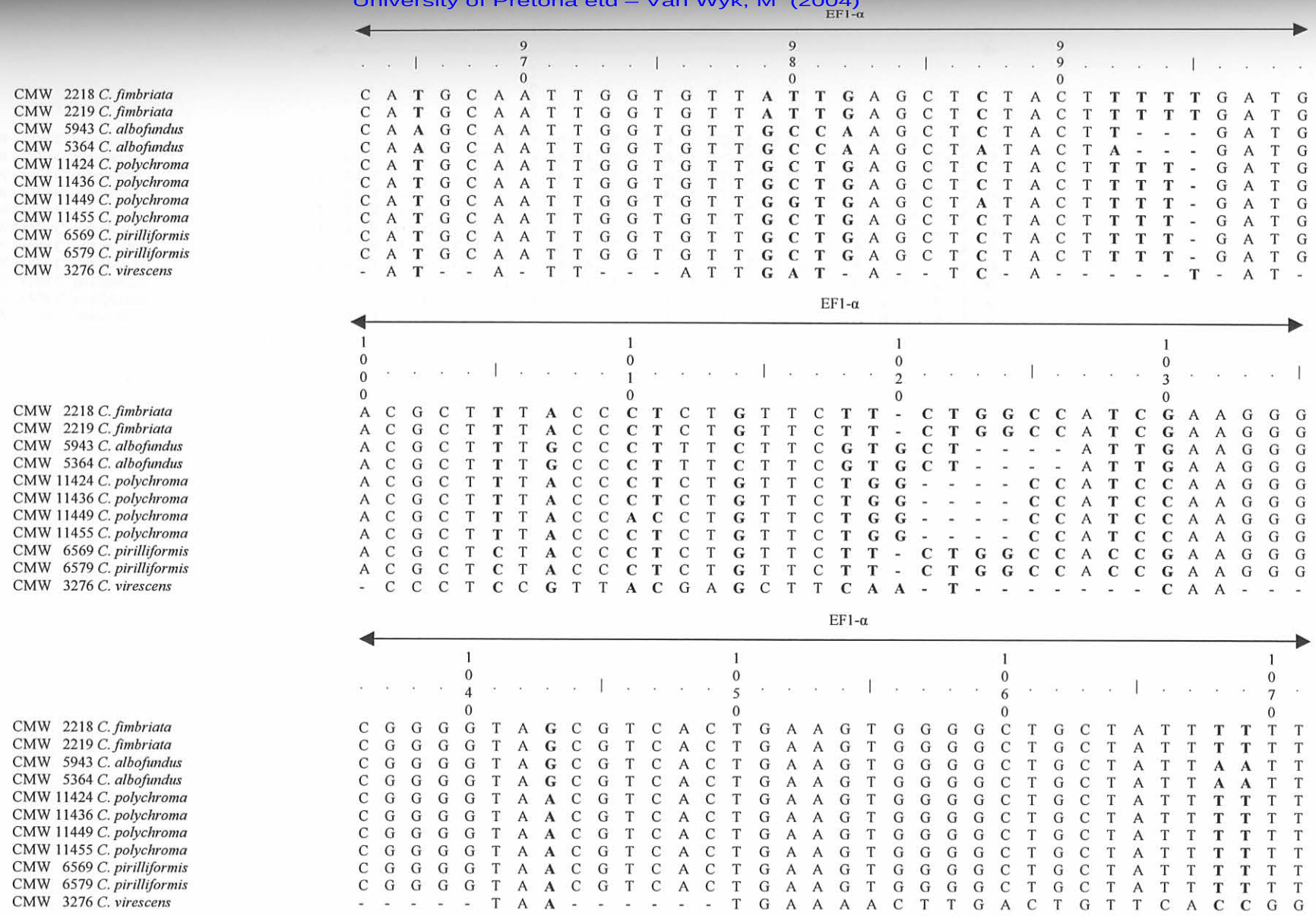
						860										870										880										890
CMW 2218 *C. fimbriata*	C	C	G	T	G	G	T	A	A	G	G	T	T	G	C	C	A	T	G	A	A	G	G	A	G	G	T	C	G	A	A	G	A	C	C	A
CMW 2219 *C. fimbriata*	C	C	G	T	G	G	T	A	A	G	G	T	T	G	C	C	A	T	G	A	A	G	G	A	G	G	T	C	G	A	A	G	A	C	C	A
CMW 5943 *C. albofundus*	C	C	G	T	G	G	T	A	A	G	G	T	T	G	C	C	A	T	G	A	A	G	G	A	G	G	T	C	G	A	A	G	A	C	C	A
CMW 5364 *C. albofundus*	C	C	G	T	G	G	T	A	A	G	G	T	T	G	C	C	A	T	G	A	A	G	G	A	G	G	T	C	G	A	A	G	A	C	C	A
CMW 11424 *C. polychroma*	C	C	G	T	G	G	T	A	A	G	G	T	T	G	C	C	A	T	G	A	A	G	G	A	G	G	T	C	G	A	A	G	A	C	C	A
CMW 11436 *C. polychroma*	C	C	G	T	G	G	T	A	A	G	G	T	T	G	C	C	A	T	G	A	A	G	G	A	G	G	T	C	G	A	A	G	A	C	C	A
CMW 11449 *C. polychroma*	C	C	G	T	G	G	T	A	A	G	G	T	T	G	C	C	A	T	G	A	A	G	G	A	G	G	T	C	G	A	A	G	A	C	C	A
CMW 11455 *C. polychroma*	C	C	G	T	G	G	T	A	A	G	G	T	T	G	C	C	A	T	G	A	A	G	G	A	G	G	T	C	G	A	A	G	A	C	C	A
CMW 6569 *C. pirilliformis*	C	C	G	T	G	G	T	A	A	G	G	T	T	G	C	C	A	T	G	A	A	G	G	A	G	G	T	C	G	A	A	G	A	C	C	A
CMW 6579 *C. pirilliformis*	C	C	G	T	G	G	T	A	A	G	G	T	T	G	C	C	A	T	G	A	A	G	G	A	G	G	T	C	G	A	A	G	A	C	C	A
CMW 3276 *C. virescens*	C	C	G	T	G	G	T	A	A	G	G	T	T	G	C	C	A	T	G	A	A	G	G	A	G	G	T	T	G	A	G	G	A	C	C	A

β-tubulin EF1-α

										900										910										920						
CMW 2218 *C. fimbriata*	G	A	T	G	C	G	C	A	A	C	G	T	**C**	C	A	**G**	A	T	C	A	T	**C**	G	A	G	A	A	G	T	T	C	G	A	G	A	A
CMW 2219 *C. fimbriata*	G	A	T	G	C	G	C	A	A	C	G	T	**C**	C	A	**G**	A	T	C	A	T	**T**	G	A	G	A	A	G	T	T	C	G	A	G	A	A
CMW 5943 *C. albofundus*	G	A	T	G	C	G	C	A	A	C	G	T	**C**	C	A	**G**	A	T	C	A	T	**C**	G	A	G	A	A	G	T	T	C	G	A	G	A	A
CMW 5364 *C. albofundus*	G	A	T	G	C	G	C	A	A	C	G	T	**C**	C	A	**G**	A	T	C	A	T	**C**	G	A	G	A	A	G	T	T	C	G	A	G	A	A
CMW 11424 *C. polychroma*	G	A	T	G	C	G	C	A	A	C	G	T	**T**	C	A	**G**	A	T	C	A	T	**C**	G	A	G	A	A	G	T	T	C	G	A	G	A	A
CMW 11436 *C. polychroma*	G	A	T	G	C	G	C	A	A	C	G	T	**T**	C	A	**G**	A	T	C	A	T	**C**	G	A	G	A	A	G	T	T	C	G	A	G	A	A
CMW 11449 *C. polychroma*	G	A	T	G	C	G	C	A	A	C	G	T	**T**	C	A	**G**	A	T	C	A	T	**C**	G	A	G	A	A	G	T	T	C	G	A	G	A	A
CMW 11455 *C. polychroma*	G	A	T	G	C	G	C	A	A	C	G	T	**T**	C	A	**G**	A	T	C	A	T	**C**	G	A	G	A	A	G	T	T	C	G	A	G	A	A
CMW 6569 *C. pirilliformis*	G	A	T	G	C	G	C	A	A	C	G	T	**C**	C	A	**A**	A	T	C	A	T	**C**	G	A	G	A	A	G	T	T	C	G	A	G	A	A
CMW 6579 *C. pirilliformis*	G	A	T	G	C	G	C	A	A	C	G	T	**C**	C	A	**A**	A	T	C	A	T	**T**	G	A	G	A	A	G	T	T	C	G	A	G	A	A
CMW 3276 *C. virescens*	G	A	T	G	C	G	C	A	A	C	G	T	**C**	C	A	**G**	A	T	C	A	T	**C**	G	A	G	A	A	G	T	T	C	G	A	G	A	A

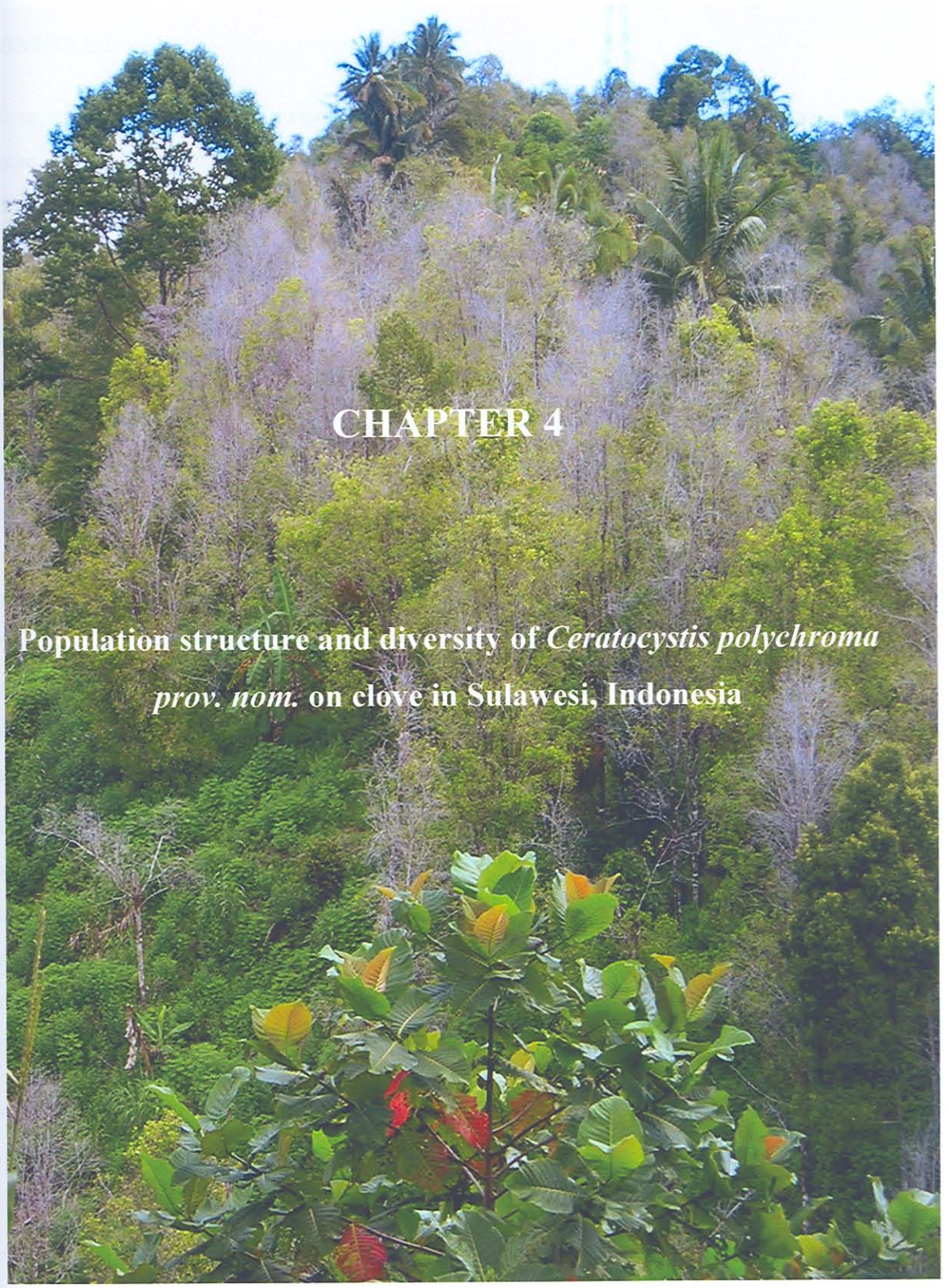
EF1-α

				930										940										950										960		
CMW 2218 *C. fimbriata*	T	A	A	G	T	C	T	C	C	C	C	T	A	T	T	T	**C**	C	C	C	T	C	A	T	T	G	**T**	T	G	T	G	G	A	C	A	G
CMW 2219 *C. fimbriata*	T	A	A	G	T	C	T	C	C	C	C	T	A	T	T	T	**C**	C	C	C	T	C	A	T	T	G	**T**	T	G	T	G	G	A	C	A	G
CMW 5943 *C. albofundus*	T	A	A	G	T	C	T	C	C	C	C	T	A	T	T	T	**C**	C	C	C	T	C	A	T	T	G	**A**	T	G	T	G	G	A	C	A	G
CMW 5364 *C. albofundus*	T	A	A	G	T	C	T	C	C	C	C	T	A	T	T	T	**C**	C	C	C	T	C	A	T	T	G	**A**	T	G	T	G	G	A	C	A	G
CMW 11424 *C. polychroma*	T	A	A	G	T	C	T	C	C	C	C	T	A	T	T	T	**C**	C	C	C	T	C	A	T	T	G	**T**	T	G	T	G	G	A	C	A	G
CMW 11436 *C. polychroma*	T	A	A	G	T	C	T	C	C	C	C	T	A	T	T	T	**C**	C	C	C	T	C	A	T	T	G	**T**	T	G	T	G	G	A	C	A	G
CMW 11449 *C. polychroma*	T	A	A	G	T	C	T	C	C	C	C	T	A	T	T	T	**C**	C	C	C	T	C	A	T	T	G	**T**	T	G	T	G	G	A	C	A	G
CMW 11455 *C. polychroma*	T	A	A	G	T	C	T	C	C	C	C	T	A	T	T	T	**C**	C	C	C	T	C	A	T	T	G	**T**	T	G	T	G	G	A	C	A	G
CMW 6569 *C. pirilliformis*	T	A	A	G	T	C	T	C	C	C	C	T	A	T	T	T	**A**	C	C	C	T	C	A	T	T	G	**T**	T	G	T	G	G	A	C	A	G
CMW 6579 *C. pirilliformis*	T	A	A	G	T	C	T	C	C	C	C	T	A	T	T	T	**A**	C	C	C	T	C	A	T	T	G	**T**	T	G	T	G	G	A	C	A	G
CMW 3276 *C. virescens*	T	A	A	G	T	C	T	C	C	C	C	C	A	-	-	T	**C**	C	A	G	T	C	-	-	-	-	**T**	T	-	-	-	-	A	C	-	-



EF1-α

									1080										1090									
CMW 2218 *C. fimbriata*	T	-	-	-	-	-	C	T	A	A	A	T	G	**A**	C	**G**	T	T	G	C	A	T	G	C	T	G
CMW 2219 *C. fimbriata*	T	-	-	-	-	-	C	T	A	A	A	T	G	**A**	C	**G**	T	T	G	C	A	T	G	C	T	G
CMW 5943 *C. albofundus*	T	**T**	**T**	-	-	-	C	T	A	A	A	T	G	**G**	C	**T**	T	T	G	C	A	T	G	C	T	G
CMW 5364 *C. albofundus*	T	**T**	**T**	**G**	-	-	C	T	A	A	A	T	G	**G**	C	**T**	T	T	G	C	A	T	G	C	T	G
CMW 11424 *C. polychroma*	T	**T**	**T**	**T**	**C**	**T**	C	T	A	A	A	T	G	**G**	C	**G**	T	T	G	C	A	T	G	C	T	G
CMW 11436 *C. polychroma*	T	**T**	**T**	-	**C**	**T**	C	T	A	A	A	T	G	**G**	C	**G**	T	T	G	C	A	T	G	C	T	G
CMW 11449 *C. polychroma*	T	**T**	**T**	**T**	**C**	**T**	C	T	A	A	A	T	G	**G**	C	**G**	T	T	G	C	A	T	G	C	T	G
CMW 11455 *C. polychroma*	T	**T**	**T**	**T**	**C**	**T**	C	T	A	A	A	T	G	**G**	C	**G**	T	T	G	C	A	T	G	C	T	G
CMW 6569 *C. pirilliformis*	T	-	-	-	**C**	**T**	C	T	A	A	A	T	G	**G**	C	**G**	T	T	G	C	A	T	G	C	T	G
CMW 6579 *C. pirilliformis*	T	-	-	-	**C**	**T**	C	T	A	A	A	T	G	**G**	C	**G**	T	T	G	C	A	T	G	C	T	G
CMW 3276 *C. virescens*	T	**T**	**T**	-	**C**	-	G	-	A	A	T	T	C	**G**	T	**T**	G	G	T	T	G	G	-	A	T	C



CHAPTER 4

Population structure and diversity of *Ceratocystis polychroma prov. nom.* on clove in Sulawesi, Indonesia

ABSTRACT

Clove trees in Northern Sulawesi, Indonesia are seriously affected by a decline disease, the cause of which is poorly understood. Clove decline, characterised by wilting, defoliation and tree death is considered to be the most serious problem affecting clove production in this area. An important component of the disease is infestation of trees by the Cerambycid woodborer *Hexamitodera semivelutina*. A newly described and pathogenic *Ceratocystis* sp., *C. polychroma prov. nom.* is also consistently associated with the dying trees. Based on culture morphology, isolates of *C. polychroma prov. nom.* can be separated into three groups, but DNA sequence comparisons for three different gene regions show no differences between them. The aim of this study was to assess the genetic diversity amongst a population of isolates of *C. polychroma prov. nom.* and thus to consider whether phenotypic differences in cultures can be defined genetically. Microsatellite markers developed for the related tree pathogen, *C. fimbriata* were assessed for their usefulness in studying *C. polychroma prov. nom.* Effective markers were then used to infer allele frequencies, which were used in population genetic analysis for 50 isolates of *C. polychroma prov. nom.* Ten of the eleven microsatellite markers developed for *C. fimbriata*, successfully amplified microsatellite regions in *C. polychroma prov. nom.*, confirming the close relatedness of these fungi. The fungus had a high gene diversity of $\overline{H}$ = 0.402. No unique alleles were observed for any of the three morphological groups. The genotypic diversity was very high, (G_{ST} = 44.46) with a maximum of 89.28 %. Linkage disequilibrium analysis revealed that the population reproduces predominantly clonally, but that outcrossing and the generation of new allelic combinations occurs at low frequency. Data from this study suggest that *C. polychroma prov. nom.* is endemic in Sulawesi.

Clove decline is considered to be the most serious problem affecting clove production in Sulawesi. In some plantations, it results in up to 80 % mortality. Dieback affects both seedlings and fully grown trees. The disease is characterised by rapid wilting, defoliation, and twig dieback from the tips that proceeds downwards. Branches and ultimately entire trees die (Liew *et al*. 2003).

Ceratocystis polychroma prov. nom. Van Wyk, Liew & Wingfield is a recently described species associated with clove decline in Northern Sulawesi (Chapter 3). The fungus is associated with rapid wilting and death of full-grown trees. A recent investigation has revealed the presence of the trunk borer, *Hexamitodera semivelutina* Hell. (Coleoptera: Cerambycidae) in association with *C. polychroma prov. nom.* (Liew *et al*. 2003, Chapter 3). This insect is a well-known pest of *S. aromaticum* L. Merr. & Perry that has invaded many Indonesian islands in the past ten years (Anonymous 2002). Preliminary tests have shown that it is an aggressive pathogen (Wingfield & Liew unpublished). It is, however, not known whether this is a specific association between a fungus and insect or a chance infection of wounds by *C. polychroma prov. nom.*

The association of *C. polychroma prov. nom.* and its apparent contribution to clove decline in Sulawesi is not surprising. This fungus is closely related to *C. fimbriata* Ellis & Halsted, which is a well-known pathogen of many woody plant species (Kile 1993). Recent studies based on DNA sequence comparisons have shown that *C. fimbriata* represents a species complex (Barnes *et al*. 2001a). Thus new species that have previously been treated under the name *C. fimbriata* have been described. The serious wilt pathogen of *Acacia mearnsii* de Wild. in South Africa, *C. albofundus* De Beer, Wingfield & Morris (Wingfield *et al*. 1996) and the *Eucalyptus* pathogen *C. pirilliformis* (Barnes *et al*. 2003) represent two good examples. *Ceratocystis polychroma prov. nom.* appears to be another cryptic species in the *C. fimbriata* species complex, and all of these fungi are evidently pathogenic.

Ceratocystis fimbriata is a homothallic fungus that undergoes unidirectional mating type switching (Olsen 1949, Webster & Butler 1967, Harrington & McNew 1997). Sexual reproduction in ascomycetes is controlled by two opposite mating type genes. In homothallic

species like *C. fimbriata*, both genes (MAT-1 and MAT-2) exist in a single individual, thus self-fertile strains contain both MAT-1 and MAT-2 genes. Single ascospore cultures produced from a selfing event result in approximately half self-sterile strains and half self-fertile strains (Harrington & McNew 1997). In self-fertile strains, the MAT-1 gene has been deleted and this is known as uni-directional mating type switching (Harrington & McNew 1997). These strains cannot self but can cross with self-fertile strains (Harrington & McNew 1997, Witthuhn *et al.* 2000). Although the sexual behaviour of *C. polychroma prov. nom.* has not been studied critically, the fungus appears to be similar to *C. fimbriata*, where single ascospores give rise to either self-sterile or self-fertile isolates.

In earlier studies (Chapter 3), variation in the morphology of *C. polychroma prov. nom.* cultures has been seen. One objective of this study was to use DNA sequence analysis to ascertain if these fungi are the same. The close phylogenetic relationship between *C. polychroma prov. nom.* and *C. fimbriata* (Chapter 3) suggests that tools used to study the latter pathogen might be useful in studying the fungus associated with clove decline. Barnes *et al.* (2001a) developed 11 microsatellite markers for population studies of *C. fimbriata*. These markers have been successfully applied in population studies with *C. albofundus* (Barnes 2002, Nakabonge 2002). Thus, a further aim was to test whether the microsatellite markers developed for *C. fimbriata* could be used in studies of *C. polychroma prov. nom.* and if so, to consider the genetic diversity of a population of *C. polychroma prov. nom.* from dying clove trees in Indonesia. It was hoped that this might resolve questions relating to the different cultural characteristics amongst isolates and to provide some indication of the probable origin of the fungus.

MATERIALS AND METHODS

Fungal isolates and DNA extractions

Fungal isolates were obtained from *Syzygium aromaticum* trees that showed symptoms of decline. Eighteen sample sites in Sulawesi were selected (Table 1 & Fig. 1) for isolation. Isolates were collected either by transferring ascospores from the necks of ascomata in the galleries of the clove borer or by carrot baiting of discoloured vascular tissue as described by Moller & De Vay (1968).

Cultures were purified by transferring masses of ascospores from the tips of ascomatal necks, mycelium or conidial masses from the primary isolation plates onto 2 % (w/v) Malt Extract Agar (MEA) (Biolab, Midrand, South Africa). All cultures 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 2).

Three different groups were identified amongst isolates based solely on cultural characteristics. Cultures were transferred three times by taking single ascospore droplets to ensure that the cultural characteristic remained constant throughout each transfer. The groups were defined based solely on this cultural character; no further studies on the morphology of isolates were considered.

For DNA extraction, isolates (Table 1) were grown on 2 % (w/v) MEA plates for 2 weeks. Mycelium was scraped from the surface of these cultures using a scalpel, and transferred to Eppendorf tubes and lyophilised for 2 days. The lyophilised mycelium was placed in liquid nitrogen, ground to a fine powder using a glass rod, and DNA was extracted using a modified version of the method described by Barnes *et al.* (2001b).

PCR amplification

For the DNA sequence analysis the gene regions chosen were the ITS regions. These gene regions were amplified with the primers ITS1 and ITS4 (White *et al.* 1990). The PCR reactions and annealing temperature were the same as for Chapter 3. Eleven microsatellite primers previously developed for *C. fimbriata* (Barnes *et al.* 2001a) were tested for use in this study. PCR reactions were specific for each primer, as determined by the optimum annealing temperature (Barnes *et al.* 2001a). The PCR reactions consisted of 2 ng genomic DNA, 1 x Expand High Fidelity Buffer containing 1.5 mM $MgCl_2$ (Roche Molecular Biochemicals), 200 µM of each dNTP, 300 nM of the forward and reverse primer (Barnes *et al.* 2001a) and 0.35 U Expand High Fidelity enzyme. Sterile water was used to adjust the final volume of each reaction to 25 µl. The PCR reaction was initiated with an initial denaturing step of 2 min at 96 °C. This was followed by 10 cycles of 20 s at 94 °C, annealing at 48 s and an extension for 45 s at 72 °C. Another 25 cycles, with a 5 s time increase on the annealing temperature followed. The final elongation cycle was at 72 °C for 10 min. The PCR products were electrophoresed on a 2 % (20 % w/v) agarose gel containing ethidium bromide and visualised

using ultra violet light. A 100 bp molecular weight marker (Roche Diagnostics, Mannheim, Germany) was included to determine the approximate size and concentrations of the amplicons.

DNA Sequencing and analyses

The ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit was used according to the manufacturer's protocols (Applied BioSystems, Foster City, California) to obtain DNA sequences of the PCR amplicons from both directions. The same primer pairs and cleaning techniques were used for the sequence reactions as for the PCR reactions. Sequence reactions were run on an ABI PRISM™ 3100 Autosequencer (Applied BioSystems, Foster City, California, U.S.A) and sequence electropherograms were analysed using Sequence Navigator version 1.0.1 (Applied BioSystems, Foster City, California).

The sequences obtained for the three different cultural groups of *Ceratocystis polychroma prov. nom.* were compared with those of morphologically similar *Ceratocystis* spp. that are available in GenBank (Table 2). Sequences were aligned manually and analysed using PAUP version 4.0b10* [Phylogenetic Analysis Using Parsimony (and other methods)] (Swofford 2002). All data were treated as described in Chapter 3. *Ceratocystis virescens* (Davids.) Moreau was used as the out-group. 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 posterior probability of nodes in the phylogenetic tree was determined using the Markov Chain Monte Carlo (MCMC) method (Larget & Simon 1999), with a Bayesian framework. One hundred thousand random trees were generated, sampling every 100th tree and printing every 10th tree. A number of trees generated (4700) had to be discarded as they might have been sampled before the convergence of the Markov chain. For the analysis of the ITS region, gamma rate heterogeneity was set, and no codon specific sites were included. For the β-tubulin and EF1-α sequences, codon specific sites were specified with a site-specific substitution rate and the site partition was treated as a by-codon.

Genescan analyses

Three different sets for genescan analyses were assembled as described by Barnes *et al.* (2001a). Each reaction consisted of 0.2 µl of the different amplicons, 0.3 µl of the loading dye (Perkin Elmer Corporation, California, USA), 1.1 µl formamide as well as 0.6 µl of an internal size standard, GENESCAN-500 TAMRA (Perkin Elmer Corporation, California, USA). PCR amplicons were size fractionated on a 4.25 % PAGE (Polyacrylamide Gel Electrophoresis) gel, on an ABI Prism 377™ DNA sequencer. The sizes of the DNA fragments were determined using a combination of the software programs GeneScan® 2.1 (Perkin Elmer Corporation, California, USA) and Genotyper® 3.0 (Perkin Elmer Corporation, California, USA).

Analyses of data

Gene diversity ($\overline{H}$) (Nei 1973) was calculated to consider the probability of sampling two different alleles at the same locus within a population. A matrix was assembled where absence "0" or presence "1" of specific alleles was noted. The frequency of each allele in the population was determined and gene diversity was calculated where

$$\overline{H} = 1 - \sum_k x_k^2$$

and x^2 is the frequency of the k^{th} genotype (Nei 1973). Genotypic diversity ($\hat{G}$) and the probability that two individuals taken at random in a population have different genotypes (Nei 1973) were calculated. The number of different genotypes present in the population was calculated by allocating an alphabetic letter to each allelic size at that specific locus. Isolates with the same profile thus had the same genotype. Genotypic diversity was calculated using the formula

$$\hat{G} = \frac{1}{\left[\sum fx\left(\frac{x}{N}\right)^2\right]}$$

where N is the sample size, and f is the number of genotypes occurring x times in the sample (Stoddart & Taylor 1988). To determine whether a sufficiently large population of isolates was used in the study, the genotypic diversity was modelled against the number of loci with 1000 resampling repetitions to produce a sigmoidal graph (Stoddart & Taylor 1988).

To determine the level of linkage disequilibrium, the index of association (I_A) was calculated (Taylor, Jacobson & Fisher 1999). The software program Multilocus (Agapow & Burt 2001) was used to randomise the data set (1000 randomisations) in order to model the observed I_A against an optimally outcrossing population of the same genetic background. Therefore, the null hypothesis assumes random mating, and can be rejected with high significance if the observed value does not conform to random mating.

RESULTS

Fungal isolates and DNA extractions

All sites where isolations were made were in the North of Sulawesi. In total, more than 200 isolates of *C. polychroma prov. nom.* were collected from 18 different trees at 18 sites (Table 1 & Fig. 1). Of these isolates, 50 were selected to represent a population of *C. polychroma prov. nom.* to be used for the purpose of this chapter. Isolates were obtained either directly from separate ascomata in the tunnels of the woodborer or from carrot baiting of the discoloured wood.

Three distinct colony morphologies were observed for isolates of *C. polychroma prov. nom.* (Fig. 2). Isolates representing Group I had a flat appearance with little to no aerial mycelium. The white appearance was a result of masses of conidia that formed on the surface of cultures. Isolates in this group were fast growing and represented the largest number obtained from any area. Isolates representing Group II had more aerial mycelium than those in Group I. Group II isolates had a greenish colour and were slower growing than Group I isolates. Isolates residing in Group III had a woolly texture when compared to the other two groups. Group II isolates had a mixture of white and green colour and these were the slowest growing of all three groups of isolates. Isolates in all three different groups produced perithecia. For further study, 20 isolates from Group I, 23 isolates from Group II and seven isolates representing Group III were chosen (Table 1).

PCR amplification

For amplification of the gene regions for DNA sequence analysis, an average amplicon size of 500 bp was obtained for both the ITS regions. PCR products were obtained for ten of the eleven microsatellite marker primers designed for *C. fimbriata* (Barnes *et al.* 2001a). The

primer pair CF 15/16 gave no results even after changing parameters and concentrations of the PCR reaction conditions. All temperatures used for the amplification of the ten *C. polychroma prov. nom.* loci were the same as those for *C. fimbriata* (Barnes *et al.* 2001a).

DNA Sequencing and analyses

The data set obtained from the ITS regions produced 538 characters (Appendix), with 75 most parsimonious trees of which one was chosen for presentation (Fig. 3). The tree had a length of 430, with a consistency index (CI) of 0.9628, a homoplasy index of 0.0372, a retention index (RI) of 0.9175 and a rescaled consistency index (RC) of 0.8834. The bootstrap values for the different clades were; 92 % for *C. polychroma prov. nom.*, 100 % for *C. pirilliformis* Barnes & Wingfield, 91 % for *C. fimbriata* and 98 % for *C. albofundus* Wingfield, De Beer & Morris. The Bayesian inference programme's posterior probability of the branch nodes supported the bootstrap values. The posterior probability for the branch nodes for the *C. pirilliformis, C. fimbriata* and *C. albofundus* clades were 99 %, 80 % and 98 %, respectively. Isolates of *C. polychroma prov. nom.* resided in a single discrete clade that grouped separately from all the other clades, with its own posterior probability of 99 % (Fig. 3). No sub-clades or separate clades were evident for the three cultural groups present.

Genescan analyses

The ten microsatellite primers amplified a total of 35 alleles for *C. polychroma nom prov.* (Table 3). One of the loci (CF 15/16) produced a monomorphic allele of 155 bp (Table 3). No unique alleles were observed for any of the three groups of isolates based on morphological differences. Thus, isolates representing these three apparent sub-groups were subsequently treated as a single population.

Analyses of data

Loci with two or three observed alleles (AG 1/2, AG 7/8, AG 15/16 & CF 21/22) exhibited a high allele frequency for one of the alleles, while the other allele(s) had a very low frequency. At loci where more than four alleles were observed (CF 5/6, CF 11/12, CF 17/18), with the exception of one locus (CF 13/14), the distribution of the alleles was interspersed. The frequency for each allele (Table 4) was used to determine the gene diversity (Nei 1973). Loci CF 5/6 and CF 17/18 had the highest gene diversity of 0.781 and 0.734, respectively. The

overall gene diversity ($\overline{H}$) of the population of isolates was $\overline{H} = 0.402$. Locus CF 23/24 was monomorphic and had a gene diversity of 0. This locus was omitted during calculation and did not influence the overall gene diversity of the population. Forty-eight different multilocus genotypes were inferred from the allelic data. Only two genotypes were observed more than once (Table 5). The maximum percentage of genotypic diversity ($\hat{G}$) was 89.28 %. A plot of genotypic diversity vs. number of loci showed that the data matrix was sufficiently large to allow population genetic inference (Fig. 4). The observed linkage disequilibrium (I_A) was 0.20 and was located within the distribution range for the randomised data sets (P = 0.20) (Fig. 5). The null hypothesis that the population is in linkage equilibrium could thus be rejected at a confidence level of P = 0.20 (80 % confidence).

DISCUSSION

In this study we have shown that a newly discovered and interesting fungus, *C. polychroma prov. nom.* associated with clove decline in Sulawesi, is probably endemic to the area. Isolates of the fungus had a high level of genetic diversity, which suggests that it has been present in Northern Sulawesi for a long time. This is in contrast to what one would expect from a newly introduced pathogen, where the population would most likely be clonal (McDonald 1997).

DNA sequence analysis for the ITS regions showed no evidence of sub-clade formation correlating with the three different groups representing culture morphology. Rather, all the isolates of *C. polychroma prov. nom.* grouped together in one clade separate from the other *Ceratocystis* spp. included in this study. This suggests that differences in culture morphology are probably due to a small number of unlinked genes, which control these morphological differences. Microsatellite marker analysis used in this study also showed no correlation to differences in colony morphology.

Based on morphology of cultures, isolates of *C. polychroma nom prov.* could be placed in one of three different groups. However, comparison of these isolates using microsatellite makers showed no clear genetic difference between them. This is consistent with the DNA sequence comparisons using the ITS gene region. There were two isolates that originated from different geographical areas and grouped within different cultural groups but had the same genotype.

This was unexpected, as the genetic differentiation is high within this population. This provides further evidence that there is no basis for grouping *C. polychroma prov. nom.* into three groups based on differences in culture morphology.

This study was facilitated by the fact that most polymorphic markers designed for *C. fimbriata* (Barnes *et al.* 2001a) could be used to study *C. polychroma prov. nom.* These results support DNA based comparisons showing that the two fungi are phylogenetically closely related (Chapter 3). These primers have also been used on the closely related *C. albofundus* (Barnes 2002, Nakabonge 2002), and it is likely that they will be useful for other cryptic species for example *C. pirilliformis* (Barnes *et al.* 2003, Marin 2003) that evidently reside in the *C. fimbriata* species complex.

A total of 35 alleles were identified in the isolates of *C. polychroma prov. nom.*, using ten microsatellite primer pairs. Differences in size of the alleles were observed between isolates of *C. fimbriata*, *C. albofundus* and *C. polychroma prov. nom.* Similar differences were also observed between *C. albofundus* and *C. polychroma prov. nom.* Although microsatellite markers and allele sizes derived from them are not typically appropriate for distinguishing between species, they clearly reflect phylogenetic relationships in the fungi mentioned above. This has also been found in various other fungi such as *Diplodia pinea* (Desm.) Kickx (Burgess, Wingfield & Wingfield 2001) species in the *C. polonica* complex (Marin 2003) and within different phylogenetic groups recognised in *C. fimbriata* (Marin 2003).

At the loci where only two or three alleles were observed, a high allele frequency was observed for one of the alleles, while the other alleles had a very low frequency. This could indicate fixation, where there is a random genetic drift. Genetic drift is the chance that allele frequency can change due to the drawing of the same gametes in the population (Hartl & Clark 1997). This can also be as a result of selection, based on the fact that the one allele is genetically more stable or favourable than the others (Hartl & Clark 1997). Alternatively, the single high allele frequency could indicate an introduction of a selectively advantageous genotype into the population.

Gene diversity for a large collection of *C. polychroma prov. nom.* isolates was high ($\overline{H}$ = 0.402) relative to studies on various other fungi (Leung & Williams 1986, Boeger, Chen & McDonald 1993, Goodwin *et al.* 1993, Barnes *et al.* 2001a). The highest gene diversity was obtained for loci CF 5/6 and CF 17/18 indicating a more equal distribution, signifying a high level of variation at this locus. The high level of gene diversity suggests that *C. polychroma prov. nom.* has been present in Sulawesi for a long time. This fungus appears to have a sexual system similar to that of *C. fimbriata*, which is homothallic but with a capacity to outcross (Olson 1949, Webster & Butler 1967, Harrington & McNew 1997). If it had been recently introduced into Sulawesi, we would have expected a much more limited genetic diversity with distinguishable clonal lineages, and this would be similar to the situation with *C. fimbriata* in Uruguay (Barnes 2002). This study included 50 isolates and only two genotypes occurred more than once. The maximum genotypic diversity (89.28 %) was very high; also supporting the view that *C. polychroma prov. nom.* is probably native to Sulawesi.

Linkage disequilibrium for *C. polychroma prov. nom.* showed that the population is predominantly clonal. This is consistent with the fact that the fungus is homothallic and able to produce perithecia from single ascospores. In this regard, it is most like *C. fimbriata* and *Cryphonectria cubensis* (Bruner) Hodges, which is homothallic but where genetic outcrossing can occur (Webster & Butler 1967, Harrington & McNew 1997, Van der Merwe 2000). The test suggests that recombination occurs only occasionally. Self-crossing as well as out-crossing are typically observed in homothallic fungi. Some of the alleles are in equilibrium while some alleles are in disequilibrium. This result is typical of a homothallic fungus. It suggests that the *C. polychroma prov. nom.* population is clonal but recombination as the result of out-crossing does occur at low frequency.

This study has shown that a large group of isolates of *C. polychroma prov. nom.* reflect a high level of genetic diversity. In this regard, they suggest that the fungus is probably native to Sumatra. The fact that the fungus has not been found elsewhere in the world also suggests that some part of Indonesia is most likely its area of origin. This study is limited by the fact that *C. polychroma prov. nom.* is known only from Sumatra and that there are no other populations of the fungus available for comparison.

Clove decline in Sumatra is a relatively new problem and it is clearly associated with damage due to the borer *Hexamitodera semivelutina*. Hell. (Coleoptera: Cerambycidae). This insect is native on cloves in their area of origin in the Molucca islands and was introduced into nearby Sulawesi, where cloves have been introduced. It would be interesting to know whether *C. polychroma prov. nom.* occurs in the Moluccas, or whether this fungus is unique in Sulawesi, capitalising on a niche provided by the borer. The absence of clove decline in the Moluccas where the insect is present suggests that the fungus might be an important component in the overall disease syndrome.

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Table 1. Isolates of *Ceratocystis polychroma* from Sulawesi, used in this study

Isolate number	Region of isolation	Cultural group allocated to
CMW 11416	Toliangoki B	2
CMW 11418	"	1
CMW 11420	"	1
CMW 11423	"	2
CMW 11429	"	2
CMW 11431	Lahendong	2
CMW 11432	"	1
CMW 11438	"	1
CMW 11439	Leilum	1
CMW 11440	"	1
CMW 11441	Kaiwa	1
CMW 11445	"	1
CMW 11446	"	1
CMW 11448	Rumoong	1
CMW 11451	"	1
CMW 11452	"	1
CMW 11456	"	1
CMW 11460	Tumpaan / Pinamorongan	2
CMW 11462	Munte	1
CMW 11463	"	1
CMW 11464	Lalumpe	2
CMW 11466	"	1
CMW 11467	Tulap	2
CMW 11468	Kakas	1
CMW 11469	"	2
CMW 11470	"	3
CMW 11477	"	2
CMW 11480	"	2
CMW 11482	"	2
CMW 11486	Tinoor	2
CMW 11487	"	3
CMW 11488	Kumelembuai	2
CMW 11490	"	1
CMW 11492	"	3
CMW 11493	Motoling A	3
CMW 11495	Motoling B	2
CMW 11497	"	3
CMW 11498	"	2
CMW 11499	Tambelang B	2
CMW 11501	"	2
CMW 11504	"	2
CMW 11507	"	3
CMW 11510	Tambelang C	2
CMW 11511	"	2
CMW 11512	"	2
CMW 11513	"	3
CMW 11517	Koka B	2
CMW 11518	"	2
CMW 11519	"	1
CMW 11520	Kembes	2

Table 2. Isolates of *Ceratocystis* used in this study.

Species	Isolate no. [a]	Alternative numbers	GenBank accession nr.	Date of isolation	Host	Geographical origin	Associated insect	Collector(s)
C. fimbriata	CMW 4835[b]	None	AF395689[c] N/A[d] N/A[e]	1998	*Gymnocladus dioica*	Colombia	None	Wingfield, M. J.
"	CMW 2219[b]	"	AF395679[c] N/A[f] AY528975[e]	1991	*Platanus*	France	"	Grosclaude, C.
C. albofundus	CMW 2475[b]	"	AF043605[c] N/A[d] N/A[e]	1992	*Acacia mearnsii*	South Africa	"	Mc Leman, S.
"	CMW 2148[b]	"	AF264910[c] N/A[d] N/A[e]	N/A	"	"	"	Morris, M. J.
C. pirilliformis	CMW 6569[b]	"	AF427104[c] N/A[d] AY528982[e]	"	*Eucalyptus nitens*	Australia	"	Wingfield, M. J.
"	CMW 6579[b]	"	AF427105[c] N/A[d] AY528983[e]	"	"	"	"	"
C. polychroma prov. nom	CMW 11418	"	N/A	2002	*Syzygium aromaticum*	Sulawesi, Indonesia	*Hexamitodera semivelutina*	Liew, E. C. Y. & Wingfield, M. J.
"	CMW 11420	"	N/A	"	"	"	"	"
"	CMW 11470	"	N/A	"	"	"	"	"
"	CMW 111487	"	N/A	"	"	"	"	"
"	CMW 11497	"	N/A	"	"	"	"	"
	CMW 11492		N/A	"	"	"	"	"
	CMW 11499		N/A	"	"	"	"	"
	CMW 11501		N/A	"	"	"	"	"
	CMW 11504		N/A	"	"	"	"	"
	CMW 11507		N/A	"	"	"	"	"
	CMW 11513		N/A	"	"	"	"	"
C. virescens	CMW 3276[b]	None	AY528984[c] AY528990[d] AY528991[e]	1963	*Quercus* sp.	USA.	None	Hinds, T.

[a, b, c, d, e] Isolates marked with [a] CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, those marked with [b] were sequenced, GenBank accession numbers that are marked with [c] reprenesnt the ITS sequences, those marked with [d]. represent the β-tubulin sequences and those marked with [e] represent the Elongation Factor sequences.

Table 3. A comparison of alleles obtained from *C. fimbriata*, *C. albofundus* and *C. polychroma* with the microsatellite primers designed for *C. fimbriata*.

Primer pairs	Alleles		
	C. fimbriata[a]	*C. albofundus*[a]	*C. polychroma*
AG 1/2	255	None	261
	263		262
	264		
	265		
	266		
	268		
AG 7/8	285	309	276
	287	322	277
	288	323	
	289	325	
	290	326	
	300	327	
	301	331	
	304	332	
	305		
AG 15/16	274	288	277
	276	293	279
AG 17/18	304	310	275
	305	311	276
	307		
	308		
	311		
	313		
CF 5/6	365	380	374
	367	387	385
	368		387
	369		398
	370		399
	371		400
	382		
	384		
	385		
CF 11/12	217	None	197
	219		198
	220		199
	222		200
			205
			207
			210
CF 13/14	400	None	369
	402		374
	403		376
	405		418
	407		432
	410		446
	413		
	414		
	415		
CF 15/16	477	288	None
	480	293	
	487		
CF 17/18	268	288	271
	271	291	272
	279	292	273
			274
CF 21/22	250	283	232
	255	284	234
	256	285	235
CF 23/24	156	166	155
	160	168	
	168		

[a] Data obtained from Barnes 2002.

Table 4. Alleles, genotype configuration and allele frequency obtained for isolates of *C. polychroma prov. nom.* at each loci.

Locus	Alleles[a]	Genotype configuration[b]	Allele frequency[c]
AG 1/2	261	A	0.68
	262	B	0.32
AG 7/8	276	A	0.70
	277	B	0.30
AG 15/16	277	A	0.94
	279	B	0.06
AG 17/18	275	A	0.20
	276	B	0.80
CF 5/6	374	A	0.26
	385	B	0.06
	387	C	0.02
	398	D	0.16
	399	E	0.26
	400	F	0.24
CF 11/12	197	A	0.06
	198	B	0.38
	199	C	0.08
	200	D	0.04
	205	E	0.08
	207	F	0.14
	210	G	0.02
CF 13/14	369	A	0.66
	374	B	0.04
	376	C	0.06
	418	D	0.06
	432	E	0.04
	446	F	0.02
CF 17/18	271	A	0.22
	272	B	0.28
	273	C	0.18
	274	D	0.32
CF 21/22	232	A	0.04
	234	B	0.90
	235	C	0.06
CF 23/24	155	A	1.00

[a] Observed allele size.

[b] A final representation of the multilocus genotype, for each genotype that has the same configuration the same character was assigned.

[c] Allele frequency is obtained by dividing the total number of occurrence of that allele by the number of isolates in the population.

Table 5. The multilocus genotype for the three *C. polychroma* populations.

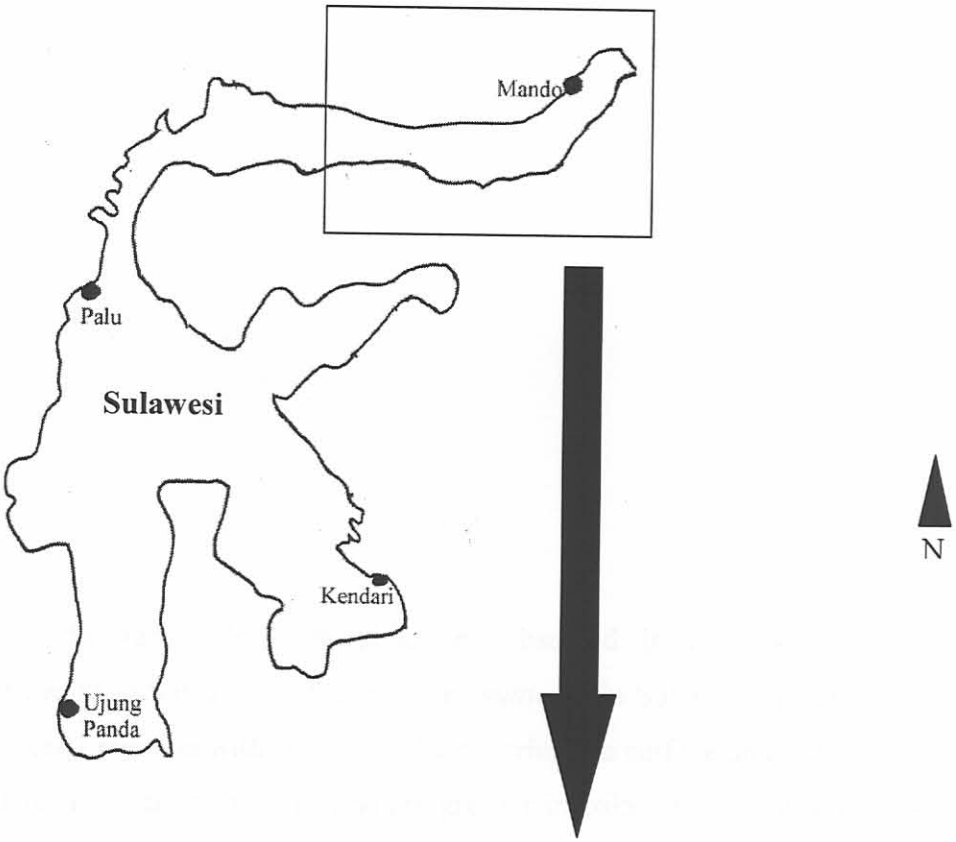
Isolate number	Multilocus genotype	Isolate genotype	Isolate number	Multilocus genotype	Isolate genotype
Group I			**Group II**		
CMW 11418	B B A B F B B B B A	a	CMW 11416	A A A B F F C B C A	u
CMW 11420	B A A A F B C B B A	b	CMW 11423	B B A B E B C B B A	v
CMW 11432	B A A B D B A D B A	c	CMW 11429	A B A B A B B D B A	w
CMW 11438	B A A A A B A D B A	d	CMW 11431	[a] A B A B A B A D B A	l
CMW 11439	A A A B F B A D B A	e	CMW 11460	A A A B A B A C B A	x
CMW 11440	A A A B E C A D B A	f	CMW 11464	B A A A E B A D A A	y
CMW 11441	A B A B E B A D B A	g	CMW 11467	A A A B E G A B B A	z
CMW 11445	A B A B E B A D A A	h	CMW 11469	A A A B F A A B B A	aa
CMW 11446	A A A B E B A C B A	i	CMW 11480	A A A B D B A D B A	bb
CMW 11448	A A A A D B A C B A	j	CMW 11482	A B A B A A A B B A	cc
CMW 11451	B A A B F C A C B A	k	CMW 11488	B A A A A C A A B A	dd
CMW 11452	[a] A B A B A B A D B A	l	CMW 11495	A B A B A E A A B A	ee
CMW 11456	A A A B A B A D B A	m	CMW 11498	A A A B E E A B B A	ff
CMW 11462	A B A B E B A A B A	n	CMW 11499	A B B B B D F C B A	gg
CMW 11463	B A A A E B A D B A	o	CMW 11501	A B A B F F X A B A	hh
CMW 11466	A A A B E B A A B A	p	CMW 11504	B A A B F E X B B A	ii
CMW 11468	[b] A A A A D B A D B A	q	CMW 11510	B B A B E F E A B A	jj
CMW 11477	A A A B A A A A B A	r	CMW 11511	A A A B F F X B B A	kk
CMW 11486	[b] A A A A D B A D B A	q	CMW 11512	A A A B A F X B B A	ll
CMW 11490	A A A A F B A A C A	s	CMW 11517	A A A B C F D B B A	mm
CMW 11519	A A A B D B D C B A	t	CMW 11518	A A A B D B D A B A	nn
			CMW 11520	B A A B A B E C B A	oo
			Group III		
			CMW 11470	A A A B A B A B B A	pp
			CMW 11487	B A A B E C A C B A	qq
			CMW 11492	B A A B D B A A B A	rr
			CMW 11493	B A A A F B A D B A	ss
			CMW 11497	A A A B F E A B B A	tt
			CMW 11507	A B B B B D X A B A	uu
			CMW 11513	B B B B B F X C C A	vv

[a] Indicates the same genotype

[b] Indicates the same genotype

Figure 1. a) Map representing Sulawesi, b) an enlargement of Northern Sulawesi, to indicate (with small dots) the 18 areas where samples were collected.

a)



b)

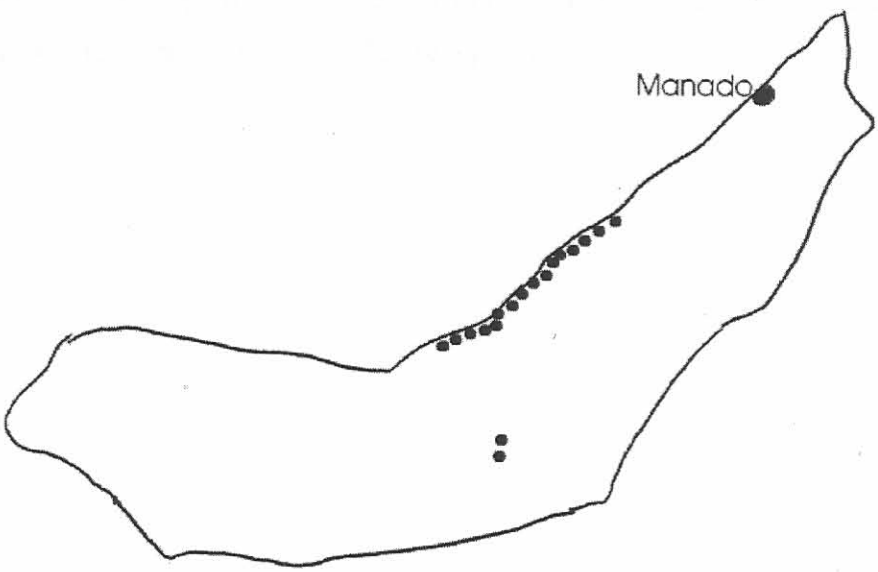


Figure 2. Three different colony morphologies observed in isolates of *Ceratocystis polychroma prov. nom.* isolated from *Syzygium aromaticum* in Sulawesi. a) Group I isolates have little to no aerial mycelia with white conidia covering the surface and a fast growth rate, b) Group II isolates have aerial mycelia and are greener in colour with a slower growth than isolates of Group I, c) Group III isolates have a woolish texture and the slowest growth when compared with isolates representing the other two groups.

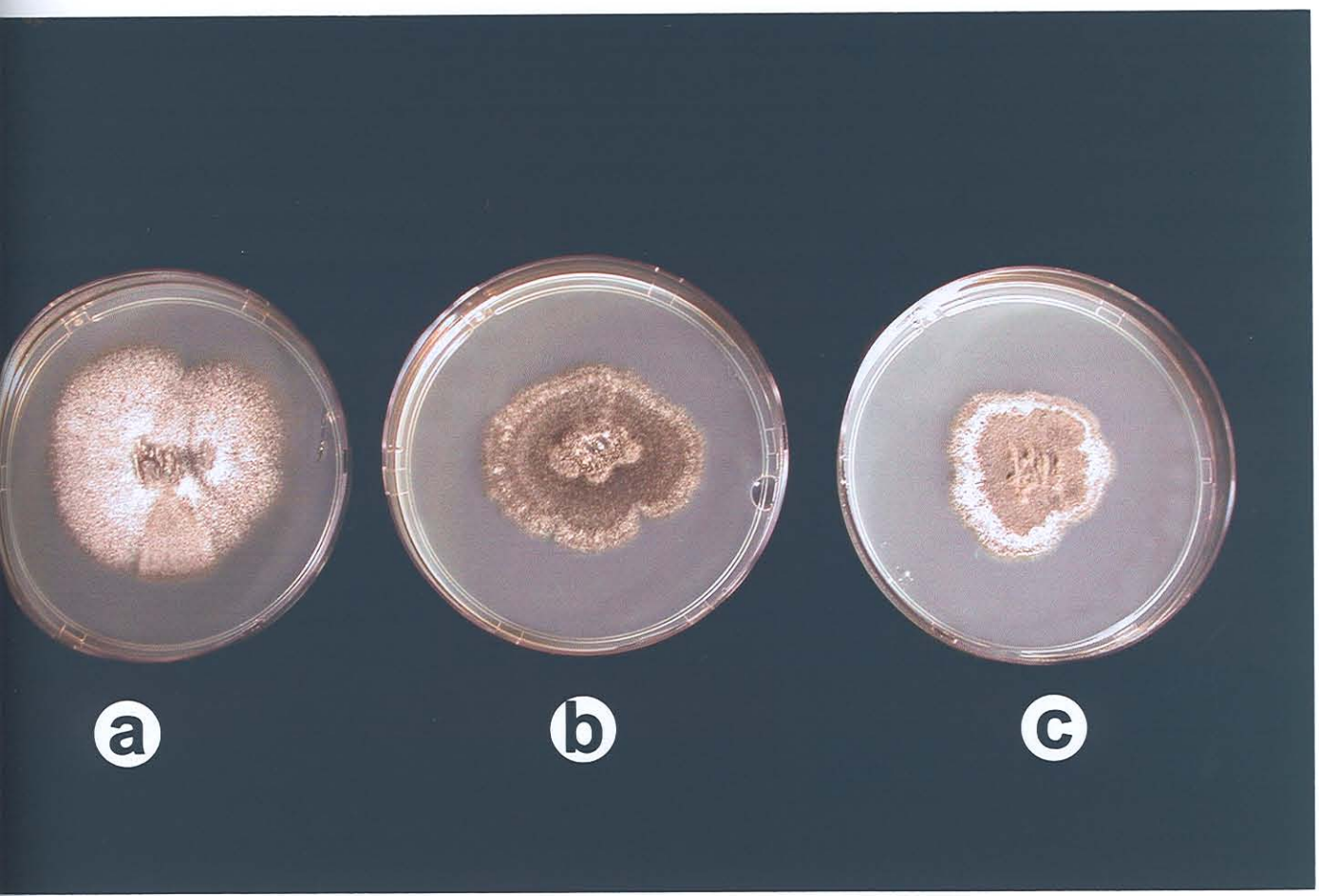


Figure 3. A phylogram representing all the different groups observed for *C. polychroma nom. prov.* Bootstrap values are indicated above the branch while Bayesian node values are indicated below the branches. *Ceratocystis virescens* is treated as the out-group.

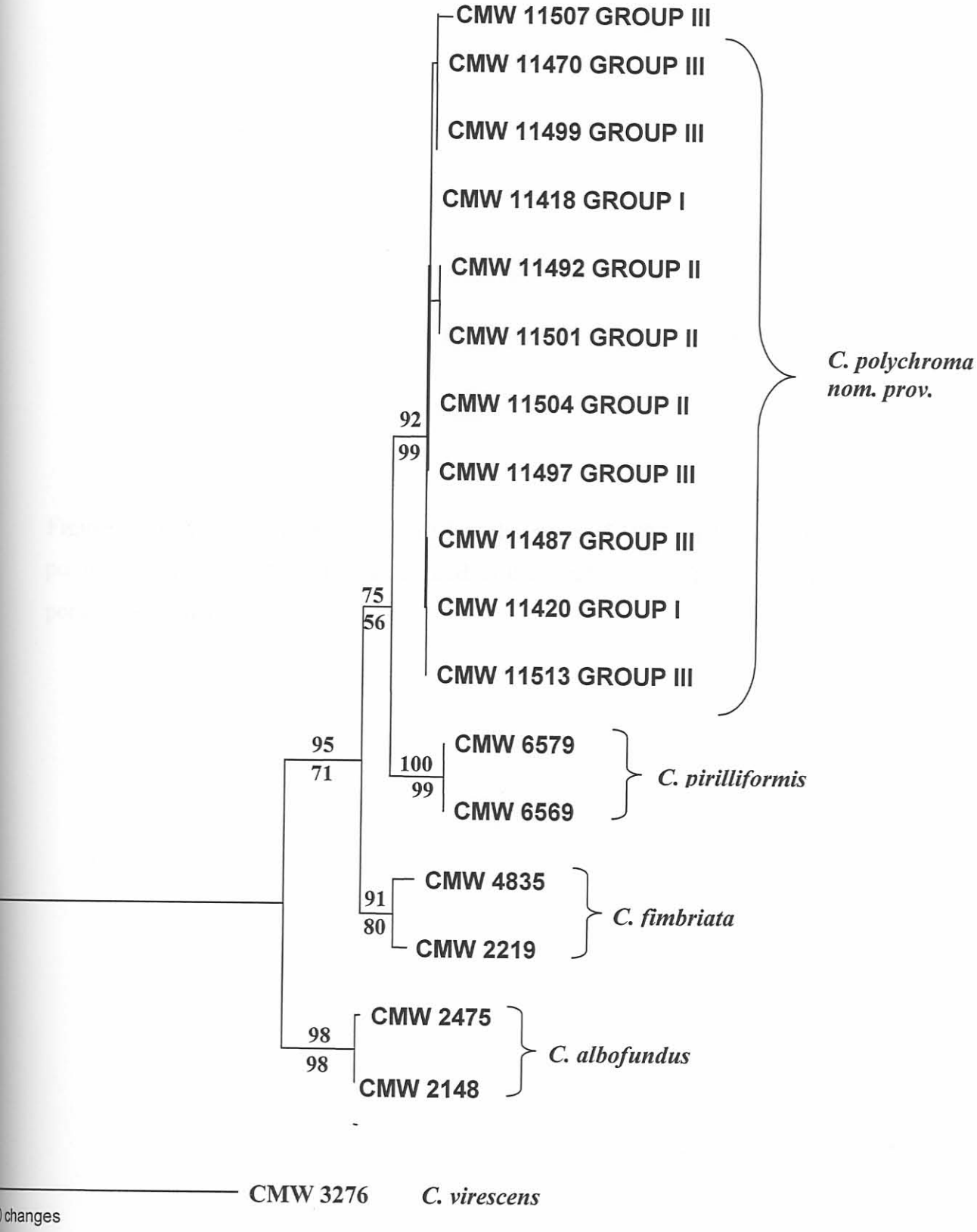

CMW 11507 GROUP III
CMW 11470 GROUP III
CMW 11499 GROUP III
CMW 11418 GROUP I
CMW 11492 GROUP II
CMW 11501 GROUP II
CMW 11504 GROUP II
CMW 11497 GROUP III
CMW 11487 GROUP III
CMW 11420 GROUP I
CMW 11513 GROUP III
C. polychroma nom. prov.
92
99
75
56
95
71
100
99
CMW 6579
CMW 6569
C. pirilliformis
91
80
CMW 4835
CMW 2219
C. fimbriata
98
98
CMW 2475
CMW 2148
C. albofundus
CMW 3276
C. virescens
changes

Figure 4. A plot of G_{ST} vs. number of loci the mean of 1000 randomisations for each data point indicating that the data matrix used in this study was sufficiently large to allow for population gametic analysis.

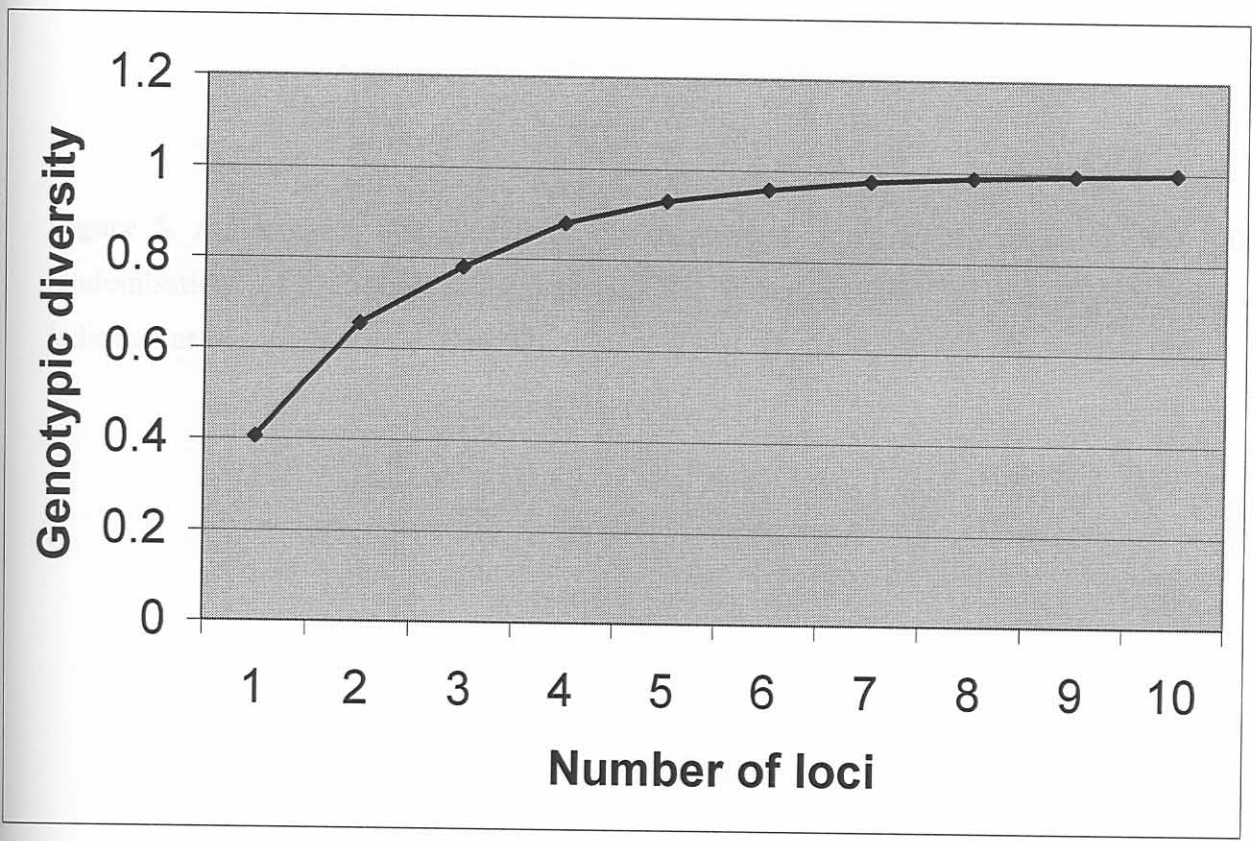
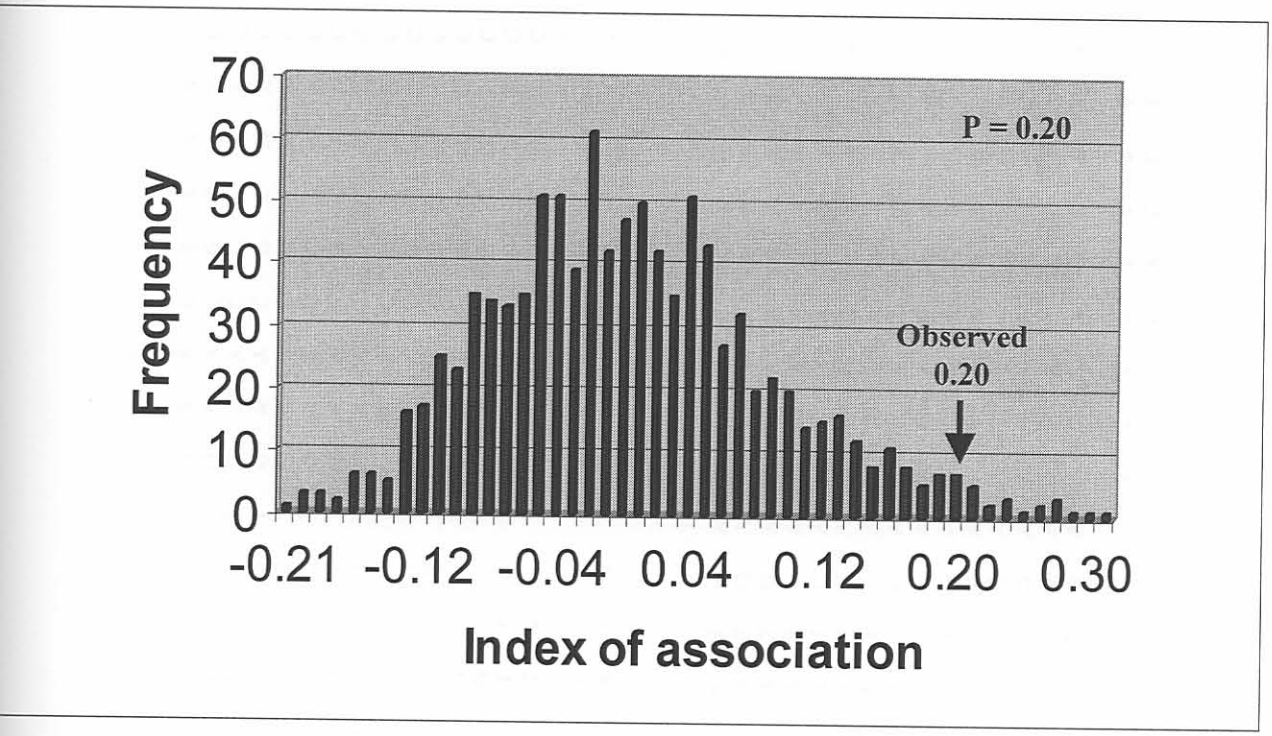


Figure 5. A histogram compiled from the frequencies of associative indices, after 1000 randomisations of the original data matrix. The index obtained for the original data is indicated as an observed vale (P=0.02).



APPENDIX

	1	.	.	.	.	|	.	.	.	.	10	.	.	.	.	|	.	.	.	.	20	.	.	.	.	|	.	.
CMW 11507 *C. polychroma prov. nom.*	C	C	A	T	**G**	T	G	T	G	A	A	C	**T**	T	A	**A**	**A**	**C**	T	A	T	C	T	T	G	T	**G**	**A**
CMW 11418 *C. polychroma prov. nom.*	C	C	A	T	**G**	T	G	T	G	A	A	C	**G**	T	A	**A**	**C**	**T**	T	A	T	C	T	T	G	T	**G**	**A**
CMW 11470 *C. polychroma prov. nom.*	C	C	A	T	**G**	T	G	T	G	A	A	C	**G**	T	A	**A**	**C**	**T**	T	A	T	C	T	T	G	T	**G**	**A**
CMW 11499 *C. polychroma prov. nom.*	C	C	A	T	**G**	T	G	T	G	A	A	C	**G**	T	A	**A**	**C**	**T**	T	A	T	C	T	T	G	T	**G**	**A**
CMW 11487 *C. polychroma prov. nom.*	C	C	A	T	**G**	T	G	T	G	A	A	C	**G**	T	A	**A**	**C**	**T**	T	A	T	C	T	T	G	T	**G**	-
CMW 11420 *C. polychroma prov. nom.*	C	C	A	T	**G**	T	G	T	G	A	A	C	**G**	T	A	**A**	**C**	**T**	T	A	T	C	T	T	G	T	**G**	-
CMW 11513 *C. polychroma prov. nom.*	C	C	A	T	**G**	T	G	T	G	A	A	C	**G**	T	A	**A**	**C**	**T**	T	A	T	C	T	T	G	T	**G**	-
CMW 11492 *C. polychroma prov. nom.*	C	C	A	T	**G**	T	G	T	G	A	A	C	**G**	T	A	**A**	**C**	**T**	T	A	T	C	T	T	G	T	**G**	**A**
CMW 11504 *C. polychroma prov. nom.*	C	C	A	T	**G**	T	G	T	G	A	A	C	**G**	T	A	**A**	**C**	**T**	T	A	T	C	T	T	G	T	**G**	**A**
CMW 11501 *C. polychroma prov. nom.*	C	C	A	T	**G**	T	G	T	G	A	A	C	**G**	T	A	**A**	**C**	**T**	T	A	T	C	T	T	G	T	**G**	**A**
CMW 11497 *C. polychroma prov. nom.*	C	C	A	T	**G**	T	G	T	G	A	A	C	**G**	T	A	**A**	**C**	**T**	T	A	T	C	T	T	G	T	**G**	**A**
CMW 4835 *C. fimbriata*	C	C	A	T	**G**	T	G	T	G	A	A	C	**A**	T	A	**C**	**C**	**C**	T	A	T	C	T	T	G	T	**A**	**G**
CMW 2219 *C. fimbriata*	C	C	A	T	**G**	T	G	T	G	A	A	C	**G**	T	A	-	**C**	**C**	T	A	T	C	T	T	G	T	**A**	**G**
CMW 6579 *C. pirilliformis*	C	C	A	T	**T**	T	G	T	G	A	A	C	**G**	T	A	**A**	**C**	**C**	T	A	T	C	T	T	G	T	**A**	**C**
CMW 6569 *C. pirilliformis*	C	C	A	T	**T**	T	G	T	G	A	A	C	**G**	T	A	**A**	**C**	**C**	T	A	T	C	T	T	G	T	**A**	**C**
CMW 2475 *C. albofundus*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CMW 2148 *C. albofundus*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CMW 3276 *C. virescens*	C	C	A	T	**A**	T	G	T	G	A	A	C	**A**	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-

	.	.	30	.	.	.	.	|	.	.	.	.	40	.	.	.	.	|	.	.	.	.	50	.	.	.	.	|
CMW 11507 *C. polychroma prov. nom.*	**A**	**G**	**A**	-	A	T	G	A	A	**C**	**A**	**A**	**A**	**A**	**A**	-	-	-	-	G	C	T	G	**C**	**T**	T	T	G
CMW 11418 *C. polychroma prov. nom.*	**A**	-	**G**	**G**	A	T	G	A	A	**C**	**A**	**A**	**A**	**A**	**A**	-	-	-	-	G	C	T	G	**C**	**T**	T	T	G
CMW 11470 *C. polychroma prov. nom.*	**A**	**G**	**A**	-	A	T	G	A	A	**C**	**A**	**A**	**A**	**A**	**A**	-	-	-	-	G	C	T	G	**C**	**T**	T	T	G
CMW 11499 *C. polychroma prov. nom.*	**A**	**G**	**A**	-	A	T	G	A	A	**C**	**A**	**A**	**A**	**A**	**A**	-	-	-	-	G	C	T	G	**C**	**T**	T	T	G
CMW 11487 *C. polychroma prov. nom.*	**A**	**A**	**G**	**G**	A	T	G	A	A	**C**	**A**	**A**	**A**	**A**	**A**	-	-	-	-	G	C	T	G	**C**	**T**	T	T	G
CMW 11420 *C. polychroma prov. nom.*	**A**	**A**	**G**	**G**	A	T	G	A	A	**C**	**A**	**A**	**A**	**A**	**A**	-	-	-	-	G	C	T	G	**C**	**T**	T	T	G
CMW 11513 *C. polychroma prov. nom.*	**A**	**A**	**G**	**G**	A	T	G	A	A	**C**	**A**	**A**	**A**	**A**	**A**	-	-	-	-	G	C	T	G	**C**	**T**	T	T	G
CMW 11492 *C. polychroma prov. nom.*	**A**	-	**G**	**G**	A	T	G	A	A	**C**	**A**	**A**	**A**	**A**	-	-	-	-	-	G	C	T	G	**C**	**T**	T	T	G
CMW 11504 *C. polychroma prov. nom.*	**A**	-	**G**	**G**	A	T	G	A	A	**C**	**A**	**A**	**A**	**A**	-	-	-	-	-	G	C	T	G	**C**	**T**	T	T	G
CMW 11501 *C. polychroma prov. nom.*	**A**	-	**G**	**G**	A	T	G	A	A	**C**	**A**	**A**	**A**	**A**	-	-	-	-	-	G	C	T	G	**C**	**T**	T	T	G
CMW 11497 *C. polychroma prov. nom.*	**A**	-	**G**	**G**	A	T	G	A	A	**C**	**A**	**A**	**A**	**A**	**A**	-	-	-	-	G	C	T	G	**C**	**T**	T	T	G
CMW 4835 *C. fimbriata*	**T**	**G**	**A**	**G**	A	T	G	A	A	**T**	-	-	-	-	-	-	-	-	-	G	C	T	G	**T**	**T**	T	T	G
CMW 2219 *C. fimbriata*	**T**	-	**G**	**G**	A	T	G	A	A	**T**	-	-	-	-	-	-	-	-	-	G	C	T	G	**T**	**T**	T	T	G
CMW 6579 *C. pirilliformis*	**T**	-	**G**	**G**	A	T	G	A	A	**T**	**A**	**A**	**A**	**C**	**A**	**A**	**T**	**A**	**T**	G	C	T	G	**C**	**T**	T	T	G
CMW 6569 *C. pirilliformis*	-	-	**G**	**G**	A	T	G	A	A	**T**	**A**	**A**	**A**	**C**	**A**	**A**	**T**	**A**	**T**	G	C	T	G	**C**	**T**	T	T	G
CMW 2475 *C. albofundus*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	C	T	G	**C**	**C**	T	T	G
CMW 2148 *C. albofundus*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	C	T	G	**C**	**C**	T	T	G
CMW 3276 *C. virescens*	-	-	-	-	-	-	-	-	-	-	-	-	**A**	**C**	**C**	**T**	**A**	**T**	**T**	A	G	C	G	**G**	**C**	T	T	T

	:	:	:	.	60	.	.	.	.	|	.	.	.	.	70	.	.	.	.	|	.	.	.	.	80	.	.	.
CMW 11507 *C. polychroma prov. nom.*	G	T	-	-	-	-	-	-	-	-	-	-	A	G	T	T	G	G	G	G	G	G	G	-	-	-	-	-
CMW 11418 *C. polychroma prov. nom.*	G	T	-	-	-	-	-	-	-	-	-	-	A	G	T	T	G	G	G	G	G	G	G	G	G	-	-	-
CMW 11470 *C. polychroma prov. nom.*	G	T	-	-	-	-	-	-	-	-	-	-	A	G	T	T	G	G	G	G	G	G	G	G	G	-	-	-
CMW 11499 *C. polychroma prov. nom.*	G	T	-	-	-	-	-	-	-	-	-	-	A	G	T	T	G	G	G	G	G	G	G	G	G	-	-	-
CMW 11487 *C. polychroma prov. nom.*	G	T	-	-	-	-	-	-	-	-	-	-	A	G	T	T	G	G	G	G	G	G	G	G	G	-	-	-
CMW 11420 *C. polychroma prov. nom.*	G	T	-	-	-	-	-	-	-	-	-	-	A	G	T	T	G	G	G	G	G	G	G	G	G	-	-	-
CMW 11513 *C. polychroma prov. nom.*	G	T	-	-	-	-	-	-	-	-	-	-	A	G	T	T	G	G	G	G	G	G	G	G	G	-	-	-
CMW 11492 *C. polychroma prov. nom.*	G	T	-	-	-	-	-	-	-	-	-	-	A	G	T	T	G	G	G	G	G	G	G	G	-	-	-	-
CMW 11504 *C. polychroma prov. nom.*	G	T	-	-	-	-	-	-	-	-	-	-	A	G	T	T	G	G	G	G	G	G	G	-	-	-	-	-
CMW 11501 *C. polychroma prov. nom.*	G	T	-	-	-	-	-	-	-	-	-	-	A	G	T	T	G	G	G	G	G	G	G	G	-	-	-	-
CMW 11497 *C. polychroma prov. nom.*	G	T	-	-	-	-	-	-	-	-	-	-	A	G	T	T	G	G	G	G	G	G	G	G	G	-	-	-
CMW 4835 *C. fimbriata*	G	T	-	-	-	-	-	-	-	-	-	-	G	G	T	T	A	G	G	G	-	-	-	-	-	-	-	-
CMW 2219 *C. fimbriata*	G	T	-	-	-	-	-	-	-	-	-	-	G	G	T	A	G	G	G	-	-	-	-	-	-	-	-	-
CMW 6579 *C. pirilliformis*	G	T	-	-	-	-	-	-	-	-	-	-	A	G	T	T	G	G	G	G	G	G	G	G	G	-	A	G
CMW 6569 *C. pirilliformis*	G	T	-	-	-	-	-	-	-	-	-	-	A	G	T	T	G	G	G	G	G	G	G	G	G	G	A	G
CMW 2475 *C. albofundus*	G	T	G	G	G	T	G	T	C	T	G	T	A	G	T	G	G	T	G	T	T	A	A	-	-	-	-	-
CMW 2148 *C. albofundus*	G	T	G	G	G	T	G	T	C	T	G	T	A	G	T	G	G	T	G	T	T	A	A	-	-	-	-	-
CMW 3276 *C. virescens*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	G	C	A	G	G	C	-	-	-

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CMW 11507 *C. polychroma prov. nom.*	-	C	A	C	C	C	C	T	T	C	T	G	T	A	A	A	G	A	A	G	T	T	-	-	-	T	T	C
CMW 11418 *C. polychroma prov. nom.*	-	C	A	C	C	C	-	T	T	C	T	G	T	A	A	A	G	A	A	G	T	T	-	-	-	T	T	C
CMW 11470 *C. polychroma prov. nom.*	-	C	A	C	C	C	-	T	T	C	T	G	T	A	A	A	G	A	A	G	T	T	-	-	-	T	T	C
CMW 11499 *C. polychroma prov. nom.*	-	C	A	C	C	C	-	T	T	C	T	G	T	A	A	A	G	A	A	G	T	T	-	-	-	T	T	C
CMW 11487 *C. polychroma prov. nom.*	-	C	A	C	C	C	-	T	T	C	T	G	T	A	A	A	G	A	A	G	T	T	-	-	-	T	T	C
CMW 11420 *C. polychroma prov. nom.*	-	C	A	C	C	C	-	T	T	C	T	G	T	A	A	A	G	A	A	G	T	T	-	-	-	T	T	C
CMW 11513 *C. polychroma prov. nom.*	-	C	A	C	C	C	-	T	T	C	T	G	T	A	A	A	G	A	A	G	T	T	-	-	-	T	T	C
CMW 11492 *C. polychroma prov. nom.*	-	C	A	C	C	C	-	T	T	C	T	G	T	A	A	A	G	A	A	G	T	T	-	-	-	T	T	C
CMW 11504 *C. polychroma prov. nom.*	-	C	A	C	C	C	-	T	T	C	T	G	T	A	A	A	G	A	A	G	T	T	-	-	-	T	T	C
CMW 11501 *C. polychroma prov. nom.*	-	C	A	C	C	C	-	T	T	C	T	G	T	A	A	A	G	A	A	G	T	T	-	-	-	T	T	C
CMW 11497 *C. polychroma prov. nom.*	-	C	A	C	C	C	-	T	T	C	T	G	T	A	A	A	G	A	A	G	T	T	-	-	-	T	T	C
CMW 4835 *C. fimbriata*	-	-	-	-	-	-	-	-	-	C	C	C	T	T	C	T	G	A	A	A	G	G	G	G	G	G	G	-
CMW 2219 *C. fimbriata*	-	-	-	-	-	-	-	-	-	C	C	C	T	T	C	T	G	A	A	G	G	G	C	A	-	-	-	-
CMW 6579 *C. pirilliformis*	A	G	C	T	C	C	G	-	C	C	T	T	G	T	G	T	G	A	A	G	T	T	T	T	T	T	T	C
CMW 6569 *C. pirilliformis*	A	G	C	T	C	C	G	-	C	C	T	T	G	T	G	T	G	A	A	G	T	T	T	T	T	T	T	C
CMW 2475 *C. albofundus*	-	-	-	-	-	C	C	T	C	T	T	T	T	T	A	T	A	A	G	G	G	G	G	C	A	G	C	C
CMW 2148 *C. albofundus*	-	-	-	-	-	C	C	T	C	T	T	T	T	T	A	T	A	A	G	G	G	G	G	C	A	G	C	C
CMW 3276 *C. virescens*	-	-	-	-	-	-	-	-	-	-	-	-	T	T	G	G	T	A	A	C	A	C	A	A	G	-	-	-

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CMW 11507 *C. polychroma prov. nom.*	T	T	T	C	C	C	A	C	T	A	C	C	A	G	C	A	G	T	A	T	A	A	-	-	-	-	G	T
CMW 11418 *C. polychroma prov. nom.*	T	T	T	C	C	C	A	C	T	A	C	C	A	G	C	A	G	T	A	T	A	A	-	-	-	-	G	T
CMW 11470 *C. polychroma prov. nom.*	T	T	T	C	C	C	A	C	T	A	C	C	A	G	C	A	G	T	A	T	A	A	-	-	-	-	G	T
CMW 11499 *C. polychroma prov. nom.*	T	T	T	C	C	C	A	C	T	A	C	C	A	G	C	A	G	T	A	T	A	A	-	-	-	-	G	T
CMW 11487 *C. polychroma prov. nom.*	T	T	T	C	C	C	A	C	T	A	C	C	A	G	C	A	G	T	A	T	A	A	-	-	-	-	G	T
CMW 11420 *C. polychroma prov. nom.*	T	T	T	C	C	C	A	C	T	A	C	C	A	G	C	A	G	T	A	T	A	A	-	-	-	-	G	T
CMW 11513 *C. polychroma prov. nom.*	T	T	T	C	C	C	A	C	T	A	C	C	A	G	C	A	G	T	A	T	A	A	-	-	-	-	G	T
CMW 11492 *C. polychroma prov. nom.*	T	T	T	C	C	C	A	C	T	A	C	C	A	G	C	A	G	T	A	T	A	A	-	-	-	-	G	T
CMW 11504 *C. polychroma prov. nom.*	T	T	T	C	C	C	A	C	T	A	C	C	A	G	C	A	G	T	A	T	A	A	-	-	-	-	G	T
CMW 11501 *C. polychroma prov. nom.*	T	T	T	C	C	C	A	C	T	A	C	C	A	G	C	A	G	T	A	T	A	A	-	-	-	-	G	T
CMW 11497 *C. polychroma prov. nom.*	T	T	T	C	C	C	A	C	T	A	C	C	A	G	C	A	G	T	A	T	A	A	-	-	-	-	G	T
CMW 4835 *C. fimbriata*	-	-	C	A	C	C	G	C	T	G	C	C	A	G	C	A	G	T	A	T	A	A	G	-	-	-	-	-
CMW 2219 *C. fimbriata*	-	-	-	-	C	C	G	C	T	G	C	C	A	G	C	A	G	T	A	T	A	-	-	-	-	-	G	T
CMW 6579 *C. pirilliformis*	T	T	T	T	C	C	A	C	T	A	C	C	A	G	C	A	G	T	A	T	A	A	A	A	-	-	G	T
CMW 6569 *C. pirilliformis*	T	T	T	T	C	C	A	C	T	A	C	C	A	G	C	A	G	T	A	T	A	A	A	A	-	-	G	T
CMW 2475 *C. albofundus*	C	A	C	T	A	C	C	G	C	T	A	G	C	C	A	C	C	A	G	C	A	G	C	A	T	A	C	A
CMW 2148 *C. albofundus*	C	A	C	T	A	C	C	G	C	T	A	G	C	C	A	C	C	A	G	C	A	G	C	A	T	A	C	A
CMW 3276 *C. virescens*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	C	T	G	C	C	G	G

	140	.	.	.	.	|	.	.	.	.	150	.	.	.	.	|	.	.	.	.	160	.	.	.	.	|	.	.
CMW 11507 *C. polychroma prov. nom.*	A	A	-	-	-	T	C	T	T	C	T	T	C	A	C	C	A	C	-	-	T	-	-	-	A	A	A	A
CMW 11418 *C. polychroma prov. nom.*	C	A	-	-	-	-	C	T	T	C	T	T	T	A	C	C	A	C	-	-	T	-	-	A	A	A	A	A
CMW 11470 *C. polychroma prov. nom.*	C	A	-	-	-	T	C	T	T	C	T	T	T	A	C	C	A	C	-	-	T	-	-	A	A	A	A	A
CMW 11499 *C. polychroma prov. nom.*	C	A	-	-	-	T	C	T	T	C	T	T	T	A	C	C	A	C	-	-	T	-	-	A	A	A	A	A
CMW 11487 *C. polychroma prov. nom.*	C	A	-	-	-	T	C	T	T	C	T	T	T	A	C	C	A	C	-	-	T	-	-	A	A	A	A	A
CMW 11420 *C. polychroma prov. nom.*	C	A	-	-	-	T	C	T	T	C	T	T	T	A	C	C	A	C	-	-	T	-	-	A	A	A	A	A
CMW 11513 *C. polychroma prov. nom.*	C	A	-	-	-	T	C	T	T	C	T	T	T	A	C	C	A	C	-	-	T	-	-	A	A	A	A	A
CMW 11492 *C. polychroma prov. nom.*	C	A	-	-	-	T	C	T	T	C	T	T	T	A	C	C	A	C	-	-	T	-	-	A	A	A	A	A
CMW 11504 *C. polychroma prov. nom.*	C	A	-	-	-	T	C	T	T	C	T	T	T	A	C	C	A	C	-	-	T	-	A	A	A	A	A	A
CMW 11501 *C. polychroma prov. nom.*	C	A	-	-	-	T	C	T	T	C	T	T	T	A	C	C	A	C	-	-	T	-	-	A	A	A	A	A
CMW 11497 *C. polychroma prov. nom.*	C	A	-	-	-	T	C	T	T	C	T	T	T	A	C	C	A	C	-	-	T	-	-	A	A	A	A	A
CMW 4835 *C. fimbriata*	-	-	-	-	-	-	-	-	T	C	T	T	C	A	C	C	A	C	-	-	T	G	G	-	A	A	A	A
CMW 2219 *C. fimbriata*	C	-	-	-	-	-	T	C	G	-	-	-	-	-	C	C	A	C	-	-	T	G	T	-	-	A	-	-
CMW 6579 *C. pirilliformis*	A	G	T	T	T	-	T	C	T	C	T	T	T	A	C	C	A	C	-	-	T	A	A	A	A	A	A	A
CMW 6569 *C. pirilliformis*	A	G	T	-	T	T	T	C	T	C	T	T	T	A	C	C	A	C	-	-	T	-	A	A	A	A	A	A
CMW 2475 *C. albofundus*	A	G	T	C	T	T	-	-	-	-	-	T	T	A	C	C	A	C	T	A	T	-	-	A	A	A	-	C
CMW 2148 *C. albofundus*	A	G	T	C	T	T	-	-	-	-	-	T	T	A	C	C	A	C	T	A	T	-	-	A	A	A	-	C
CMW 3276 *C. virescens*	T	A	G	T	A	T	-	-	-	-	T	T	T	-	-	-	-	-	-	-	-	-	-	A	A	A	A	C

	170	180	190
CMW 11507 *C. polychroma prov. nom.*	C T C - - - - - T T T T T A T T T T T T G T T A G A A A T
CMW 11418 *C. polychroma prov. nom.*	C T C - - - - T T T T T A T T T T T T G T T A G A A A T
CMW 11470 *C. polychroma prov. nom.*	C T C - - - - T T T T T A T T T T T T G T T A G A A A T
CMW 11499 *C. polychroma prov. nom.*	C T C - - - - T T T T T A T T T T T T G T T A G A A A T
CMW 11487 *C. polychroma prov. nom.*	C T C - - - - T T T T T A T T T T T T G T T A G A A A T
CMW 11420 *C. polychroma prov. nom.*	C T C - - - - T T T T T A T T T T T T G T T A G A A A T
CMW 11513 *C. polychroma prov. nom.*	C T C - - - - T T T T T A T T T T T T G T T A G A A A T
CMW 11492 *C. polychroma prov. nom.*	C T C - - - - T T T T T A T T T T T T G T T A G A A A T
CMW 11504 *C. polychroma prov. nom.*	C T C - - - - T T T T T A T T T T T T G T T A G A A A T
CMW 11501 *C. polychroma prov. nom.*	C T C - - - - T T T T T A T T T T T T G T T A G A A A T
CMW 11497 *C. polychroma prov. nom.*	C T C - - - - T T T T T A T T T T T T G T T A G A A A T
CMW 4835 *C. fimbriata*	C T C - T T T T T T A T A T T T T C T - - - A G A - - T
CMW 2219 *C. fimbriata*	C T C - - - - - T T A T A T T T T T C G - - A G A - - T
CMW 6579 *C. pirilliformis*	C T C - - - T T T A A T A T T T T T A T - - A G A A A T
CMW 6569 *C. pirilliformis*	C T C - - - T T T A A T A T T T T T A T - - A G A A A T
CMW 2475 *C. albofundus*	C T T C - - - T G T A T A T T T T T T A - - A A A T T T
CMW 2148 *C. albofundus*	C T T C - - - T G T A T A T T T T T T A - - A A A T T T
CMW 3276 *C. virescens*	T C - T T T T T T T T A T T G T A A - - - A G A A T T A

	200	210	220
CMW 11507 *C. polychroma prov. nom.*	T T T G A T T T C A T T G C T G A G T G G C - - A T A A
CMW 11418 *C. polychroma prov. nom.*	T T T G A T T T C A T T G C T G A G T G G C - - A T A A
CMW 11470 *C. polychroma prov. nom.*	T T T G A T T T C A T T G C T G A G T G G C - - A T A A
CMW 11499 *C. polychroma prov. nom.*	T T T G A T T T C A T T G C T G A G T G G C - - A T A A
CMW 11487 *C. polychroma prov. nom.*	T T T G A T T T C A T T G C T G A G T G G C - - A T A A
CMW 11420 *C. polychroma prov. nom.*	T T T G A T T T C A T T G C T G A G T G G C - - A T A A
CMW 11513 *C. polychroma prov. nom.*	T T T G A T T T C A T T G C T G A G T G G C - - A T A A
CMW 11492 *C. polychroma prov. nom.*	T T T G A T T T C A T T G C T G A G T G G C - - A T A A
CMW 11504 *C. polychroma prov. nom.*	T T T G A T T T C A T T G C T G A G T G G C - - A T A A
CMW 11501 *C. polychroma prov. nom.*	T T T G A T T T C A T T G C T G A G T G G C - - A T A A
CMW 11497 *C. polychroma prov. nom.*	T T T G A T T T C A T T G C T G A G T G G C - - A T A A
CMW 4835 *C. fimbriata*	T T - - - T T T C A T T G C T G A G T G G C - - A T A -
CMW 2219 *C. fimbriata*	T T T - - T T T C A T T G C T G A G T G G C - - A T A A
CMW 6579 *C. pirilliformis*	T T T G A T T T C A T T G C T G A G T G A A - - A T A A
CMW 6569 *C. pirilliformis*	T T T G A T T T C A T T G C T G A G T G A A G C A T A A
CMW 2475 *C. albofundus*	T T T - - A A A A A T T G C T G A G T G G C G C A T A -
CMW 2148 *C. albofundus*	T T T - - A A A A A T T G C T G A G T G G C - - A T A -
CMW 3276 *C. virescens*	A T T - - - - C A T T G C T G A G T G - G C A T T A A C

	.		.	.	.	.	230	.	.	.	.		.	.	.	.	240	.	.	.	.		.	.	.	.	250	.
CMW 11507 *C. polychroma prov. nom.*	**A**	**C**	T	A	**T**	**A**	A	A	**A**	-	-	-	-	**G**	**T**	T	A	A	A	A	C	T	T	T	C	A	A	C
CMW 11418 *C. polychroma prov. nom.*	**A**	**C**	T	A	**T**	**A**	A	A	**A**	**A**	-	-	-	**G**	**T**	T	A	A	A	A	C	T	T	T	C	A	A	C
CMW 11470 *C. polychroma prov. nom.*	**A**	**C**	T	A	**T**	**A**	A	A	**A**	**A**	-	-	-	**G**	**T**	T	A	A	A	A	C	T	T	T	C	A	A	C
CMW 11499 *C. polychroma prov. nom.*	**A**	**C**	T	A	**T**	**A**	A	A	**A**	**A**	-	-	-	**G**	**T**	T	A	A	A	A	C	T	T	T	C	A	A	C
CMW 11487 *C. polychroma prov. nom.*	**A**	**C**	T	A	**T**	**A**	A	A	**A**	**A**	-	-	-	**G**	**T**	T	A	A	A	A	C	T	T	T	C	A	A	C
CMW 11420 *C. polychroma prov. nom.*	**A**	**C**	T	A	**T**	**A**	A	A	**A**	**A**	-	-	-	**G**	**T**	T	A	A	A	A	C	T	T	T	C	A	A	C
CMW 11513 *C. polychroma prov. nom.*	**A**	**C**	T	A	**T**	**A**	A	A	**A**	**A**	-	-	-	**G**	**T**	T	A	A	A	A	C	T	T	T	C	A	A	C
CMW 11492 *C. polychroma prov. nom.*	**A**	**C**	T	A	**T**	**A**	A	A	**A**	**A**	-	-	-	**G**	**T**	T	A	A	A	A	C	T	T	T	C	A	A	C
CMW 11504 *C. polychroma prov. nom.*	**A**	**C**	T	A	**T**	**A**	A	A	**A**	**A**	-	-	-	**G**	**T**	T	A	A	A	A	C	T	T	T	C	A	A	C
CMW 11501 *C. polychroma prov. nom.*	**A**	**C**	T	A	**T**	**A**	A	A	**A**	**A**	-	-	-	**G**	**T**	T	A	A	A	A	C	T	T	T	C	A	A	C
CMW 11497 *C. polychroma prov. nom.*	**A**	**C**	T	A	**T**	**A**	A	A	**A**	**A**	-	-	-	**G**	**T**	T	A	A	A	A	C	T	T	T	C	A	A	C
CMW 4835 *C. fimbriata*	**A**	**C**	T	A	**T**	**A**	A	A	**A**	**A**	**A**	**A**	**A**	**G**	**T**	T	A	A	A	A	C	T	T	T	C	A	A	C
CMW 2219 *C. fimbriata*	-	**C**	T	A	**T**	**A**	A	A	**A**	**A**	-	-	-	**G**	**T**	T	A	A	A	A	C	T	T	T	C	A	A	C
CMW 6579 *C. pirilliformis*	**A**	**A**	T	A	**A**	**T**	A	A	-	-	-	-	-	**G**	**T**	T	A	A	A	A	C	T	T	T	C	A	A	C
CMW 6569 *C. pirilliformis*	**A**	**A**	T	A	**A**	**T**	A	A	-	-	-	-	-	**G**	**T**	T	A	A	A	A	C	T	T	T	C	A	A	C
CMW 2475 *C. albofundus*	**A**	**C**	T	A	**T**	**A**	A	A	**A**	**A**	**A**	-	-	**G**	**T**	T	A	A	A	A	C	T	T	T	C	A	A	C
CMW 2148 *C. albofundus*	**A**	**C**	T	A	**T**	**A**	A	A	**A**	**A**	**A**	-	-	**G**	**T**	T	A	A	A	A	C	T	T	T	C	A	A	C
CMW 3276 *C. virescens*	**A**	**T**	A	A	**T**	**A**	A	-	-	-	-	-	**G**	**T**	**T**	A	A	A	A	C	T	T	T	C	A	A	C	A

	.	.	.		.	.	.	260	.	.	.	.		.	.	.	.	270	.	.	.	.		.	.	.	.	280	.
CMW 11507 *C. polychroma prov. nom.*	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G
CMW 11418 *C. polychroma prov. nom.*	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G
CMW 11470 *C. polychroma prov. nom.*	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G
CMW 11499 *C. polychroma prov. nom.*	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G
CMW 11487 *C. polychroma prov. nom.*	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G
CMW 11420 *C. polychroma prov. nom.*	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G
CMW 11513 *C. polychroma prov. nom.*	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G
CMW 11492 *C. polychroma prov. nom.*	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G
CMW 11504 *C. polychroma prov. nom.*	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G
CMW 11501 *C. polychroma prov. nom.*	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G
CMW 11497 *C. polychroma prov. nom.*	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G
CMW 4835 *C. fimbriata*	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G
CMW 2219 *C. fimbriata*	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G
CMW 6579 *C. pirilliformis*	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G
CMW 6569 *C. pirilliformis*	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G
CMW 2475 *C. albofundus*	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G
CMW 2148 *C. albofundus*	A	A	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G
CMW 3276 *C. virescens*	A	-	C	G	G	A	T	C	T	C	T	T	G	G	C	T	C	T	A	G	C	A	T	C	G	A	T	G

										290									300								
CMW 11507 *C. polychroma prov. nom.*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T
CMW 11418 *C. polychroma prov. nom.*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T
CMW 11470 *C. polychroma prov. nom.*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T
CMW 11499 *C. polychroma prov. nom.*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T
CMW 11487 *C. polychroma prov. nom.*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T
CMW 11420 *C. polychroma prov. nom.*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T
CMW 11513 *C. polychroma prov. nom.*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T
CMW 11492 *C. polychroma prov. nom.*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T
CMW 11504 *C. polychroma prov. nom.*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T
CMW 11501 *C. polychroma prov. nom.*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T
CMW 11497 *C. polychroma prov. nom.*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T
CMW 4835 *C. fimbriata*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T
CMW 2219 *C. fimbriata*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T
CMW 6579 *C. pirilliformis*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T
CMW 6569 *C. pirilliformis*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T
CMW 2475 *C. albofundus*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T
CMW 2148 *C. albofundus*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T
CMW 3276 *C. virescens*	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	A	G	T	A	A	T

		310									320									330								
CMW 11507 *C. polychroma prov. nom.*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G
CMW 11418 *C. polychroma prov. nom.*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G
CMW 11470 *C. polychroma prov. nom.*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G
CMW 11499 *C. polychroma prov. nom.*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G
CMW 11487 *C. polychroma prov. nom.*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G
CMW 11420 *C. polychroma prov. nom.*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G
CMW 11513 *C. polychroma prov. nom.*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G
CMW 11492 *C. polychroma prov. nom.*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G
CMW 11504 *C. polychroma prov. nom.*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G
CMW 11501 *C. polychroma prov. nom.*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G
CMW 11497 *C. polychroma prov. nom.*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G
CMW 4835 *C. fimbriata*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G
CMW 2219 *C. fimbriata*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G
CMW 6579 *C. pirilliformis*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G
CMW 6569 *C. pirilliformis*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G
CMW 2475 *C. albofundus*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G
CMW 2148 *C. albofundus*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G
CMW 3276 *C. virescens*	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	A	G	T	G	A	A	T	C	A	T	C	G

	340							350									360								
CMW 11507 *C. polychroma prov. nom.*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A
CMW 11418 *C. polychroma prov. nom.*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A
CMW 11470 *C. polychroma prov. nom.*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A
CMW 11499 *C. polychroma prov. nom.*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A
CMW 11487 *C. polychroma prov. nom.*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A
CMW 11420 *C. polychroma prov. nom.*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A
CMW 11513 *C. polychroma prov. nom.*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A
CMW 11492 *C. polychroma prov. nom.*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A
CMW 11504 *C. polychroma prov. nom.*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A
CMW 11501 *C. polychroma prov. nom.*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A
CMW 11497 *C. polychroma prov. nom.*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A
CMW 4835 *C. fimbriata*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A
CMW 2219 *C. fimbriata*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A
CMW 6579 *C. pirilliformis*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A
CMW 6569 *C. pirilliformis*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A
CMW 2475 *C. albofundus*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A
CMW 2148 *C. albofundus*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A
CMW 3276 *C. virescens*	A	A	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	T	G	G	C	A

	370									380								390							
CMW 11507 *C. polychroma prov. nom.*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C
CMW 11418 *C. polychroma prov. nom.*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C
CMW 11470 *C. polychroma prov. nom.*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C
CMW 11499 *C. polychroma prov. nom.*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C
CMW 11487 *C. polychroma prov. nom.*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C
CMW 11420 *C. polychroma prov. nom.*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C
CMW 11513 *C. polychroma prov. nom.*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C
CMW 11492 *C. polychroma prov. nom.*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C
CMW 11504 *C. polychroma prov. nom.*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C
CMW 11501 *C. polychroma prov. nom.*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C
CMW 11497 *C. polychroma prov. nom.*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C
CMW 4835 *C. fimbriata*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C
CMW 2219 *C. fimbriata*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C
CMW 6579 *C. pirilliformis*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C
CMW 6569 *C. pirilliformis*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C
CMW 2475 *C. albofundus*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C
CMW 2148 *C. albofundus*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C
CMW 3276 *C. virescens*	G	T	A	T	T	C	T	G	C	C	A	G	G	C	A	T	G	C	C	T	G	T	C	C	G	A	G	C

	.	.	|	.	.	.	.	400	.	.	.	.	|	.	.	.	.	410	.	.	.	.	|	.	.	.	.	420
CMW 11507 *C. polychroma prov. nom.*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	**A**	**A**	**C**	T	**C**	-	**T**	T	T	**T**
CMW 11418 *C. polychroma prov. nom.*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	**A**	**A**	**C**	T	**C**	-	**T**	T	T	**T**
CMW 11470 *C. polychroma prov. nom.*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	**A**	**A**	**C**	T	**C**	-	**T**	T	T	**T**
CMW 11499 *C. polychroma prov. nom.*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	**A**	**A**	**C**	T	**C**	-	**T**	T	T	**T**
CMW 11487 *C. polychroma prov. nom.*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	**A**	**A**	**C**	T	**C**	-	**T**	T	T	**T**
CMW 11420 *C. polychroma prov. nom.*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	**A**	**A**	**C**	T	**C**	-	**T**	T	T	**T**
CMW 11513 *C. polychroma prov. nom.*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	**A**	**A**	**C**	T	**C**	-	**T**	T	T	**T**
CMW 11492 *C. polychroma prov. nom.*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	**A**	**A**	**C**	T	**C**	-	**T**	T	T	**T**
CMW 11504 *C. polychroma prov. nom.*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	**A**	**A**	**C**	T	**C**	-	**T**	T	T	**T**
CMW 11501 *C. polychroma prov. nom.*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	**A**	**A**	**C**	T	**C**	-	**T**	T	T	**T**
CMW 11497 *C. polychroma prov. nom.*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	**A**	**A**	**C**	T	**C**	-	**T**	T	T	**T**
CMW 4835 *C. fimbriata*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	**A**	C	**C**	T	**C**	**T**	**T**	T	T	**G**
CMW 2219 *C. fimbriata*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	**G**	**A**	**C**	T	**C**	**C**	**T**	T	T	**G**
CMW 6579 *C. pirilliformis*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	**A**	**A**	**C**	T	**A**	-	-	T	T	**T**
CMW 6569 *C. pirilliformis*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	**A**	**A**	**C**	T	**A**	-	**T**	T	T	**T**
CMW 2475 *C. albofundus*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	**A**	C	**T**	T	**G**	**C**	**T**	T	T	**A**
CMW 2148 *C. albofundus*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	**A**	C	**T**	T	**G**	**C**	**T**	T	T	**A**
CMW 3276 *C. virescens*	G	T	C	A	T	T	T	C	A	C	C	A	C	T	C	A	A	G	-	-	-	-	-	-	**C**	T	C	**C**

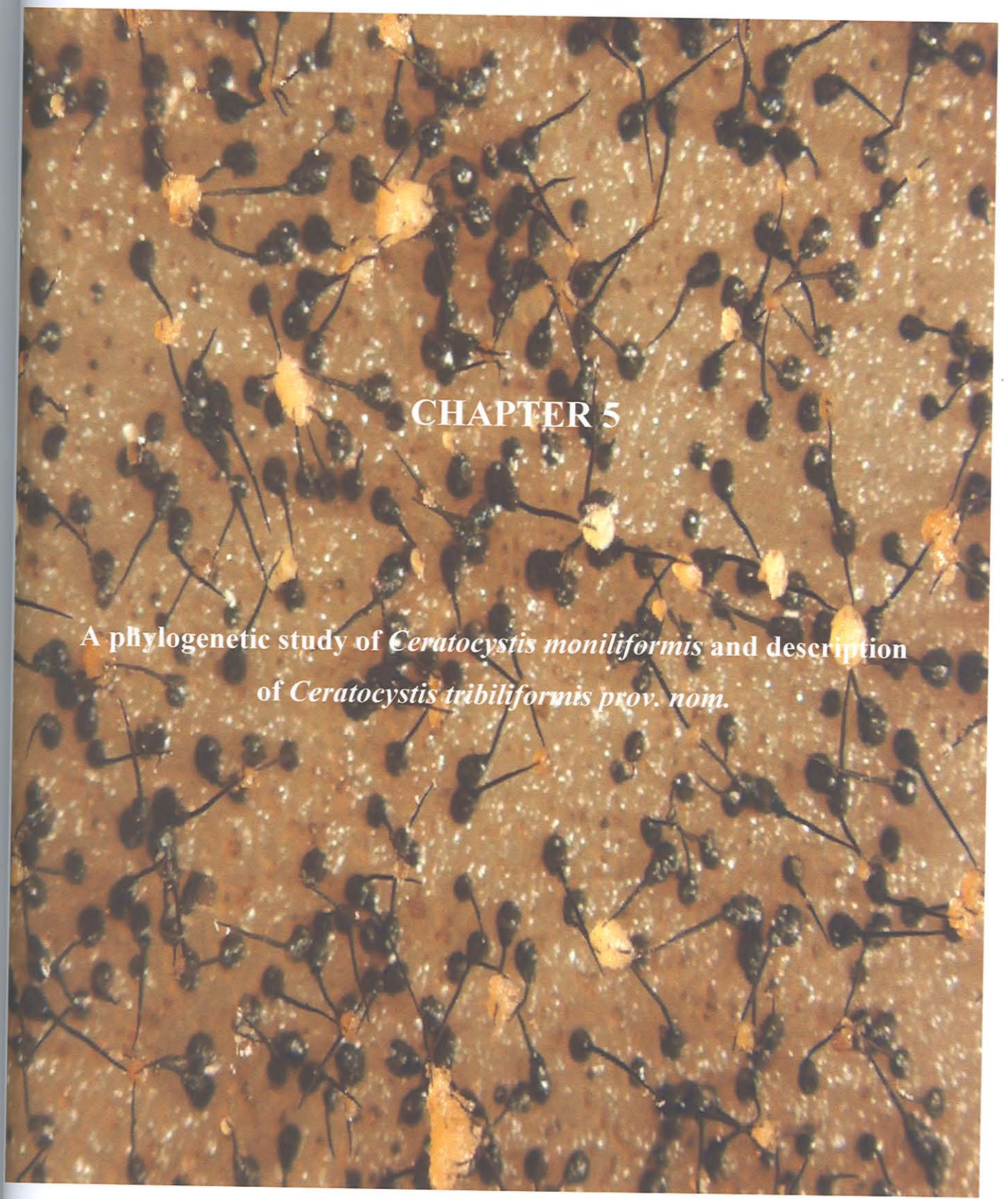
	.	.	.	.	|	.	.	.	.	430	.	.	.	.	|	.	.	.	.	440	.	.	.	.	|	.	.	.
CMW 11507 *C. polychroma prov. nom.*	**T**	T	**C**	T	T	G	G	**T**	G	T	T	G	G	A	G	G	T	C	C	T	G	T	T	C	T	-	-	-
CMW 11418 *C. polychroma prov. nom.*	**T**	T	**C**	T	T	G	G	**T**	G	T	T	G	G	A	G	G	T	C	C	T	G	T	T	C	T	-	-	-
CMW 11470 *C. polychroma prov. nom.*	**T**	T	**C**	T	T	G	G	**T**	G	T	T	G	G	A	G	G	T	C	C	T	G	T	T	C	T	-	-	-
CMW 11499 *C. polychroma prov. nom.*	**T**	T	**C**	T	T	G	G	**T**	G	T	T	G	G	A	G	G	T	C	C	T	G	T	T	C	T	-	-	-
CMW 11487 *C. polychroma prov. nom.*	**T**	T	**C**	T	T	G	G	**T**	G	T	T	G	G	A	G	G	T	C	C	T	G	T	T	C	T	-	-	-
CMW 11420 *C. polychroma prov. nom.*	**T**	T	**C**	T	T	G	G	**T**	G	T	T	G	G	A	G	G	T	C	C	T	G	T	T	C	T	-	-	-
CMW 11513 *C. polychroma prov. nom.*	**T**	T	**C**	T	T	G	G	**T**	G	T	T	G	G	A	G	G	T	C	C	T	G	T	T	C	T	-	-	-
CMW 11492 *C. polychroma prov. nom.*	**T**	T	**C**	T	T	G	G	**T**	G	T	T	G	G	A	G	G	T	C	C	T	G	T	T	C	T	-	-	-
CMW 11504 *C. polychroma prov. nom.*	**T**	T	**C**	T	T	G	G	**T**	G	T	T	G	G	A	G	G	T	C	C	T	G	T	T	C	T	-	-	-
CMW 11501 *C. polychroma prov. nom.*	**T**	T	**C**	T	T	G	G	**T**	G	T	T	G	G	A	G	G	T	C	C	T	G	T	T	C	T	-	-	-
CMW 11497 *C. polychroma prov. nom.*	**T**	T	**C**	T	T	G	G	**T**	G	T	T	G	G	A	G	G	T	C	C	T	G	T	T	C	T	-	-	-
CMW 4835 *C. fimbriata*	**T**	T	**C**	T	T	G	G	**C**	G	T	T	G	G	A	G	G	T	C	C	T	G	T	T	C	T	-	-	-
CMW 2219 *C. fimbriata*	**T**	T	**C**	T	T	G	G	**C**	G	T	T	G	G	A	G	G	T	C	C	T	G	T	T	C	T	-	-	-
CMW 6579 *C. pirilliformis*	**T**	T	**C**	T	T	G	G	**T**	G	T	T	G	G	A	G	G	T	C	C	T	G	T	T	C	T	**T**	-	-
CMW 6569 *C. pirilliformis*	**T**	T	**C**	T	T	G	G	**T**	G	T	T	G	G	A	G	G	T	C	C	T	G	T	T	C	T	**T**	-	-
CMW 2475 *C. albofundus*	**G**	T	**T**	T	T	G	G	**T**	G	T	T	G	G	A	G	G	T	C	C	T	G	T	T	C	T	**T**	**A**	**C**
CMW 2148 *C. albofundus*	**G**	T	**T**	T	T	G	G	**T**	G	T	T	G	G	A	G	G	T	C	C	T	G	T	T	C	T	**T**	**A**	**C**
CMW 3276 *C. virescens*	**G**	C	**T**	T	T	G	G	**T**	G	T	T	G	G	A	G	A	C	C	T	G	T	T	G	T	T	**T**	-	-

Isolate	.	450	.	.	.	.	|	.	.	.	.	460	.	.	.	.	|	.	.	.	.	470	.	.	.	.	|	.
CMW 11507 *C. polychroma prov. nom.*	-	C	C	C	C	T	G	A	A	C	A	G	G	C	C	G	C	C	G	A	A	A	T	G	T	A	T	C
CMW 11418 *C. polychroma prov. nom.*	-	C	C	C	C	T	G	A	A	C	A	G	G	C	C	G	C	C	G	A	A	A	T	G	T	A	T	C
CMW 11470 *C. polychroma prov. nom.*	-	C	C	C	C	T	G	A	A	C	A	G	G	C	C	G	C	C	G	A	A	A	T	G	T	A	T	C
CMW 11499 *C. polychroma prov. nom.*	-	C	C	C	C	T	G	A	A	C	A	G	G	C	C	G	C	C	G	A	A	A	T	G	T	A	T	C
CMW 11487 *C. polychroma prov. nom.*	-	C	C	C	C	T	G	A	A	C	A	G	G	C	C	G	C	C	G	A	A	A	T	G	T	A	T	C
CMW 11420 *C. polychroma prov. nom.*	-	C	C	C	C	T	G	A	A	C	A	G	G	C	C	G	C	C	G	A	A	A	T	G	T	A	T	C
CMW 11513 *C. polychroma prov. nom.*	-	C	C	C	C	T	G	A	A	C	A	G	G	C	C	G	C	C	G	A	A	A	T	G	T	A	T	C
CMW 11492 *C. polychroma prov. nom.*	-	C	C	C	C	T	G	A	A	C	A	G	G	C	C	G	C	C	G	A	A	A	T	G	T	A	T	C
CMW 11504 *C. polychroma prov. nom.*	-	C	C	C	C	T	G	A	A	C	A	G	G	C	C	G	C	C	G	A	A	A	T	G	T	A	T	C
CMW 11501 *C. polychroma prov. nom.*	-	C	C	C	C	T	G	A	A	C	A	G	G	C	C	G	C	C	G	A	A	A	T	G	T	A	T	C
CMW 11497 *C. polychroma prov. nom.*	-	C	C	C	C	T	G	A	A	C	A	G	G	C	C	G	C	C	G	A	A	A	T	G	T	A	T	C
CMW 4835 *C. fimbriata*	-	C	C	C	C	T	G	A	A	C	A	G	G	C	C	G	C	C	G	A	A	A	T	G	T	A	T	C
CMW 2219 *C. fimbriata*	-	C	C	C	C	T	G	A	A	C	A	G	G	C	C	G	C	C	G	A	A	A	T	G	T	A	T	C
CMW 6579 *C. pirilliformis*	-	-	-	-	C	T	G	A	G	C	A	G	G	C	C	G	C	C	G	A	A	A	T	G	T	A	T	C
CMW 6569 *C. pirilliformis*	-	-	-	-	C	T	G	A	G	C	A	G	G	C	C	G	C	C	G	A	A	A	T	G	T	A	T	C
CMW 2475 *C. albofundus*	C	C	T	T	C	T	G	A	A	C	A	G	G	C	C	G	C	C	G	A	A	A	T	G	T	A	T	C
CMW 2148 *C. albofundus*	C	C	T	T	C	C	G	A	A	C	A	G	G	C	C	G	C	C	G	A	A	A	T	G	T	A	T	C
CMW 3276 *C. virescens*	-	C	C	C	T	C	A	A	C	A	G	G	C	C	A	C	C	-	G	A	A	A	T	G	T	A	T	C

Isolate	.	.	.	480	.	.	.	.	|	.	.	.	.	490	.	.	.	.	|	.	.	.	.	500	.	.	.	.
CMW 11507 *C. polychroma prov. nom.*	G	G	C	T	G	T	T	A	-	-	-	-	T	A	C	T	T	G	C	-	C	A	A	C	T	C	C	C
CMW 11418 *C. polychroma prov. nom.*	G	G	C	T	G	T	T	A	-	-	-	-	T	A	C	T	T	G	C	-	C	A	A	C	T	C	C	C
CMW 11470 *C. polychroma prov. nom.*	G	G	C	T	G	T	T	A	-	-	-	-	T	A	C	T	T	G	C	-	C	A	A	C	T	C	C	C
CMW 11499 *C. polychroma prov. nom.*	G	G	C	T	G	T	T	A	-	-	-	-	T	A	C	T	T	G	C	-	C	A	A	C	T	C	C	C
CMW 11487 *C. polychroma prov. nom.*	G	G	C	T	G	T	T	A	-	-	-	-	T	A	C	T	T	G	C	-	C	A	A	C	T	C	C	C
CMW 11420 *C. polychroma prov. nom.*	G	G	C	T	G	T	T	A	-	-	-	-	T	A	C	T	T	G	C	-	C	A	A	C	T	C	C	C
CMW 11513 *C. polychroma prov. nom.*	G	G	C	T	G	T	T	A	-	-	-	-	T	A	C	T	T	G	C	-	C	A	A	C	T	C	C	C
CMW 11492 *C. polychroma prov. nom.*	G	G	C	T	G	T	T	A	-	-	-	-	T	A	C	T	T	G	C	T	C	A	A	C	T	C	C	C
CMW 11504 *C. polychroma prov. nom.*	G	G	C	T	G	T	T	A	-	-	-	-	T	A	C	T	T	G	C	-	C	A	A	C	T	C	C	C
CMW 11501 *C. polychroma prov. nom.*	G	G	C	T	G	T	T	A	-	-	-	-	T	A	C	T	T	G	C	T	C	A	A	C	T	C	C	C
CMW 11497 *C. polychroma prov. nom.*	G	G	C	T	G	T	T	A	-	-	-	-	T	A	C	T	T	G	C	-	C	A	A	C	T	C	C	C
CMW 4835 *C. fimbriata*	G	G	C	T	G	T	T	A	-	-	-	-	T	A	C	T	T	G	C	C	C	A	A	C	T	C	C	C
CMW 2219 *C. fimbriata*	G	G	C	T	G	T	T	A	-	-	-	-	T	A	C	T	T	G	C	-	C	A	A	C	T	C	C	C
CMW 6579 *C. pirilliformis*	G	G	C	T	G	T	T	A	A	-	-	-	-	A	C	T	T	G	C	-	C	A	A	C	T	C	C	C
CMW 6569 *C. pirilliformis*	G	G	C	T	G	T	T	A	A	-	-	-	-	A	C	T	T	G	C	-	C	A	A	C	T	C	C	C
CMW 2475 *C. albofundus*	G	G	C	T	G	T	T	A	T	T	T	T	T	A	C	T	T	G	C	-	C	A	A	C	T	C	C	C
CMW 2148 *C. albofundus*	G	G	C	T	G	T	T	A	T	T	T	T	T	A	C	T	T	G	C	-	C	A	A	C	T	C	C	C
CMW 3276 *C. virescens*	G	G	C	T	G	T	T	-	-	-	-	-	A	C	C	T	T	G	-	A	G	C	T	T	C	C	C	T

	510									520											530		
CMW 11507 *C. polychroma prov. nom.*	C	T	G	T	G	T	A	G	T	A	**T**	A	A	**A**	**A**	-	-	-	-	-	-	-	-	-	-	-	**T**
CMW 11418 *C. polychroma prov. nom.*	C	T	G	T	G	T	A	G	T	A	**T**	A	A	**A**	**A**	-	-	-	-	-	-	-	-	-	-	-	**T**
CMW 11470 *C. polychroma prov. nom.*	C	T	G	T	G	T	A	G	T	A	**T**	A	A	**A**	**A**	-	-	-	-	-	-	-	-	-	-	-	**T**
CMW 11499 *C. polychroma prov. nom.*	C	T	G	T	G	T	A	G	T	A	**T**	A	A	**A**	**A**	**G**	-	-	-	-	-	-	-	-	-	-	**T**
CMW 11487 *C. polychroma prov. nom.*	C	T	G	T	G	T	A	G	T	A	**T**	A	A	**A**	**A**	-	-	-	-	-	-	-	-	-	-	-	**T**
CMW 11420 *C. polychroma prov. nom.*	C	T	G	T	G	T	A	G	T	A	**T**	A	A	**A**	**A**	-	-	-	-	-	-	-	-	-	-	-	**T**
CMW 11513 *C. polychroma prov. nom.*	C	T	G	T	G	T	A	G	T	A	**T**	A	A	**A**	**A**	-	-	-	-	-	-	-	-	-	-	-	**T**
CMW 11492 *C. polychroma prov. nom.*	C	T	G	T	G	T	A	G	T	A	**C**	A	A	**A**	**A**	-	-	-	-	-	-	-	-	-	-	-	**T**
CMW 11504 *C. polychroma prov. nom.*	C	T	G	T	G	T	A	G	T	A	**T**	A	A	**A**	**A**	-	-	-	-	-	-	-	-	-	-	-	**T**
CMW 11501 *C. polychroma prov. nom.*	C	T	G	T	G	T	A	G	T	A	**C**	A	A	**A**	**A**	-	-	-	-	-	-	-	-	-	**T**	**T**	**T**
CMW 11497 *C. polychroma prov. nom.*	C	T	G	T	G	T	A	G	T	A	**T**	A	A	**A**	**A**	-	-	-	-	-	-	-	-	-	-	-	**T**
CMW 4835 *C. fimbriata*	C	T	G	T	G	T	A	G	T	A	**T**	A	A	**A**	**A**	-	-	-	-	-	-	-	-	-	-	**T**	**T**
CMW 2219 *C. fimbriata*	C	T	G	T	G	T	A	G	T	A	**T**	A	A	**A**	**A**	-	-	-	-	-	-	-	-	-	-	-	-
CMW 6579 *C. pirilliformis*	C	T	G	T	G	T	A	G	T	A	**T**	A	A	**A**	**A**	-	-	-	-	-	-	-	-	-	-	-	-
CMW 6569 *C. pirilliformis*	C	T	G	T	G	T	A	G	T	A	**T**	A	A	**A**	**G**	**G**	**A**	**A**	**T**	**A**	**A**	**T**	**T**	**T**	**T**	**T**	**T**
CMW 2475 *C. albofundus*	C	T	G	T	G	T	A	G	T	A	**T**	A	A	**A**	**G**	**G**	**A**	**A**	**T**	**A**	**A**	**T**	**T**	**T**	**T**	**T**	**T**
CMW 2148 *C. albofundus*	C	T	G	T	G	T	A	G	T	A	**C**	A	A	**G**	**A**	-	-	-	-	-	-	-	-	-	-	-	**T**
CMW 3276 *C. virescens*	G	T	G	T	A	G	T	A	A	T	**A**	T	C	T	A	**T**	**T**	**T**	**A**	**C**	**A**	**C**	**T**	**T**	**T**	-	-	-

			540									550												
CMW 11507 *C. polychroma prov. nom.*	**T**	**T**	**T**	**C**	**C**	A	A	T	T	T	T	T	-	A	C	**A**	**C**	T	T	T	G	**A**	A	G	T
CMW 11418 *C. polychroma prov. nom.*	**T**	**T**	**T**	**C**	**C**	A	A	T	T	T	T	T	-	A	C	**A**	**C**	T	T	T	G	**A**	A	G	T
CMW 11470 *C. polychroma prov. nom.*	**T**	**T**	**T**	**C**	**C**	A	A	T	T	T	T	T	-	A	C	**A**	**C**	T	T	T	G	**A**	A	G	T
CMW 11499 *C. polychroma prov. nom.*	**T**	**T**	**T**	**C**	**C**	A	A	T	T	T	T	T	-	A	C	**A**	**C**	T	T	T	G	**A**	A	G	T
CMW 11487 *C. polychroma prov. nom.*	**T**	**T**	**T**	**C**	**C**	A	A	T	T	T	T	T	-	A	C	**A**	**C**	T	T	T	G	**A**	A	G	T
CMW 11420 *C. polychroma prov. nom.*	**T**	**T**	**T**	**C**	**C**	A	A	T	T	T	T	T	-	A	C	**A**	**C**	T	T	T	G	**A**	A	G	T
CMW 11513 *C. polychroma prov. nom.*	**T**	**T**	**T**	**C**	**C**	A	A	T	T	T	T	T	-	A	C	**A**	**C**	T	T	T	G	**A**	A	G	T
CMW 11492 *C. polychroma prov. nom.*	**C**	**T**	**T**	**C**	**T**	A	A	T	T	T	T	T	-	A	C	**A**	**C**	T	T	T	G	**A**	A	G	T
CMW 11504 *C. polychroma prov. nom.*	**T**	**T**	**T**	**C**	**C**	A	A	T	T	T	T	T	-	A	C	**A**	**C**	T	T	T	G	**A**	A	G	T
CMW 11501 *C. polychroma prov. nom.*	**C**	**T**	**T**	**C**	**T**	A	A	T	T	T	T	T	-	A	C	**A**	**C**	T	T	T	G	**A**	A	G	T
CMW 11497 *C. polychroma prov. nom.*	**T**	**T**	**T**	**C**	**C**	A	A	T	T	T	T	T	-	A	C	**A**	**C**	T	T	T	G	**A**	A	G	T
CMW 4835 *C. fimbriata*	**T**	**T**	**T**	**C**	**T**	A	A	T	T	T	T	T	-	A	C	**A**	**C**	T	T	T	G	**A**	A	G	T
CMW 2219 *C. fimbriata*	**T**	**T**	**T**	**C**	**T**	A	A	T	T	T	T	T	-	A	C	**A**	**C**	T	T	T	G	**A**	A	G	T
CMW 6579 *C. pirilliformis*	**T**	**T**	**T**	**C**	**C**	A	A	T	T	T	T	T	**T**	A	C	**A**	**T**	T	T	T	G	**A**	A	G	T
CMW 6569 *C. pirilliformis*	**T**	**T**	**T**	**C**	**C**	A	A	T	T	T	T	T	**T**	A	C	**A**	**T**	T	T	T	G	**A**	A	G	T
CMW 2475 *C. albofundus*	**T**	**T**	**T**	**T**	**T**	A	A	T	T	T	T	T	**T**	A	C	**G**	**C**	T	T	T	G	**G**	A	G	T
CMW 2148 *C. albofundus*	**T**	**T**	**T**	**T**	**T**	A	A	T	T	T	T	T	**T**	A	C	**G**	**C**	T	T	T	G	**G**	A	G	T
CMW 3276 *C. virescens*	-	-	-	**G**	**A**	A	A	C	T	C	T	T	**G**	T	-	-	-	-	-	-	-	-	-	-	-	-



CHAPTER 5

A phylogenetic study of *Ceratocystis moniliformis* and description of *Ceratocystis tribiliformis prov. nom.*

ABSTRACT

Ceratocystis moniliformis is a colonist of fresh wounds on trees, mainly in the tropics. The fungus is not known to be a pathogen and it has thus not been widely studied. *Ceratocystis moniliformis* has been found on many taxonomically different plants and in many different climatic zones. It is thought to represent a complex of morphologically similar but different species, of which some have recently been described. The aim of this study was to consider the phylogenetic relationships of isolates of *C. moniliformis* from various hosts and origins, based on DNA sequence comparisons of three gene regions. Four recently described species, closely related to *C. moniliformis*, were included in the study and confirmed to be phylogenetically distinct. Results showed that the isolates of *C. moniliformis* used in this study represent a monophyletic group. A collection of isolates initially identified as *C. moniliformis*, from *Quercus* in Sumatra represented a distinct clade. These isolates are also morphologically distinct from *C. moniliformis* and this fungus is described here as *Ceratocystis tribilliformis prov. nom.*

INTRODUCTION

Ceratocystis moniliformis Hedgcock is a cosmopolitan fungus, which has been reported from many hosts and continents (Davidson 1935, Bakshi 1951, Hunt 1956, Upadhyay 1981) (Table 1). The fungus was first collected from gumwood (*Liquidambar styraciflua* L.) in Texas (Von Schrenk 1903) and was initially described as *Ceratostomella moniliformis* (Hedgcock 1906). The species was transferred to *Ceratocystis* in 1952 (Moreau 1952).

Ceratocystis moniliformis is one of six species belonging to the genus that has hat-shaped ascospores. It can, however, easily be distinguished from the other *Ceratocystis* spp. This is generally based on the presence of well-developed conical spines that cover the ascomatal bases of *C. moniliformis* isolates (Hedgcock 1906, Luc 1952, Hunt 1956, Upadhyay 1981). Another distinguishing characteristic are the disc-formed bases of the ascomatal necks (Bakshi 1951, Hunt 1956). Two types of conidia, one cylindrical in shape and the other more barrel-shaped, have been reported for the *Thielaviopsis* anamorph of *C. moniliformis* (Davidson 1935, Bakshi 1951, Paulin-Mahady, Harrington & McNew 2002). *Ceratocystis moniliformis* is one of the few *Ceratocystis* spp. known not to produce chlamydospores (Davidson 1935, Paulin-Mahady *et al.* 2002).

Descriptions for *C. moniliformis* have tended to be somewhat disparate. For example, the conical spines on ascomatal bases were not noted by some authors (Kitajima 1936, Luc 1952, Roldan 1962) who rather referred to hyphal ornamentation. The ascomatal bases have also been described variably as globose (Hedgcock 1906), elongate to pear-shaped (Davidson 1935) and spherical or elongated (Luc 1952). Dimensions for neck length and ascomatal base widths have also varied (Hedgcock 1906, Kitajima 1936, Bakshi 1951, Hunt 1956, Upadhyay 1981) (Table 2). Likewise, there has been little agreement regarding the morphology of the conidiophores in various descriptions (Davidson 1935, Kitajima 1936, Bakshi 1951, Hunt 1956, Upadhyay 1981) (Table 3).

Ceratocystis moniliformis commonly infects wounds on woody plants and especially trees (Grylls & Seifert 1993, Kile 1993). It is not considered to be a pathogen, causing only sap stain. There is also no evidence of *C. moniliformis* imparting structural changes to infected wood, but discolouration does lead to reduction of timber value (Davidson 1935).

The very wide host range and geographic distribution of *C. moniliformis* has led us to question whether this fungus might represent a species complex. This would be similar to the view that is emerging for the important tree pathogen, *C. fimbriata* Ell. & Halst. (Barnes 2002, Baker *et al.* 2003). The recent description of *C. moniliformopsis* Yuan & Mohammed (Yuan & Mohammed 2002), a species morphologically very similar to *C. moniliformis*, provided the first support for the view that various species might have been aggregated with *C. moniliformis*. Recent phylogenetic studies have thus led to the discovery of two additional new species, *C. bhutanensis prov. nom.* Van Wyk, Krisits & Wingfield (Chapter 2) and *C. omanensis prov. nom.* Al-Alsubhi, Deadman & Wingfield (Al-Alsubhi *et al.* 2003) that are morphologically very similar to *C. moniliformis*.

The aim of this study was to compare a collection of *C. moniliformis sensu lato* isolates based on multiple gene sequences. Recently described species such as *C. moniliformopsis*, *C. bhutanensis* and *C. omanensis* were also included to provide perspective.

MATERIALS & METHODS

Isolates

Isolates collected for this study were identified as *C. moniliformis* based on morphological characteristics such as the presence of spines on the ascomatal bases. Isolates were collected over a relatively long period (7 years) from various tree species in South Africa, Bhutan, Ecuador, Costa Rica and Indonesia (Table 4). Closely related species, *C. moniliformopsis*, *C. bhutanensis prov. nom.* and *C. omanensis prov. nom.*, originally identified as *C. moniliformis* due to their morphological similarities, were also included (Table 4). 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. Representative isolates have also been deposited with the Centraalbureau voor Schimmelcutures (CBS), Utrecht, The Netherlands.

DNA extraction

Cultures for DNA extraction were grown on 2 % Malt Extract Agar (MEA) (20 % w/v) (Biolab, Midrand, South Africa) at 25 °C for two weeks. Masses of fungal mycelium and ascomata were scraped directly from the actively growing cultures and transferred to Eppendorf tubes and lyophilised for two days. The lyophilised mycelium was placed in liquid nitrogen, ground to a fine powder using a glass rod, and DNA was extracted using the method described by Barnes *et al.* (2001).

PCR amplification

Three gene regions were amplified using the polymerase chain reaction (PCR). The two Internal Transcribed Spacer regions (ITS1 and ITS2) and the 5.8S gene of the ribosomal DNA (rDNA) operon were amplified using primers ITS1 and ITS4 (White *et al.* 1990). The β-tubulin gene was partially amplified using primers βt1a and βt1b (Glass & Donaldson 1995) and the EF1-α gene of the rDNA operon was amplified using primers EF1-728F and EF1-986R (Carbone & Kohn 1999).

PCR reaction 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 x 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 °C for 2 min for DNA denaturation, followed by 10 cycles at 94 °C for 20 s and 55 °C for 40 s for annealing and 72 °C for 45 s for elongation. A further 30 cycles at 94 °C for 20 s, 55 °C for 40 s with a 5 s extension after each cycle were included. A final step of 10 min at 72 °C completed the programme. Amplification of the respective genes was confirmed under UV illumination using 2 % agarose (Roche diagnostics, Mannheim, Germany) gel electrophoresis in the presence of ethidium bromide. After amplification, amplicons were purified using Sephadex G-50 columns (1 g in 15 ml H_2O, SIGMA, Steinheim, Germany).

Sequencing and analysis

PCR amplicons were sequenced in both directions using the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California). The same primers as those used in the PCR reactions were used for sequencing.

Sequencing reactions were run on an ABI PRISM™ 3100 Autosequencer (Applied BioSystems, Foster City, California, U.S.A) and sequences were analysed using Sequence Navigator version 1.0.1 (Applied BioSystems, Foster City, California).

Sequences were aligned manually and analysed using PAUP version 4.0b10* [Phylogenetic Analysis Using Parsimony (and other methods)] (Swofford 2002). A partition homogeneity test (Swofford 2002) was used to determine whether the sequence data sets for the three different genome regions could be combined for the analysis. Gaps were treated as "newstate" and trees were obtained via stepwise addition of 1000 replicates with the Mulpar option effective. The heuristic search option based on parsimony with tree bisection reconnection was used to obtain the phylogram. Confidence intervals using 1000 bootstrap replicates were calculated. *Ceratocystis virescens* (Davidson) Moreau was used as a monophyletic sister out-group with respect to the in-group. All sequences derived from this study have been deposited in GenBank (Table 4).

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 were generated using the MCMC procedure, sampling every 100th tree and saving every 10th tree. To avoid including trees sampled before convergence of the Markov chain, the first 4700 trees were discarded. For the combined dataset of the three gene sequences, gamma rate heterogeneity was set, and no codon specific sites were included for the ITS gene. For β-tubulin and EF1-α sequences, codon specific sites were specified with a site-specific substitution rate and the site partition was treated as a by-codon.

Cultural Characteristics

To supplement DNA sequence data, isolates were compared based on morphology and growth in culture. For growth comparisons, three isolates from each of the different countries were chosen (Table 4). Three representative isolates of the closely related species, *C. moniliformopsis*, *C. bhutanensis prov. nom.* and *C. omanensis prov. nom.* were also included.

Prior to commencing the growth tests, the fungi were grown on 2 % MEA. After a two-week incubation period at 25 °C, mycelial plugs were taken from the margins of the actively

growing cultures using a 5 mm diameter cork borer, and these were transferred to the centres of 90 mm Petri dishes containing 2 % MEA. Five plates of each isolate were incubated at 4 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C and 35 °C, respectively. Growth rates were assessed by taking two measurements of colony diameter at right angles to each other, every day for 3 days. Averages and standard deviations were computed in Microsoft Excel for all growth measurements. The entire experiment was repeated once. The colony colour of the isolates was determined using the colour tables of Rayner (1970) and colony textures were noted.

Morphology

Isolates were further compared by studying fungal structures using a Zeiss Model light microscope. These comparisons showed that there was a group of isolates that were distinctly different to other isolates of *C. moniliformis*. These morphologically different cultures were subjected to further detailed studies.

Morphological characteristics of the isolates residing in a unique clade, related to *C. moniliformis* (Fig. 3), were described from 10-day-old cultures on 2 % MEA. For microscopic examination, fungal structures were mounted in lactophenol on glass slides. Fifty measurements were taken for each taxonomically relevant structure of isolate CMW 13013, which was chosen to represent the fungus reflected by this unique clade. In addition, ten measurements were made of each of the relevant structures for three other isolates chosen from this unique group (Table 4). Ranges, averages, and standard deviations of all measurements were calculated in Microsoft Excel.

RESULTS

PCR amplification

Amplification of the ITS regions and the 5.8S gene of the rDNA operon resulted in fragments of ~500 base pairs (bp) in size. Amplification of the β-tubulin gene resulted in fragments of ~500 bp in size, while the amplification of the EF1-α gene resulted in fragments of ~300 bp in size. All three gene regions were successfully amplified for all isolates.

Sequencing and analyses

The partition homogeneity test showed that the sequence data sets for β-tubulin, ITS and EF 1-α could be combined (P = 0.05). The combined data set resulted in 1310 characters (Appendix), of which 349 characters were variable, 213 were parsimony-uninformative and 186 were parsimony informative. Analyses of the data set resulted in two most parsimonious trees, with a tree length of 632, a consistency index (CI) of 0.908, homoplasy index (HI) of 0.09, retention index (RI) of 0.894 and a rescaled consistency index (RC) of 0.812.

Five distinct clades, representing four different phylogenetic species were observed in the combined gene tree, each supported by a 100 % bootstrap value (Fig. 1). The *C. moniliformis* group was separated into two distinct clades. The one clade included only isolates from Sumatra, which were from *Quercus* trees, while the second clade represented all the other *C. moniliformis* isolates used in this study, originating from Bhutan, South Africa, Ecuador and Costa Rica. The posterior probability of the branch nodes of the combined tree, generated with the Bayesian inference programme, supported the bootstrap values (Fig. 1).

Cultural characteristics

There were distinct differences in growth and culture for the *C. moniliformis* isolates studied. The isolates from Sumatra grew rapidly, reaching a colony diameter of 90 mm in 5 days at 25 ˚C (Fig. 2). No growth was observed at 4 ˚C or at 35 ˚C. At 10 ˚C and at 30 ˚C, the growth was diminished. The growth for the other *C. moniliformis* isolates varied significantly (Fig. 2). At 4 °C and 35 °C there was no growth for any of the isolates. At 10 °C, 15 °C, 20 °C, 25 °C and 30 °C there were considerable differences between isolates (Fig. 2). Isolates of *C. moniliformopsis* grew slowly compared to *C. moniliformis*. They displayed optimal growth at 20 °C and did not grow at temperatures above this (Fig. 2). Isolates of *C. omanensis prov. nom.* grew faster than any other species at most of the temperatures, including 35 °C (Fig. 2). Optimum growth was observed between 25 °C to 30 °C (Fig. 2). Isolates of *C. bhutanensis prov. nom.* grew well at most temperatures and had optimum growth at 20 °C. This species displayed much better growth than any other species at temperatures below 20 °C (Fig. 2).

Ceratocystis moniliformopsis cultures had buff-yellow (19d) aerial mycelium with isabella colour (19''i) submerged mycelium, with older cultures having fawn (13''') aerial and

submerged mycelium, with few ascomata observed while *C. bhutanensis prov. nom.* culture's submerged mycelium was umber (15 m) with the well-developed aerial mycelium being ecru-drab (13""""d). *Ceratocystis omanensis prov. nom.* cultures were buff-yellow (19d) in colour with masses of ascomata. The *C. moniliformis* isolates could be separated into two broad groups that were consistent with those emerging from the phylogenetic comparisons. Isolates from Sumatra differed from others in having virtually no aerial mycelium and masses of ascomata covering the plates. The remaining *C. moniliformis* isolates had abundant buff-yellow (19d) (Rayner 1970) aerial mycelium with ascomata tending to occur below the mycelium, which is consistent with the description of this species (Hedgcock 1906, Upadhyay 1981).

Morphology

Phylogenetic analyses and differences in cultural characteristics for the Sumatran isolates prompted a more detailed morphological comparison between them and those thought to represent typical *C. moniliformis*. The ascomatal bases for the Sumatran isolates were black and obpyriform (Fig. 3a) and the ostiolar hyphae were divergent (Fig. 3b). The bases of the necks at the points of attachment resembled those of *C. moniliformis* (Fig. 3c). The ascomatal bases also resembled those of typical *C. moniliformis*, which were covered in short conical spines (Fig. 3d). Masses of hat-shaped ascospores were observed in both groups of isolates (Fig. 3e). The anamorph was typical of *Thielaviopsis*, with phialidic conidiogenous cells (Fig. 3f). Both smooth-walled hyphae and those having a granular appearance were observed (Fig. 3g) and both barrel-shaped (Fig. 3h) and cylindrical conidia were present (Fig. 3i).

TAXONOMY

DNA Sequence comparisons as well as growth characteristics in culture provided robust support for the view that the isolates thought to represent *C. moniliformis* from Sumatra included in this study, represent a new and previously undescribed species of *Ceratocystis*. The fungus is, therefore, described here as a new taxon:

Ceratocystis tribiliformis Van Wyk & Wingfield *prov. nom.*

(Fig. 3)

Etymology: Ascomata similar in shape to fruit of the plant *Tribulus terrestris*, known in the Afrikaans language as the "dubbeltjie".

Stat.conid.: *Thielaviopsis*

Coloniae colore albae. *Mycelium* rarum, praecipue in medio immersum. *Temperatura faustissima* 25 °C, supra 30 °C non crescit, minime crescit ad 4 °C et 30 °C. *Hyphae* laeves vel granulatae, in septis non constrictae, 2-4 µm latae. *Bases ascomatum* atrobrunneae vel nigrae, obpyriformes, spinis hyphibusque ornatae, spinis atrobrunneis vel nigris, (4-) 6-10 (-12) µm longis, bases (196-) 203-249 (-264) µm diametro. *Colla ascomatum* atrobrunnea vel nigra, (741-) 782-986 (-1047) µm longa, basi discoidea, (43-) 44-50 (-53) µm lata, apice (13-) 14-18 (-20) µm lata. *Hyphae ostiolares* divergentes, hyalinae, (22-) 25-31 (-32) µm longae. *Asci* non visi. *Ascosporae* lateraliter visae cucullatae, aseptatae, hyalinae, in vagina investitae, cum vagina 5-6 x 2-3 µm, sine illa 4-5 x 2-3 µm. Ascosporae in massis bubalino-luteis mucilaginis in apicibus collorum ascomatum convenientes. *Anamorpha Thielaviopsis*: conidiophora singula in mycelio crescentia, apicem versus angustata, (21-) 22-40 (-46) µm longa, basi 3-4 µm, apice 1-3 µm lata. Evolutio *conidii* phialidici per parietes annulares faciendas, *conidia* biformiia: conidia primaria hyalina, aseptata, cylindrica 7-9 x 2 µm, conidia secondaria hyalina, aseptata, doliiformia, 7-9 x 3-4 µm.

Typus: **Indonesia**: Sumatra, isolated from *Quercus* sp., 1996, M. J. Wingfield (PREM 57827 – holotypus, living culture: CMW 13013).

Colonies white in colour. *Mycelium* sparse, mostly submerged in medium. *Optimal temperature* for growth 25 °C, no growth above 30 °C, diminished growth at 4 °C and 30 °C. *Hyphae* smooth or granulated, not constricted at septa, 2-4 µm wide. *Ascomatal bases* dark brown to black, globose to obpyriform, ornamented with spines and hyphae, spines dark brown to black, (4-) 6-10 (-12) µm long, bases (196-) 203-249 (-264) µm in diameter. *Ascomatal necks* dark brown to black, (741-) 782-986 (-1047) µm long, (43-) 44-50 (-53) µm wide at the base, (13-) 14-18 (-20) µm wide at the apex, with a disc-like base. *Ostiolar hyphae* divergent, hyaline, (22-) 25-31 (-32) µm long. *Asci* not observed. *Ascospores* cucullate in side view, aseptate, hyaline, invested in sheath, 5-6 x 2-3 µm with sheath, 4-5 x 2-3 µm without sheath. Ascospores accumulating in buff-yellow (19d) mucilaginous masses on the apices of ascomatal necks. *Thielaviopsis anamorph*: conidiophores occurring singly on mycelium,

hyaline, swollen at the base, tapering towards the apex, (21-) 22-40 (-46) µm long, 3-4 µm wide at base, 1-3 µm wide at the apices. *Conidium development* through ring wall building, *conidia* of two types: primary conidia hyaline, aseptate, cylindrical 7-9 x 2 µm, secondary conidia hyaline, aseptate, barrel-shaped 7-9 x 3-4 µm.

Additional specimens examined: **Indonesia**: Sumatra, isolated from *Quercus* sp., 1996, M. J. Wingfield (culture CMW 13 011, PREM 57825); same collection data (culture CMW 13 012, PREM 57826); same collection data (culture CMW 13 015, PREM 57828).

DISCUSSION

In this study, we investigated the taxonomic status of a group of isolates reported to represent *C. moniliformis*. This was achieved using both morphological and DNA sequence data. Our results show that a set of isolates identified as *C. moniliformis sensu stricto* in fact represent two discrete phylogenetic lineages. One of the clades, consisting of isolates from a wide geographic and host range represent true *C. moniliformis sensu stricto*. The second group of isolates, from Sumatra, Indonesia, isolated from *Quercus* trees, clearly represent a separate and undescribed species. We have now described this species as *C. tribiliformis prov. nom.* We have also provided additional support for the separation of *C. bhutanensis prov. nom.*, *C. omanensis prov. nom.* and *C. moniliformopsis* into separate species, most closely related to *C. moniliformis*. We consider these species to form part of the larger *C. moniliformis sensu lato* complex, characterised by the formation of hat-shaped ascospores, a disk-shaped basal ascomatal neck and short conical spines on their ascomatal bases.

Ceratocystis tribiliformis prov. nom. is morphologically very similar to *C. moniliformis*. Small but distinct differences in the morphology of fruiting structures could be found to separate them. The ascomatal bases of *C. tribiliformis prov. nom.* are very characteristically obpyriform to globose while *C. moniliformis*, *C. omanensis prov. nom.*, *C. bhutanensis prov. nom.* and *C. moniliformopsis* all have distinctly globose bases. *Ceratocystis tribiliformis prov. nom.*, *C. omanensis prov. nom.* and *C. bhutanensis prov. nom.* have both smooth hyphae such as in *C. moniliformis* and *C. moniliformopsis*, and hyphae with granular surfaces. These fungi can also easily be distinguished from each other based on growth characteristics in culture. In

culture, *C. tribiliformis prov. nom.* has very little, if any, aerial mycelium, while all the other species in the *C. moniliformis sensu lato* complex produce prolific aerial mycelium (Hedgcock 1906, Davidson 1935, Yuan & Mohammed 2002, Chapter 2, Al-Alsubhi *et al.* 2003). Ascomata cover the agar in cultures of *C. tribiliformis prov. nom.*, while *C. moniliformis sensu lato* sporulates less prolifically and generally requires the addition of thiamine to enhance the production of ascomata (Robbins & Ma 1942, Hawker 1966, Upadhyay 1981). The growth of *C. tribiliformis prov. nom.* and *C. moniliformopsis* isolates is similar at 15 °C - 25 °C and both species have diminished growth at temperatures below 10 °C and above 30 °C. In contrast, *C. bhutanensis prov. nom.* isolates grow rapidly at temperatures below 10 °C and *C. omanensis prov. nom.* grows rapidly at temperatures above 30 °C. The optimum growth temperature for *C. moniliformis sensu lato* is 25 °C - 30 °C.

Sequence data for the ITS regions alone did not provide convincing separation between isolates of *C. bhutanensis prov. nom., C. moniliformis, C. moniliformopsis, C. omanensis prov. nom.* and *C. tribiliformis prov. nom.* However, addition of sequences for the β-tubulin and EF1-α gene areas provided clear resolution of these five species into distinct clades with robust bootstrap and Bayesian support. These phylogenetic differences support the morphological and cultural differences observed between these species and emphasize the importance of considering multiple gene regions in taxonomic and phylogenetic studies.

Species of *Ceratocystis* reside in two distinct phylogenetic groups (Witthuhn *et al.* 1999). These include the *C. fimbriata* group where species are primary plant pathogens and the *C. coerulescens* group, where most species are not plant pathogens (Witthuhn *et al.* 1999). *Ceratocystis moniliformis sensu stricto* resides in the latter group and together with *C. bhutanensis prov. nom., C. moniliformopsis, C. omanensis prov. nom.* and *C. tribiliformis prov. nom.* these are the only species in the *C. coerulescens* group with hat-shaped ascospores. For the purpose of this study these species are collectively known as *C. moniliformis sensu lato* as isolates of *C. bhutanensis prov. nom.* and *C. omanensis prov. nom.* were previously identified as *C. moniliformis*, because of their morphological similarities. Results of our phylogenetic study clearly showed that these three species are more closely related to each other than to any of the other *Ceratocystis* spp. within the *C. coerulescens* clade, suggesting that they originate from the same ancestor.

This study provides the first phylogenetic comparison for a collection of isolates, many of which have previously been treated as *C. moniliformis*. Clearly, *C. moniliformis sensu stricto* has a wide geographic distribution. Other apparently cryptic species that have emerged from this and other recent studies, however, appear to be restricted to specific geographic areas. Thus, *C. moniliformopsis* is found only in Australia (Yuan & Mohammed 2002), *C. bhutanensis prov. nom.* is associated with *Ips schmutzenhoferi* and found only in Bhutan, *C. omanensis prov. nom.* occurs in Oman and *C. tribiliformis prov. nom.* is restricted to Sumatra. Additional collections are likely to increase the distribution of some of these species but some, such as *C. bhutanensis prov. nom.*, appear to be ecologically adapted to their areas of origin and hosts. This study has also provided the first DNA sequence data showing that *C. moniliformopsis* represents a discrete species. It has also identified additional isolates of this species from mainland Australia, and from two different hosts.

Results of this study have shown clearly that the fungus that has for many years been treated as *C. moniliformis* represents a species complex. Recently, three new species, *C. bhutanensis prov. nom.*, *C. omanensis prov. nom.* and *C. moniliformopsis* thought to represent *C. moniliformis*, have been described as separate species (Yuan & Mohammed 2002, Chapter 2, Al-Alsubhi *et al.* 2003). In this study a fourth new species, *C. tribiliformis prov. nom.* has been recognised as belonging to the *C. moniliformis sensu lato* complex. We have included isolates from our own collections and others from various other sources. Given the high number of new species that have been recognised from a relatively small number of collections, it seems likely that additional new species will emerge as fungi resembling *C. moniliformis* are collected from various parts of the world.

Very little is known regarding the ecology of the species in the *C. moniliformis sensu lato* complex. Where inoculation experiments have been attempted (Davidson 1935), it has been concluded that the fungus is a wound inhabiting saprophyte. Now that additional species are being recognised, pathogenicity tests should be carried out with them, on their hosts of origin and in the areas where they have been found. This will lead to a greatly expanded understanding of the *C. moniliformis* species complex and possibly also the discovery of new pathogens.

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Table 1. Distribution and host range of *Ceratocystis moniliformis* causing staining.

Host	Geographical distribution	Reference
Macaranga capensis	South Africa	Van Wyk *et al.* 1991
Theobroma sp.	France	Paulin-Mahady *et al.* 2002
Quercus robur	Poland	Kowalski & Butin 1989
Liquidamber styraciflua	Texas, USA	Von Schrenk 1903
Pinus ponderosa	Texas, USA	Hedgcock 1906
Pinus palustris, *P. echinata*, *P. taeda*, *L. styraciflua*, *Liriodendron tulipifera*, *Nyssa aquatica*, *Fagus grandifolia*, *Magnolia* sp., *Quercus* sp.	USA	Davidson 1935
F. crenata, *Q. glandulifera*, *M. hyplleuca*, *Kalopanax ricinifolius*, *Ptercarya rhoifolia*, *Cercidiphyllum japanicum*	Tokyo	Kitajima 1936
Quercus sp.	Scotland	Bakshi 1951
Pycnanthus kombo	Cameroon	Luc 1952
Theobromae sp.	Madagascar	Luc 1952
Calamus maximus, *Endospermum peltatum*, *Parkia javanica*	Philippines	Roldan 1962
Hevea sp.	China	Witthuhn *et al.* 1999
Erythrina sp.	South Africa	Witthuhn *et al.* 1999

Table 2. Morphology of the teleomorph of *Ceratocystis moniliformis* as described by various authors[a].

Character	**Hedgcock 1906**	**Davidson 1935**	**Kitajima 1936**	**Bakshi 1951**	**Luc 1952 Pycnanthi[b]**	**Luc 1952 Theobromae[b]**	**Hunt 1956**	**Roldan 1962**	**Upadhyay 1981**
Colour	Brown/ black	Black	-	Transparent green tinge	Brown/ black	Dark brown	Black	Black	Brown/ black
Diameter	90 – 180	160 – 235	104 –244	190 – 245	210 – 270	135 – 190	120 -160	150 – 250	90 – 210
Ornamentation	Conical spines (sparce), 12 – 16 x 6 (base)	Brown bristles, 18 – 60 x 3 – 4	Brown bristles (numerous), 85 – 124 long	2 types: (1) 65 x1µm (2) 11 – 36 x 7 – 15 (Base) 2 – 3 (Tip)	Dark brown conical spines, 7 – 20 x 6 – 12 (Base)	Elongated, Setae, straight, 12 – 57 x 6 – 11 (Base), 2 – 4 (Tip)	Brown, short, conical spines, 30 x 8	Dark, hyaline tip, 10 – 65 x 2 – 3	Brown conical spines, 12 – 45 x 3 – 8 (Base)
Shape	Globose	Elongate/ pear-shaped	Flask-shaped/ spherical	Round/ elongate	Sub-spherical/ oval	Spherical/ elongated	Globose/ pear shaped	Globose/ Pear shaped	Globose/ Pear shaped
ASCOMATAL NECK									
Colour	-	Black	Black	Black	-	-	Black	Black	Black
Length	-	550 – 1000	305 – 609	731 – 896	600 – 900	500 – 700	900	920	550 –1000
Width: base	-	30 – 36	-	39 – 52	29 – 42	21 – 30	20 – 30	20 – 45	20 – 35
Width: tip	-	14 – 15	-	14	16 – 19	10 – 13	10 – 15	10 – 14	11 – 15

[a] All measurements were made in microns (µ).
[b] Luc described *C. moniliformis* as having two different forms; the pycanti and the theobromae from.

Table 2. Morphology of the teleomorph of *Ceratocystis moniliformis* as described by various authors (Continued)[a].

Character	Hedgcock 1906	Davidson 1935	Kitajima 1936	Bakshi 1951	Luc 1952 Pycnanthi[b]	Luc 1952 Theobromae[b]	Hunt 1956	Roldan 1962	Upadhyay 1981
Ostiolar hyphae	Brown-black, short, thick, 12 – 18 x 2	8 – 12 hyphae, hyaline, filaments 15 – 25	7 – 12 hyphae, hyaline, filaments 11 – 63	2 – 14 hyphae, hyaline, 34 – 41 x 2 – 3 (Base)	8 – 15 hyphae, hyaline setae, 19 – 46 x 2	7 – 12 hyphae, 12 – 21	8 – 10 hyphae, hyaline, 25 x 2	10 – 16 hyphae, hyaline, bent, 10 – 30 x 2	1 – 25 hyphae, hyaline, divergent, 2 – 3
ASCUS									
Colour	Hyaline	-	-	-	Barely visible	Poorly visible	Not seen	Evanescent	Not seen
ASCOSPORES									
Colour	Hyaline	-	Hyaline	Hyaline	Hyaline/ yellowish	Hyaline/ yellowish	-	-	Hyaline
Shape: side view	Oval, one side flat	Broad ovoid (hat)	Kidney shaped	Oval, brim (hat)	Oval, flattened	Oval, flattened	Hat-shaped	Hat-shaped	Oblong reniform
Length	4 – 5	4 – 5	2 – 4	6 – 8	4 – 6	4 – 5	3 - 6	4 - 6	-
Width	3 – 4	2 – 3	4 – 5	3 – 4	3 – 5	3 – 5	2 - 3	3 - 4	-
Texture	Long, slimy, grey mass	Gelatinous sheath	Mucilagi-nous substance	Oval globule, mucilaginous	Pinkish yellow in mass	Pinkish yellow in mass	Gelati-nous sheath	Gelati-nous sheath	Gelatinous sheath

[a] All measurements were made in microns (μ).
[b] Luc described *C. moniliformis* as having two different forms; the pycanti and the theobromae from.

Table 3. Morphology of the anamorph state of *Ceratocystis moniliformis* as described by various authors [a].

Character	Hedgcock 1906	Davidson 1935	Kitajima 1936	Bakshi 1951	Luc 1952 Pycnanthi[b]	Luc 1952 Theobromae[b]	Hunt 1956	Roldan 1962	Upadhyay 1981
CONIDIOPHORES									
Length	-	(1) 3 (2) 5 – 6	(1) 28 – 30 (2) 63 – 70	7 – 14	18 – 52	33 – 65	(1) 60 (2) 35	100	18 – 77
Width	-	-	(1) 5 (2) 7 – 8	(1) 4 – 9 (2) 5	7 – 8	4 – 5	(1) 4 – 6 (2) 6	4 – 6	2 – 3
CONIDIA									
Shape	Cylindrical	(1) Cylindrical (2) Barrel	(1) Cylindrical (2) Barrel	Cylindrical	Cylindrical to barrel	Cylindrical	(1) Cylindrical (2) Barrel	Cylindrical	(1) Cylindrical (2) Shorter
Length	6 – 8	(1) 6 – 10 (2) 5 – 7	(1) 7 – 8 (2) 8 – 14	4 – 16	5 – 8	6 – 20	(1) 6 – 19 (2) 6 – 9	6 – 20	(1) 16 – 20 (2) 6 – 9
Width	2	(1) 3 (2) 5– 6	(1) 1 – 2 (2) 7 – 10	1 – 2	2 – 4	2 – 4	(1) 2 – 3 (2) 4 – 6	2 – 4	(1) 2 – 3 (2) 4 – 6
CULTURE									
Growth rate	-	38 mm in 5 days	-	60mm in 10 days	-	-	45 mm in 5 days	25 mm in 5 days	70 mm in 12 days
Colour	Hyaline / grey /black	Hyaline / light brown	Hyaline / light brown	Hyaline / light brown	Hyaline / brown	-	White / grey / brown	White / brown	Hyaline / pale brown
Odour	-	Banana oil	-	Pear-drops	-	Ethyl acetate	Banana oil	Banana oil	Banana oil
Mycelium width	2 – 8	-	2 – 3	2 – 5	3 – 7	2 – 4	2 – 8	2 – 4	2 – 8

[a] All measurements were made in microns (μ).
[b] Luc described *C. moniliformis* as having two different forms; the pycanti and the theobromae from.

Table 4. *Ceratocystis* isolates used in this study.

Ceratocystis spp.	Isolate no. [d]	Alternative numbers [e, f]	GenBank accession nr.	Date of isolation	Host	Geographical origin	Associated insect	Collector(s)
C. moniliformis	CMW 13011[a]		AY528991[g] AY529001[h] AY529012[i]	1996	*Quercus* sp.	Sumatra, Indonesia	None	M.J. Wingfield
"	CMW 13012[a, b]		AY528992[g] AY529002[h] AY529013[i]	"	"	"	"	"
"	CMW 13013[a, b, c]		AY528993[g] AY529003[h] AY529014[i]	"	"	"	"	"
"	CMW 13015[a, b, c]		AY528994[g] AY529004[h] AY529015[i]	"	"	"	"	"
"	CMW 9590[a, c]		AY528985[g] AY528996[h] AY529006[i]	2002	*Eucalyptus grandis*	Mpumalanga, South Africa	"	J. Roux
"	CMW 10134[a, c]		N/A	"	"	"	"	M. van Wyk
"	CMW 4114[a]		AY528986[g] AY528997[h] AY529007[i]	1997	*Schizolobium parahybum*	Ecuador, South America	"	M. J. Wingfield
"	CMW 9990[a]	CBS 155.62	N/A	1962	*Theobroma cacao*	Costa Rica	"	A. J. Hansen
"	CMW 8379[a]		AY528995[g] AY529005[h] AY529016[i]	2001	*Cassia fistula*	Punaka, Bhutan	"	M. J. Wingfield, T. Kirisits & D. B. Chhetri
"	CMW 8240[a]		AY528989[g] AY529000[h] AY529010[i]	"	"	Wangdi, Bhutan	"	"
"	CMW 8238[c]		N/A	"	"	"	"	"
C. moniliformopsis	CMW 9986[a, c]	CBS 109441	AY528987[g] AY528998[h] AY529008[i]	1999	*Eucalyptus obliqua*	Tazmania, Australia	"	Z. Q. Yuan
"	CMW 10214[a, c]		AY528988[g] AY528999[h] AY529009[i]	1989	*E. sieberi*	"	"	M. J. Dudzinski

Table 4. *Ceratocystis* isolates used in this study (Continued).

Ceratocystis spp.	Isolate no. [d]	Alternative numbers [e, f]	GenBank accession nr.	Date of isolation	Host	Geographical origin	Associated insect	Collector(s)
C. bhutanensis	CMW 8217[a, c]	PREM 57807	AY528952[g] AY528957[h] AY528962[i]	2001	*Pinus spinulosa*	Jeleka, Bhutan	*Ips schmutzen-hoferi*	M. J. Wingfield, T. Kirisits & D. B. Chhetri
"	CMW 8242[a]	CBS 112907	AY528951[g] AY528956[h] AY528961[i]	2001	*Pinus spinulosa*	Jeleka, Bhutan	*Ips schmutzenhoferi*	"
"	CMW 8244[c]	PREM 57811	N/A	"	"	"	"	"
"	CMW 8241[c]	PREM 57808	N/A	"	"	"	"	"
C. omanensis	CMW 11048[a, c]	None	N/A	2003	*Mangifera* sp.	Oman	*Cryphalus scabrecollis*	A. Al-Adawi
"	CMW 3800[a]	"	N/A	"	"	"	"	"
C. virescens	CMW 3276 [a]	"	AY528984[g] AY528990[h] AY528991[i]	1963	*Quercus* sp.	N.Y., USA	None	Hinds, T.

[a, b, c, d, e, f, g, h, i] Isolates marked with [a] were sequenced, those marked with [b] were used for morphological descriptions and those marked with [c] were 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 to the National Fungal Herbarium (PREM), Pretoria, South Africa. GenBank accession numbers that are marked with [g] represents the ITS sequences, those marked with an [h] represent the β-tubulin sequences and those marked with a [i] represent the elongation factor sequences.

Figure 1. A phylogenetic tree based on the combined sequence data from three gene regions; ITS, β-tubulin and EF1-α, showing the monophyletic nature of *C. moniliformis* sensu lato. The phylogram was obtained using the heuristic search option based on parsimony. Bootstrap values are indicated above the branches and Bayesian values are indicated below the branches. *Ceratocystis virescens* was treated as the out-group.

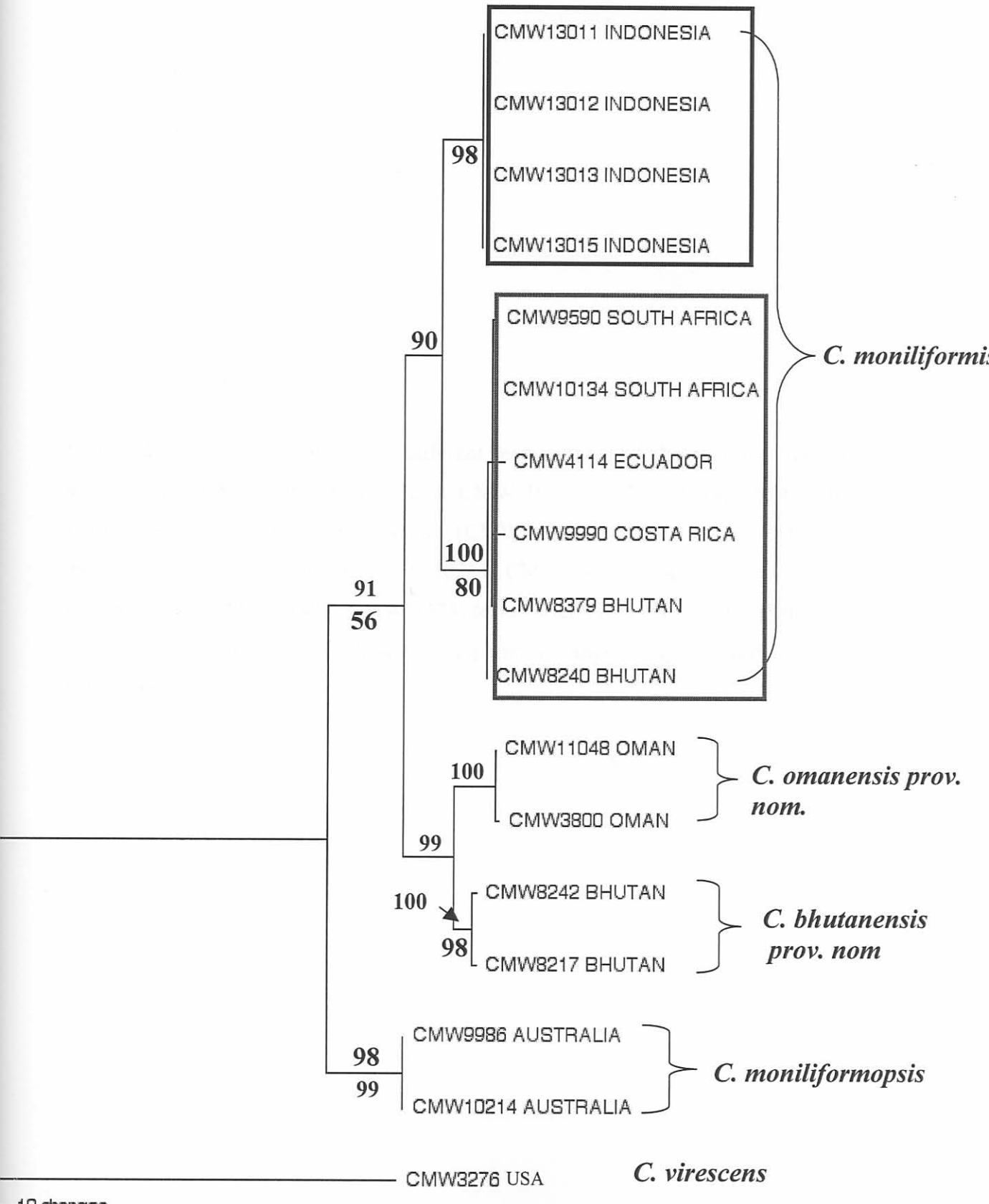
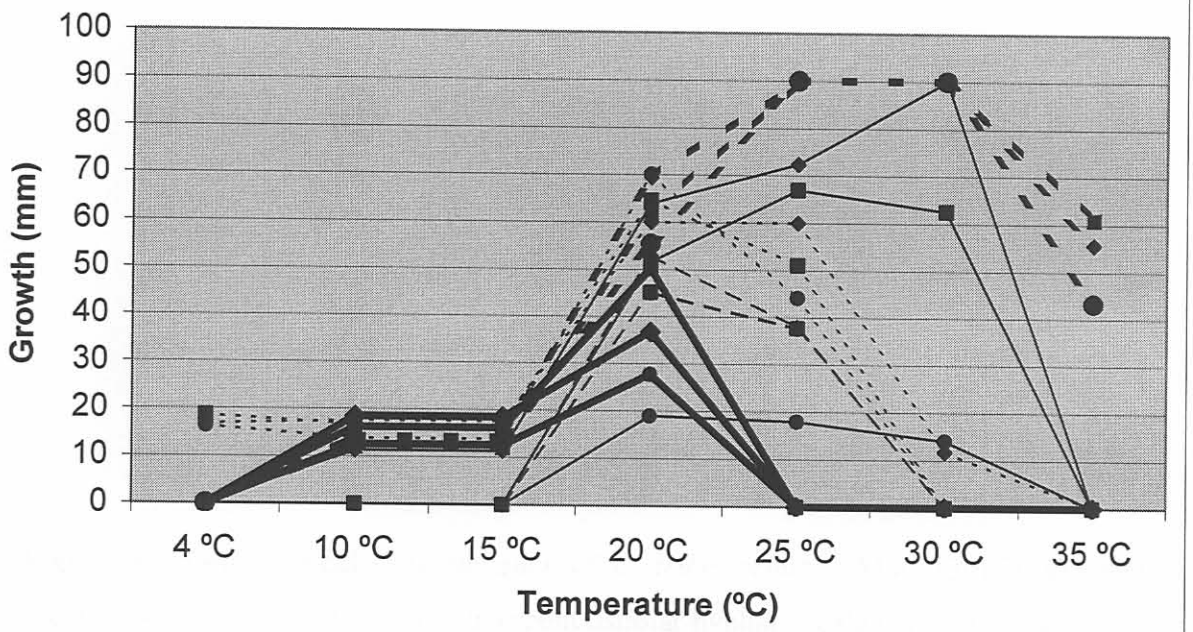
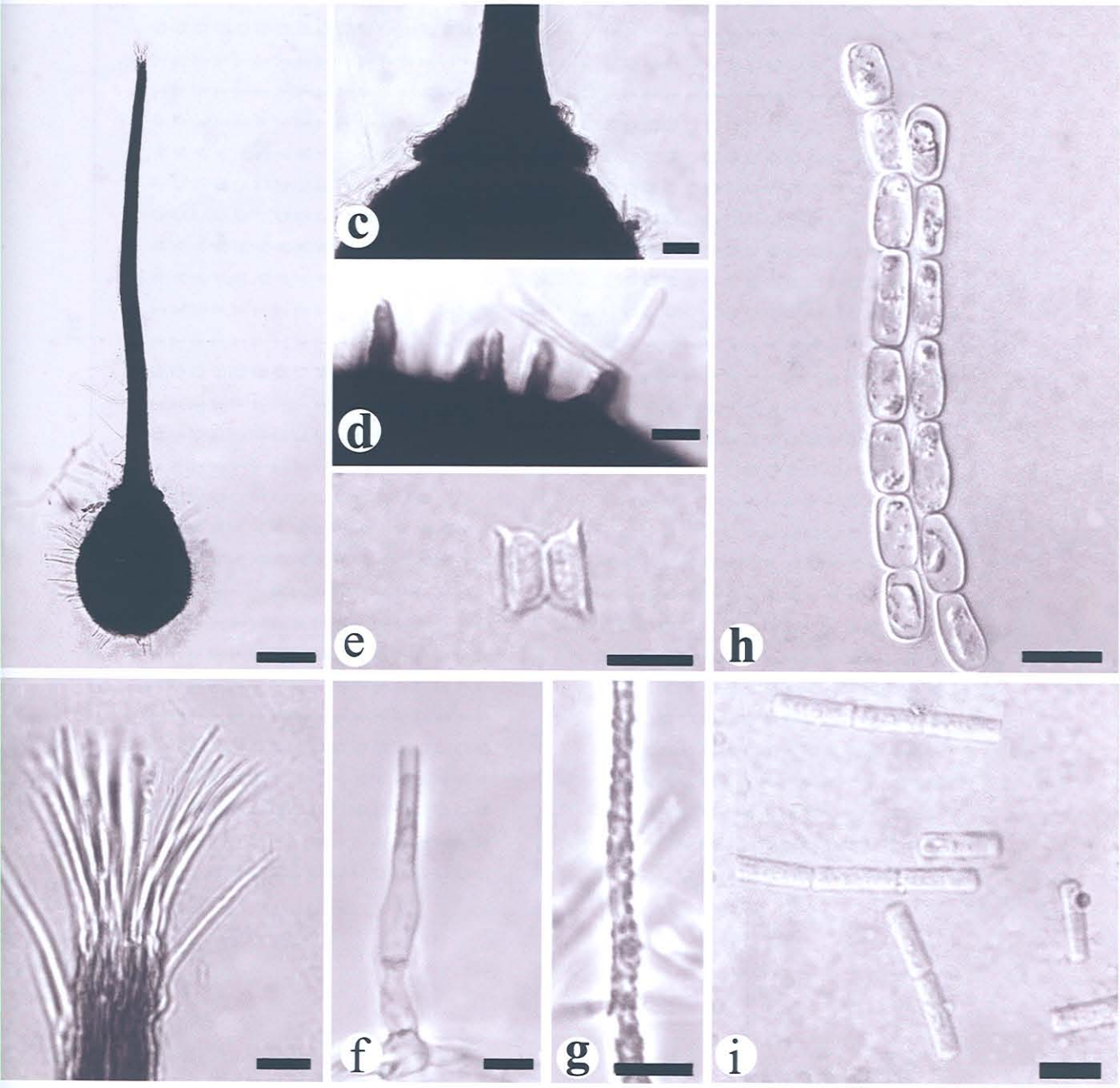

CMW13011 INDONESIA
CMW13012 INDONESIA
CMW13013 INDONESIA
CMW13015 INDONESIA
98
CMW9590 SOUTH AFRICA
CMW10134 SOUTH AFRICA
CMW4114 ECUADOR
CMW9990 COSTA RICA
CMW8379 BHUTAN
CMW8240 BHUTAN
90
100
80
91
56
C. moniliformis
CMW11048 OMAN
CMW3800 OMAN
100
C. omanensis prov. nom.
99
CMW8242 BHUTAN
CMW8217 BHUTAN
100
98
C. bhutanensis prov. nom
CMW9986 AUSTRALIA
CMW10214 AUSTRALIA
98
99
C. moniliformopsis
CMW3276 USA
C. virescens
10 changes

Figure 2. Graphs of growth at 7 different temperatures of the four different species, *C. moniliformis* (CMW 9590, CMW 8238, CMW 10134, CMW 13014, CMW 13013, CMW 13015), *C. bhutanensis prov. nom* .(CMW 8217, CMW 8244, CMW 8241), *C. moniliformopsis* (CMW 9986, CMW 10214, CMW 10215) and *C. omanensis prov. nom.* (CMW 11408, CMW 11407, CMW 3374) tested after 72 hours of incubation. The standard deviation for each isolate at all seven temperatures respectively, are indicated in the table below the graph.



Temperature/ Isolate	4 °C	10 °C	15 °C	20 °C	25 °C	30 °C	35 °C
CMW 9590	0.0	3.2	3.2	1.9	0.8	0.0	0.0
CMW 8238	0.0	0.0	0.0	2.4	1.4	2.9	0.0
CMW 10134	0.0	0.0	0.0	4.1	5.0	3.4	0.0
CMW 13014	0.0	0.0	0.0	3.0	2.1	0.0	0.0
CMW 13013	0.0	0.0	0.0	1.9	2.7	0.0	0.0
CMW 13015	0.0	0.0	0.0	1.7	2.4	0.0	0.0
CMW 8217	1.3	0.5	0.5	3.9	3.7	2.0	0.0
CMW 8244	1.1	0.6	0.6	7.3	3.2	0.0	0.0
CMW 8241	0.6	0.8	0.8	5.1	4.1	0.0	0.0
CMW 9986	0.0	1.7	1.8	1.1	0.0	0.0	0.0
CMW 10214	0.0	6.6	6.6	0.7	0.0	0.0	0.0
CMW 12215	0.0	1.2	1.2	5.8	0.0	0.0	0.0
CMW 11408	0.0	2.7	2.7	1.9	0.0	0.0	1.1
CMW 11407	0.0	2.4	2.4	7.0	0.0	0.0	7.2
CMW 3374	0.0	2.3	2.3	7.4	0.0	0.0	16.6

Figure 3. Morphological characteristics of *C. tribiliformis* (CMW 13015). a) Obpyriform ascoma (scale bar = 100 µm), b) Divergent ostiolar hyphae (scale bar = 10 µm), c) Ascomatal neck disc-shaped at base (scale bar = 10 µm), d) Ascomatal base with short, conical spines and hyphal ornamentation (scale bar = 10 µm), e) Hat-shaped ascospore in side view, oval in face view (scale bar = 10 µm), f) Phialidic conidiogenous cell (scale bar = 5 µm), g) Hyphae with rough edges (scalebar = 5 µm), h) Barrel-shaped conidia (scale bar = 10 µm), i) Cylindrical conidia (scale bar = 10 µm).



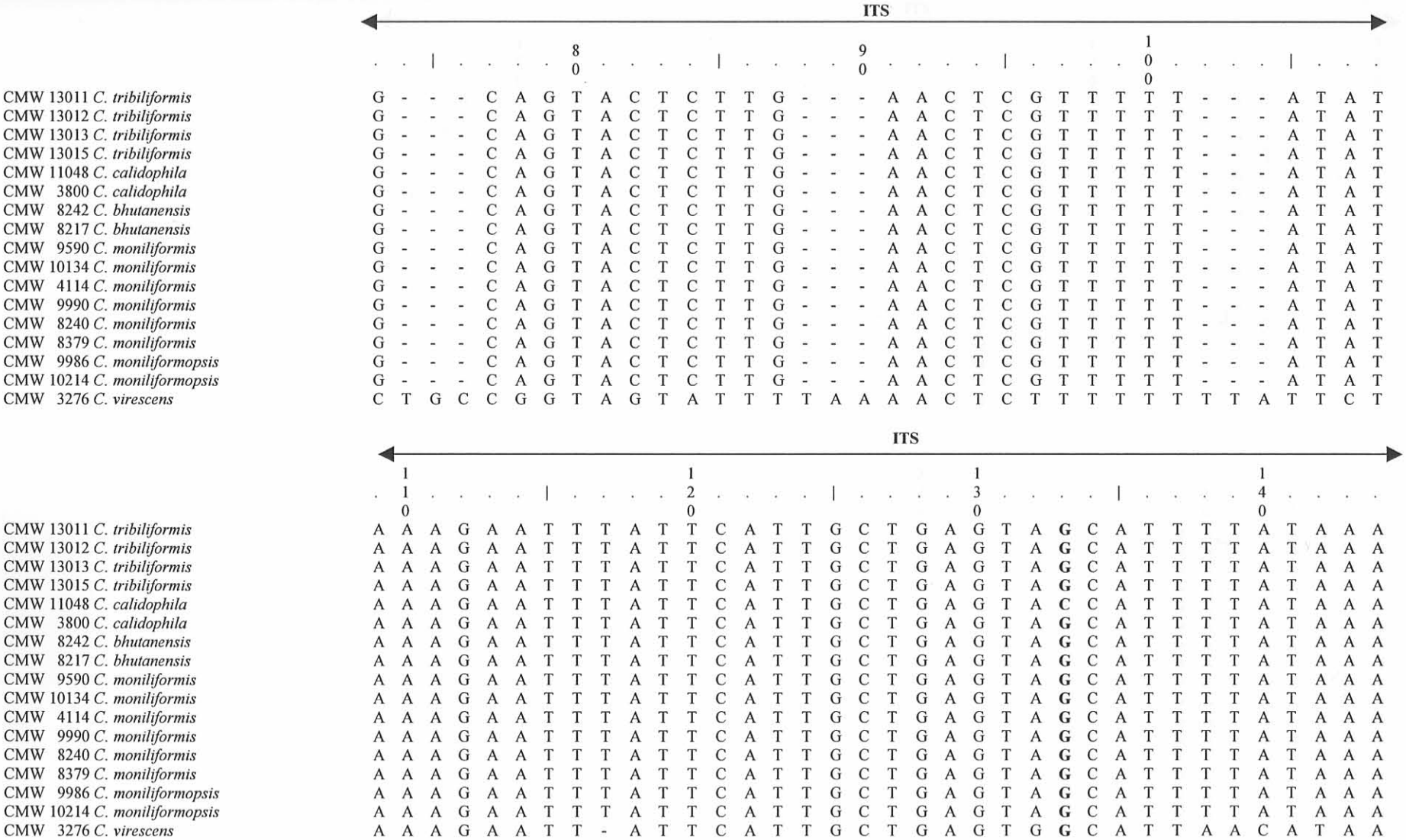
APPENDIX

ITS

	1	.	.	.	|	.	.	.	.	10	.	.	.	.	|	.	.	.	.	20	.	.	.	.	|	.	.	.	.	30	.	.	.	.	|	.
CMW 13011 *C. tribiliformis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 13012 *C. tribiliformis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 13013 *C. tribiliformis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 13015 *C. tribiliformis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 11048 *C. calidophila*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 3800 *C. calidophila*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 8242 *C. bhutanensis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 8217 *C. bhutanensis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 9590 *C. moniliformis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 10134 *C. moniliformis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 4114 *C. moniliformis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 9990 *C. moniliformis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 8240 *C. moniliformis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 8379 *C. moniliformis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 9986 *C. moniliformopsis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 10214 *C. moniliformopsis*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	G	T	T	A	A	C	C	A	T	T	T	G	T	G	A	A	T	T	T	A
CMW 3276 *C. virescens*	C	T	G	A	G	T	T	T	T	T	A	A	C	T	C	T	-	T	A	A	A	C	C	A	T	A	T	G	T	G	A	A	C	A	T	A

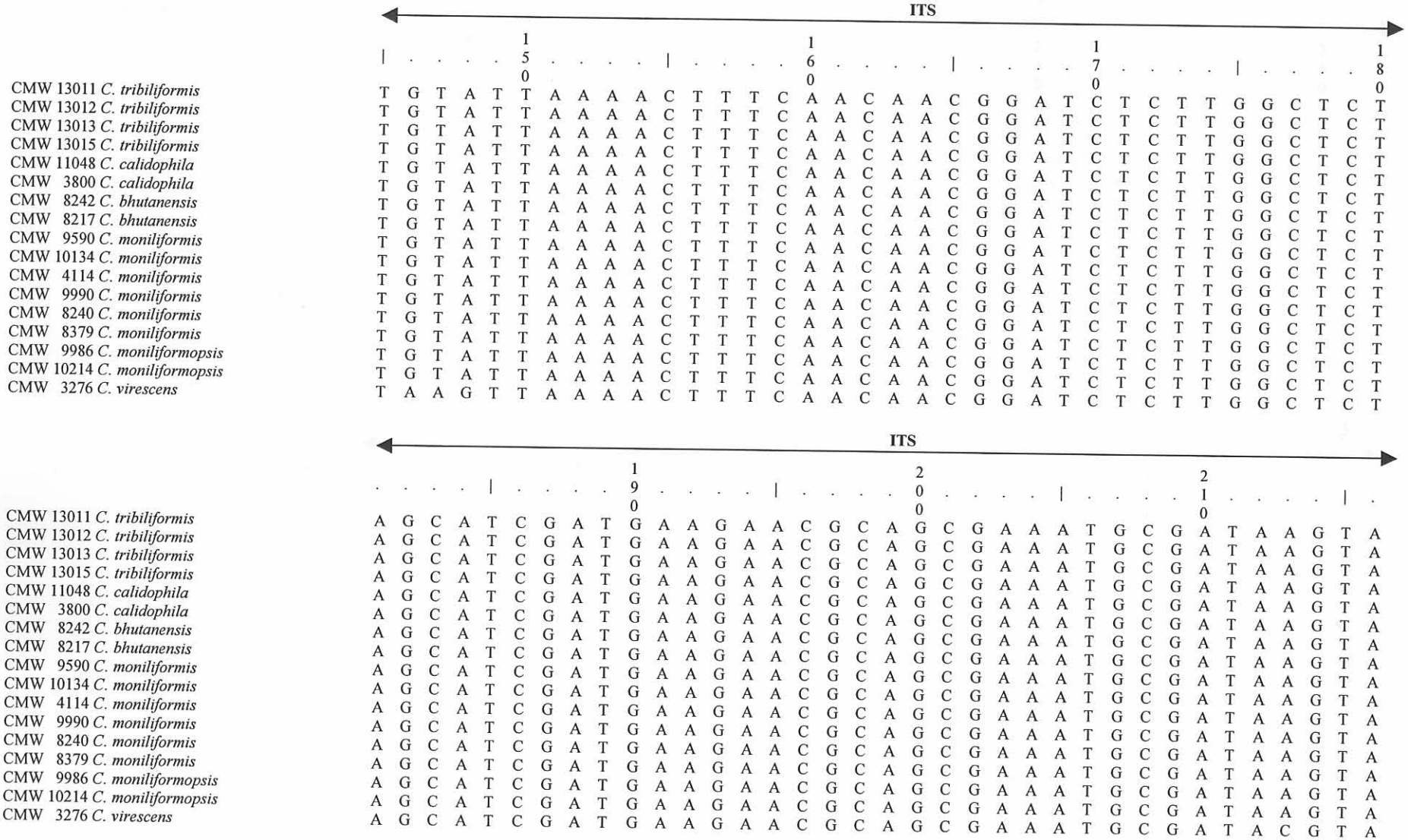
ITS

	.	.	.	40	.	.	.	.	|	.	.	.	.	50	.	.	.	.	|	.	.	.	.	60	.	.	.	.	|	.	.	.	.	70	.	.
CMW 13011 *C. tribiliformis*	C	C	A	A	A	C	A	T	G	A	A	C	T	G	C	A	T	**A**	**C**	G	G	C	G	G	G	T	C	T	C	C	C	-	G	C	C	**A**
CMW 13012 *C. tribiliformis*	C	C	A	A	A	C	A	T	G	A	A	C	T	G	C	A	T	**A**	**C**	G	G	C	G	G	G	T	C	T	C	C	C	-	G	C	C	**A**
CMW 13013 *C. tribiliformis*	C	C	A	A	A	C	A	T	G	A	A	C	T	G	C	A	T	**A**	**C**	G	G	C	G	G	G	T	C	T	C	C	C	-	G	C	C	**A**
CMW 13015 *C. tribiliformis*	C	C	A	A	A	C	A	T	G	A	A	C	T	G	C	A	T	**A**	**C**	G	G	C	G	G	G	T	C	T	C	C	C	-	G	C	C	**A**
CMW 11048 *C. calidophila*	C	C	A	A	A	C	A	T	G	A	A	C	T	G	C	A	T	**T**	-	G	G	C	G	G	G	T	C	T	C	C	C	-	G	C	C	**G**
CMW 3800 *C. calidophila*	C	C	A	A	A	C	A	T	G	A	A	C	T	G	C	A	T	**T**	-	G	G	C	G	G	G	T	C	T	C	C	C	-	G	C	C	**G**
CMW 8242 *C. bhutanensis*	C	C	A	A	A	C	A	T	G	A	A	C	T	G	C	A	T	-	**C**	G	G	C	G	G	G	T	C	T	C	C	C	-	G	C	C	**A**
CMW 8217 *C. bhutanensis*	C	C	A	A	A	C	A	T	G	A	A	C	T	G	C	A	T	-	**C**	G	G	C	G	G	G	T	C	T	C	C	C	-	G	C	C	**A**
CMW 9590 *C. moniliformis*	C	C	A	A	A	C	A	T	G	A	A	C	T	G	C	A	T	**A**	**C**	G	G	C	G	G	G	T	C	T	C	C	C	-	G	C	C	**A**
CMW 10134 *C. moniliformis*	C	C	A	A	A	C	A	T	G	A	A	C	T	G	C	A	T	**A**	**C**	G	G	C	G	G	G	T	C	T	C	C	C	-	G	C	C	**A**
CMW 4114 *C. moniliformis*	C	C	A	A	A	C	A	T	G	A	A	C	T	G	C	A	T	**A**	**C**	G	G	C	G	G	G	T	C	T	C	C	C	-	G	C	C	**A**
CMW 9990 *C. moniliformis*	C	C	A	A	A	C	A	T	G	A	A	C	T	G	C	A	T	-	**C**	G	G	C	G	G	G	T	C	T	C	C	C	-	G	C	C	**A**
CMW 8240 *C. moniliformis*	C	C	A	A	A	C	A	T	G	A	A	C	T	G	C	A	T	**A**	**C**	G	G	C	G	G	G	T	C	T	C	C	C	-	G	C	C	**A**
CMW 8379 *C. moniliformis*	C	C	A	A	A	C	A	T	G	A	A	C	T	G	C	A	T	**A**	**C**	G	G	C	G	G	G	T	C	T	C	C	C	-	G	C	C	**A**
CMW 9986 *C. moniliformopsis*	C	C	A	A	A	C	A	T	G	A	A	C	T	G	C	A	T	**A**	**T**	G	G	C	G	G	G	T	C	T	C	C	C	-	G	C	C	**A**
CMW 10214 *C. moniliformopsis*	C	C	A	A	A	C	A	T	G	A	A	C	T	G	C	A	T	**A**	**T**	G	G	C	G	G	G	T	C	T	C	C	C	-	G	C	C	**A**
CMW 3276 *C. virescens*	C	C	T	A	T	T	A	G	C	T	G	C	T	T	T	G	G	**C**	**A**	G	G	C	T	T	G	G	T	A	A	C	A	C	A	A	G	**T**



	80	90	100
CMW 13011 *C. tribiliformis*	G - - - C A G T A C T C T T G - - - A A C T C G T T T T T - - - A T A T
CMW 13012 *C. tribiliformis*	G - - - C A G T A C T C T T G - - - A A C T C G T T T T T - - - A T A T
CMW 13013 *C. tribiliformis*	G - - - C A G T A C T C T T G - - - A A C T C G T T T T T - - - A T A T
CMW 13015 *C. tribiliformis*	G - - - C A G T A C T C T T G - - - A A C T C G T T T T T - - - A T A T
CMW 11048 *C. calidophila*	G - - - C A G T A C T C T T G - - - A A C T C G T T T T T - - - A T A T
CMW 3800 *C. calidophila*	G - - - C A G T A C T C T T G - - - A A C T C G T T T T T - - - A T A T
CMW 8242 *C. bhutanensis*	G - - - C A G T A C T C T T G - - - A A C T C G T T T T T - - - A T A T
CMW 8217 *C. bhutanensis*	G - - - C A G T A C T C T T G - - - A A C T C G T T T T T - - - A T A T
CMW 9590 *C. moniliformis*	G - - - C A G T A C T C T T G - - - A A C T C G T T T T T - - - A T A T
CMW 10134 *C. moniliformis*	G - - - C A G T A C T C T T G - - - A A C T C G T T T T T - - - A T A T
CMW 4114 *C. moniliformis*	G - - - C A G T A C T C T T G - - - A A C T C G T T T T T - - - A T A T
CMW 9990 *C. moniliformis*	G - - - C A G T A C T C T T G - - - A A C T C G T T T T T - - - A T A T
CMW 8240 *C. moniliformis*	G - - - C A G T A C T C T T G - - - A A C T C G T T T T T - - - A T A T
CMW 8379 *C. moniliformis*	G - - - C A G T A C T C T T G - - - A A C T C G T T T T T - - - A T A T
CMW 9986 *C. moniliformopsis*	G - - - C A G T A C T C T T G - - - A A C T C G T T T T T - - - A T A T
CMW 10214 *C. moniliformopsis*	G - - - C A G T A C T C T T G - - - A A C T C G T T T T T - - - A T A T
CMW 3276 *C. virescens*	C T G C C G G T A G T A T T T T A A A A C T C T T T T T T T T A T T C T

	110	120	130	140
CMW 13011 *C. tribiliformis*	A A A G A A T T T A T T C A T T G C T G A G T A **G** C A T T T T A T A A A
CMW 13012 *C. tribiliformis*	A A A G A A T T T A T T C A T T G C T G A G T A **G** C A T T T T A T A A A
CMW 13013 *C. tribiliformis*	A A A G A A T T T A T T C A T T G C T G A G T A **G** C A T T T T A T A A A
CMW 13015 *C. tribiliformis*	A A A G A A T T T A T T C A T T G C T G A G T A **G** C A T T T T A T A A A
CMW 11048 *C. calidophila*	A A A G A A T T T A T T C A T T G C T G A G T A **C** C A T T T T A T A A A
CMW 3800 *C. calidophila*	A A A G A A T T T A T T C A T T G C T G A G T A **G** C A T T T T A T A A A
CMW 8242 *C. bhutanensis*	A A A G A A T T T A T T C A T T G C T G A G T A **G** C A T T T T A T A A A
CMW 8217 *C. bhutanensis*	A A A G A A T T T A T T C A T T G C T G A G T A **G** C A T T T T A T A A A
CMW 9590 *C. moniliformis*	A A A G A A T T T A T T C A T T G C T G A G T A **G** C A T T T T A T A A A
CMW 10134 *C. moniliformis*	A A A G A A T T T A T T C A T T G C T G A G T A **G** C A T T T T A T A A A
CMW 4114 *C. moniliformis*	A A A G A A T T T A T T C A T T G C T G A G T A **G** C A T T T T A T A A A
CMW 9990 *C. moniliformis*	A A A G A A T T T A T T C A T T G C T G A G T A **G** C A T T T T A T A A A
CMW 8240 *C. moniliformis*	A A A G A A T T T A T T C A T T G C T G A G T A **G** C A T T T T A T A A A
CMW 8379 *C. moniliformis*	A A A G A A T T T A T T C A T T G C T G A G T A **G** C A T T T T A T A A A
CMW 9986 *C. moniliformopsis*	A A A G A A T T T A T T C A T T G C T G A G T A **G** C A T T T T A T A A A
CMW 10214 *C. moniliformopsis*	A A A G A A T T T A T T C A T T G C T G A G T A **G** C A T T T T A T A A A
CMW 3276 *C. virescens*	A A A G A A T T - A T T C A T T G C T G A G T G **G** C A T T T A A C A T A A

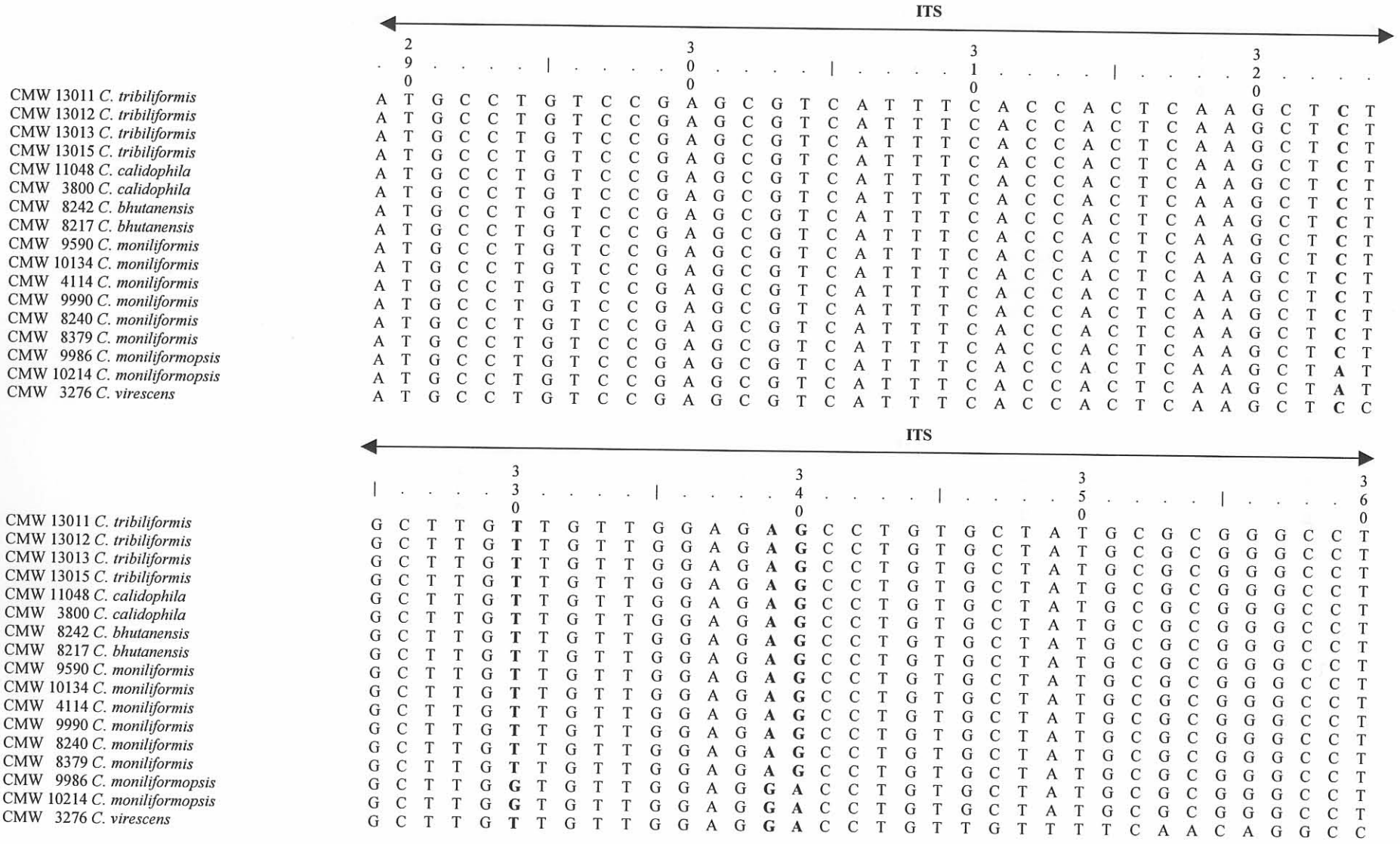


ITS

	210	220	230	240	250
CMW 13011 *C. tribiliformis*	ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT				
CMW 13012 *C. tribiliformis*	ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT				
CMW 13013 *C. tribiliformis*	ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT				
CMW 13015 *C. tribiliformis*	ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT				
CMW 11048 *C. calidophila*	ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT				
CMW 3800 *C. calidophila*	ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT				
CMW 8242 *C. bhutanensis*	ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT				
CMW 8217 *C. bhutanensis*	ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT				
CMW 9590 *C. moniliformis*	ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT				
CMW 10134 *C. moniliformis*	ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT				
CMW 4114 *C. moniliformis*	ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT				
CMW 9990 *C. moniliformis*	ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT				
CMW 8240 *C. moniliformis*	ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT				
CMW 8379 *C. moniliformis*	ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT				
CMW 9986 *C. moniliformopsis*	ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT				
CMW 10214 *C. moniliformopsis*	ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT				
CMW 3276 *C. virescens*	ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT				

ITS

	260	270	280
CMW 13011 *C. tribiliformis*	TGAACGCACATTGCGCCCCAGCAGTACTCTGCTTGGGC		
CMW 13012 *C. tribiliformis*	TGAACGCACATTGCGCCCCAGCAGTACTCTGCTTGGGC		
CMW 13013 *C. tribiliformis*	TGAACGCACATTGCGCCCCAGCAGTACTCTGCTTGGGC		
CMW 13015 *C. tribiliformis*	TGAACGCACATTGCGCCCCAGCAGTACTCTGCTTGGGC		
CMW 11048 *C. calidophila*	TGAACGCACATTGCGCCCCAGCAGTACTCTGCTTGGGC		
CMW 3800 *C. calidophila*	TGAACGCACATTGCGCCCCAGCAGTACTCTGCTTGGGC		
CMW 8242 *C. bhutanensis*	TGAACGCACATTGCGCCCCAGCAGTACTCTGCTTGGGC		
CMW 8217 *C. bhutanensis*	TGAACGCACATTGCGCCCCAGCAGTACTCTGCTTGGGC		
CMW 9590 *C. moniliformis*	TGAACGCACATTGCGCCCCAGCAGTACTCTGCTTGGGC		
CMW 10134 *C. moniliformis*	TGAACGCACATTGCGCCCCAGCAGTACTCTGCTTGGGC		
CMW 4114 *C. moniliformis*	TGAACGCACATTGCGCCCCAGCAGTACTCTGCTTGGGC		
CMW 9990 *C. moniliformis*	TGAACGCACATTGCGCCCCAGCAGTACTCTGCTTGGGC		
CMW 8240 *C. moniliformis*	TGAACGCACATTGCGCCCCAGCAGTACTCTGCTTGGGC		
CMW 8379 *C. moniliformis*	TGAACGCACATTGCGCCCCAGCAGTACTCTGCTTGGGC		
CMW 9986 *C. moniliformopsis*	TGAACGCACATTGCGCCCCAGCAGTACTCTGCTTGGGC		
CMW 10214 *C. moniliformopsis*	TGAACGCACATTGCGCCCCAGCAGTACTCTGCTTGGGC		
CMW 3276 *C. virescens*	TGAACGCACATTGCGCCTCAGGCAGTATTCTGCCAGGC		


ITS
ITS
CMW 13011 C. tribiliformis
CMW 13012 C. tribiliformis
CMW 13013 C. tribiliformis
CMW 13015 C. tribiliformis
CMW 11048 C. calidophila
CMW 3800 C. calidophila
CMW 8242 C. bhutanensis
CMW 8217 C. bhutanensis
CMW 9590 C. moniliformis
CMW 10134 C. moniliformis
CMW 4114 C. moniliformis
CMW 9990 C. moniliformis
CMW 8240 C. moniliformis
CMW 8379 C. moniliformis
CMW 9986 C. moniliformopsis
CMW 10214 C. moniliformopsis
CMW 3276 C. virescens

ITS

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CMW 13011 *C. tribiliformis*	C	T	-	G	A	A	A	T	G	**T**	A	T	C	G	G	C	T	G	T	G	T	T	A	C	A	T	G	C	A	G	T	T	T	C	C	C
CMW 13012 *C. tribiliformis*	C	T	-	G	A	A	A	T	G	**T**	A	T	C	G	G	C	T	G	T	G	T	T	A	C	A	T	G	C	A	G	T	T	T	C	C	C
CMW 13013 *C. tribiliformis*	C	T	-	G	A	A	A	T	G	**T**	A	T	C	G	G	C	T	G	T	G	T	T	A	C	A	T	G	C	A	G	T	T	T	C	C	C
CMW 13015 *C. tribiliformis*	C	T	-	G	A	A	A	T	G	**T**	A	T	C	G	G	C	T	G	T	G	T	T	A	C	A	T	G	C	A	G	T	T	T	C	C	C
CMW 11048 *C. calidophila*	C	T	-	G	A	A	A	T	G	**C**	A	T	C	G	G	C	T	G	T	G	T	T	A	C	A	T	G	C	A	G	T	T	T	C	C	C
CMW 3800 *C. calidophila*	C	T	-	G	A	A	A	T	G	**C**	A	T	C	G	G	C	T	G	T	G	T	T	A	C	A	T	G	C	A	G	T	T	T	C	C	C
CMW 8242 *C. bhutanensis*	C	T	-	G	A	A	A	T	G	**C**	A	T	C	G	G	C	T	G	T	G	T	T	A	C	A	T	G	C	A	G	T	T	T	C	C	C
CMW 8217 *C. bhutanensis*	C	T	-	G	A	A	A	T	G	**C**	A	T	C	G	G	C	T	G	T	G	T	T	A	C	A	T	G	C	A	G	T	T	T	C	C	C
CMW 9590 *C. moniliformis*	C	T	-	G	A	A	A	T	G	**T**	A	T	C	G	G	C	T	G	T	G	T	T	A	C	A	T	G	C	A	G	T	T	T	C	C	C
CMW 10134 *C. moniliformis*	C	T	-	G	A	A	A	T	G	**T**	A	T	C	G	G	C	T	G	T	G	T	T	A	C	A	T	G	C	A	G	T	T	T	C	C	C
CMW 4114 *C. moniliformis*	C	T	-	G	A	A	A	T	G	**T**	A	T	C	G	G	C	T	G	T	G	T	T	A	C	A	T	G	C	A	G	T	T	T	C	C	C
CMW 9990 *C. moniliformis*	C	T	-	G	A	A	A	T	G	**C**	A	T	C	G	G	C	T	G	T	G	T	T	A	C	A	T	G	C	A	G	T	T	T	C	C	C
CMW 8240 *C. moniliformis*	C	T	-	G	A	A	A	T	G	**T**	A	T	C	G	G	C	T	G	T	G	T	T	A	C	A	T	G	C	A	G	T	T	T	C	C	C
CMW 8379 *C. moniliformis*	C	T	-	G	A	A	A	T	G	**T**	A	T	C	G	G	C	T	G	T	G	T	T	A	C	A	T	G	C	A	G	T	T	T	C	C	C
CMW 9986 *C. moniliformopsis*	C	T	-	G	A	A	A	T	G	**T**	A	T	C	G	G	C	T	G	T	G	T	T	A	C	A	T	G	C	A	G	T	T	T	C	C	C
CMW 10214 *C. moniliformopsis*	C	T	-	G	A	A	A	T	G	**T**	A	T	C	G	G	C	T	G	T	G	T	T	A	C	A	T	G	C	A	G	T	T	T	C	C	C
CMW 3276 *C. virescens*	A	C	C	G	A	A	A	T	G	**C**	A	T	C	G	G	C	T	G	T	T	A	T	A	C	T	T	G	C	A	G	C	T	T	C	C	C

ITS

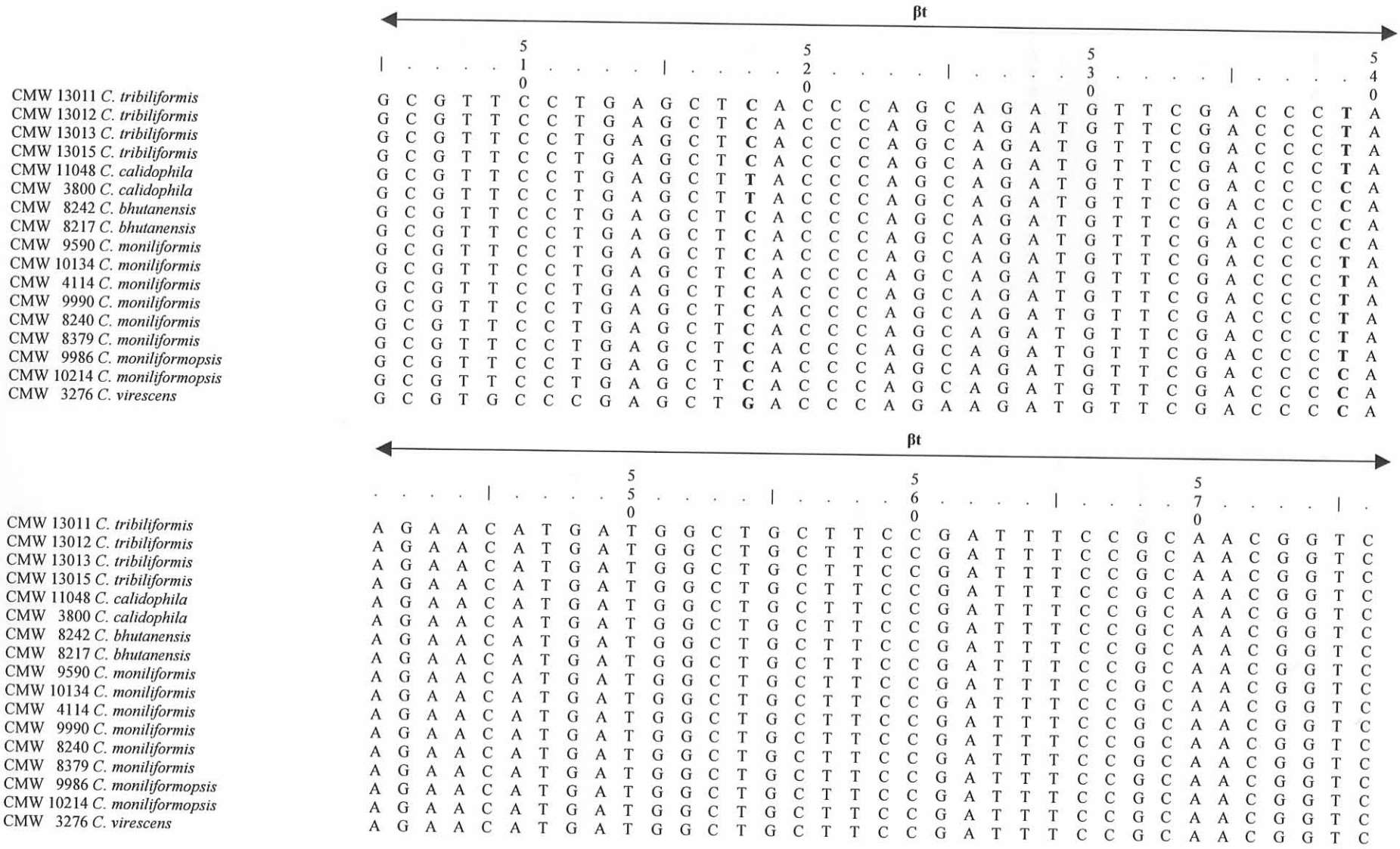
	.	.	.	400	.	.	.	.	|	.	.	.	.	410	.	.	.	.	|	.	.	.	.	420	.	.	.	.	|	.	.	.	.	430	.	.
CMW 13011 *C. tribiliformis*	T	G	T	G	T	A	G	T	A	A	A	A	C	T	T	T	A	**C**	*T*	G	T	T	G	C	A	C	T	T	T	G	A	A	A	T	T	C
CMW 13012 *C. tribiliformis*	T	G	T	G	T	A	G	T	A	A	A	A	C	T	T	T	A	**C**	*T*	G	T	T	G	C	A	C	T	T	T	G	A	A	A	T	T	C
CMW 13013 *C. tribiliformis*	T	G	T	G	T	A	G	T	A	A	A	A	C	T	T	T	A	**C**	*T*	G	T	T	G	C	A	C	T	T	T	G	A	A	A	T	T	C
CMW 13015 *C. tribiliformis*	T	G	T	G	T	A	G	T	A	A	A	A	C	T	T	T	A	**C**	*T*	G	T	T	G	C	A	C	T	T	T	G	A	A	A	T	T	C
CMW 11048 *C. calidophila*	T	G	T	G	T	A	G	T	A	A	A	A	C	T	T	T	A	**T**	*T*	G	T	T	G	C	A	C	T	T	T	G	A	A	A	T	T	C
CMW 3800 *C. calidophila*	T	G	T	G	T	A	G	T	A	A	A	A	C	T	T	T	A	**T**	*T*	G	T	T	G	C	A	C	T	T	T	G	A	A	A	T	T	C
CMW 8242 *C. bhutanensis*	T	G	T	G	T	A	G	T	A	A	A	A	C	T	T	T	A	**T**	*T*	G	T	T	G	C	A	C	T	T	T	G	A	A	A	T	T	C
CMW 8217 *C. bhutanensis*	T	G	T	G	T	A	G	T	A	A	A	A	C	T	T	T	A	**T**	*T*	G	T	T	G	C	A	C	T	T	T	G	A	A	A	T	T	C
CMW 9590 *C. moniliformis*	T	G	T	G	T	A	G	T	A	A	A	A	C	T	T	T	A	**C**	*T*	G	T	T	G	C	A	C	T	T	T	G	A	A	A	T	T	C
CMW 10134 *C. moniliformis*	T	G	T	G	T	A	G	T	A	A	A	A	C	T	T	T	A	**C**	*T*	G	T	T	G	C	A	C	T	T	T	G	A	A	A	T	T	C
CMW 4114 *C. moniliformis*	T	G	T	G	T	A	G	T	A	A	A	A	C	T	T	T	A	**C**	*T*	G	T	T	G	C	A	C	T	T	T	G	A	A	A	T	T	C
CMW 9990 *C. moniliformis*	T	G	T	G	T	A	G	T	A	A	A	A	C	T	T	T	A	**T**	*T*	G	T	T	G	C	A	C	T	T	T	G	A	A	A	T	T	C
CMW 8240 *C. moniliformis*	T	G	T	G	T	A	G	T	A	A	A	A	C	T	T	T	A	**C**	*T*	G	T	T	G	C	A	C	T	T	T	G	A	A	A	T	T	C
CMW 8379 *C. moniliformis*	T	G	T	G	T	A	G	T	A	A	A	A	C	T	T	T	A	**C**	*T*	G	T	T	G	C	A	C	T	T	T	G	A	A	A	T	T	C
CMW 9986 *C. moniliformopsis*	T	G	T	G	T	A	G	T	A	A	A	A	C	T	T	T	A	**C**	*T*	G	T	T	G	C	A	C	T	T	T	G	A	A	A	T	T	C
CMW 10214 *C. moniliformopsis*	T	G	T	G	T	A	G	T	A	A	A	A	C	T	T	T	A	**C**	*T*	G	T	T	G	C	A	C	T	T	T	G	A	A	A	T	T	C
CMW 3276 *C. virescens*	T	G	T	G	T	A	G	T	A	A	-	-	-	T	A	T	C	**T**	*A*	T	T	T	A	C	A	C	T	T	T	G	A	A	A	C	T	C

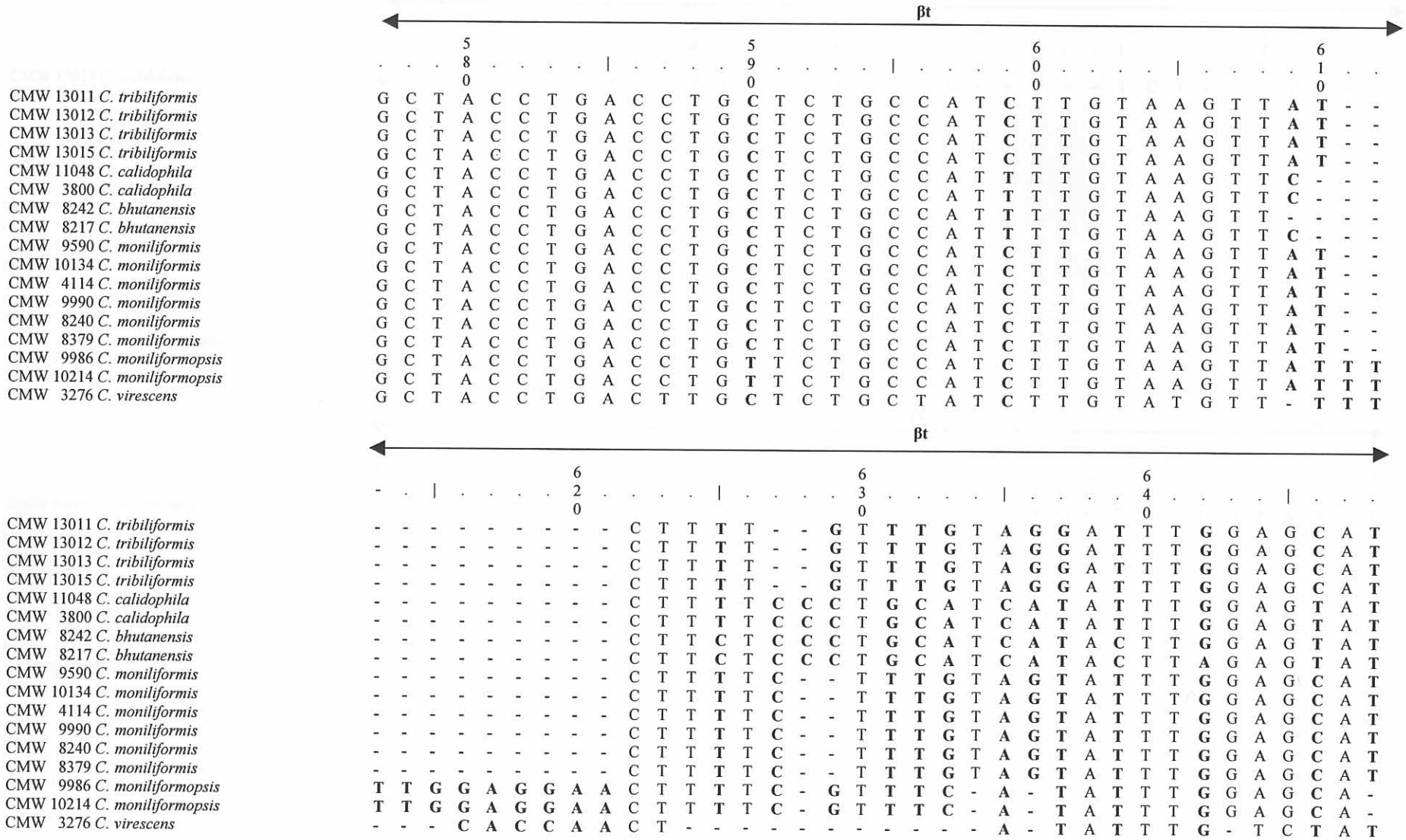
ITS ←→ βt

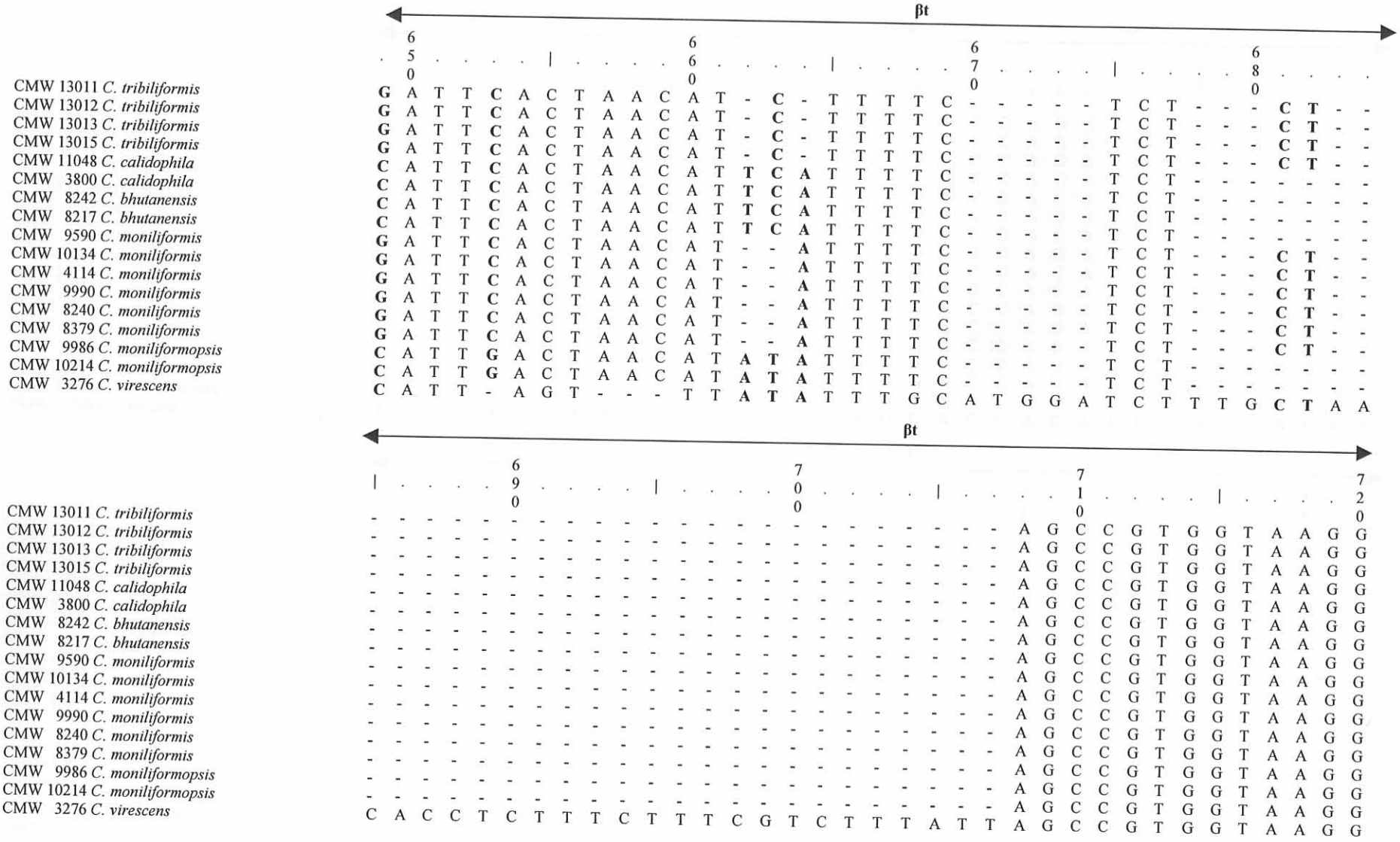
								440										450										460								
CMW 13011 *C. tribiliformis*	T	T	G	T	A	C	T	A	C	A	T	G	C	C	G	**T**	T	A	A	A	C	A	A	C	C	C	T	T	**C**	G	C	T	C	C	T	C
CMW 13012 *C. tribiliformis*	T	T	G	T	A	C	T	A	C	A	T	G	C	C	G	**T**	T	A	A	A	C	A	A	C	C	C	T	T	**C**	G	C	T	C	C	T	C
CMW 13013 *C. tribiliformis*	T	T	G	T	A	C	T	A	C	A	T	G	C	C	G	**T**	T	A	A	A	C	A	A	C	C	C	T	T	**C**	G	C	T	C	C	T	C
CMW 13015 *C. tribiliformis*	T	T	G	T	A	C	T	A	C	A	T	G	C	C	G	**T**	T	A	A	A	C	A	A	C	C	C	T	T	**C**	G	C	T	C	C	T	C
CMW 11048 *C. calidophila*	T	T	G	T	A	C	T	A	C	A	T	G	C	C	G	**T**	T	A	A	A	C	A	A	C	C	C	T	T	**C**	G	C	T	C	C	T	C
CMW 3800 *C. calidophila*	T	T	G	T	A	C	T	A	C	A	T	G	C	C	G	**T**	T	A	A	A	C	A	A	C	C	C	T	T	**C**	G	C	T	C	C	T	C
CMW 8242 *C. bhutanensis*	T	T	G	T	A	C	T	A	C	A	T	G	C	C	G	**T**	T	A	A	A	C	A	A	C	C	C	T	T	**C**	G	C	T	C	C	T	C
CMW 8217 *C. bhutanensis*	T	T	G	T	A	C	T	A	C	A	T	G	C	C	G	**T**	T	A	A	A	C	A	A	C	C	C	T	T	**C**	G	C	T	C	C	T	C
CMW 9590 *C. moniliformis*	T	T	G	T	A	C	T	A	C	A	T	G	C	C	G	**C**	T	A	A	A	C	A	A	C	C	C	T	T	**C**	G	C	T	C	C	T	C
CMW 10134 *C. moniliformis*	T	T	G	T	A	C	T	A	C	A	T	G	C	C	G	**C**	T	A	A	A	C	A	A	C	C	C	T	T	**C**	G	C	T	C	C	T	C
CMW 4114 *C. moniliformis*	T	T	G	T	A	C	T	A	C	A	T	G	C	C	G	**C**	T	A	A	A	C	A	A	C	C	C	T	T	**C**	G	C	T	C	C	T	C
CMW 9990 *C. moniliformis*	T	T	G	T	A	C	T	A	C	A	T	G	C	C	G	**T**	T	A	A	A	C	A	A	C	C	C	T	T	**C**	G	C	T	C	C	T	C
CMW 8240 *C. moniliformis*	T	T	G	T	A	C	T	A	C	A	T	G	C	C	G	**C**	T	A	A	A	C	A	A	C	C	C	T	T	**C**	G	C	T	C	C	T	C
CMW 8379 *C. moniliformis*	T	T	G	T	A	C	T	A	C	A	T	G	C	C	G	**C**	T	A	A	A	C	A	A	C	C	C	T	T	**C**	G	C	T	C	C	T	C
CMW 9986 *C. moniliformopsis*	T	T	G	T	A	C	T	A	C	A	T	G	C	C	G	**T**	T	A	A	A	C	A	A	C	C	C	T	T	**T**	G	C	T	C	C	T	C
CMW 10214 *C. moniliformopsis*	T	T	G	T	A	C	T	A	C	A	T	G	C	C	G	**T**	T	A	A	A	C	A	A	C	C	C	T	T	**T**	G	C	T	C	C	T	C
CMW 3276 *C. virescens*	T	T	G	T	A	C	T	A	C	A	T	G	C	C	G	**T**	-	A	A	-	-	A	A	C	C	C	T	T	**T**	G	C	T	C	C	C	C

βt

		470										480										490										500				
CMW 13011 *C. tribiliformis*	T	G	A	C	C	A	G	C	C	G	T	G	G	C	G	C	**T**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A
CMW 13012 *C. tribiliformis*	T	G	A	C	C	A	G	C	C	G	T	G	G	C	G	C	**T**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A
CMW 13013 *C. tribiliformis*	T	G	A	C	C	A	G	C	C	G	T	G	G	C	G	C	**T**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A
CMW 13015 *C. tribiliformis*	T	G	A	C	C	A	G	C	C	G	T	G	G	C	G	C	**T**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A
CMW 11048 *C. calidophila*	T	G	A	C	C	A	G	C	C	G	T	G	G	C	G	C	**C**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A
CMW 3800 *C. calidophila*	T	G	A	C	C	A	G	C	C	G	T	G	G	C	G	C	**C**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A
CMW 8242 *C. bhutanensis*	T	G	A	C	C	A	G	C	C	G	T	G	G	C	G	C	**C**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A
CMW 8217 *C. bhutanensis*	T	G	A	C	C	A	G	C	C	G	T	G	G	C	G	C	**C**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A
CMW 9590 *C. moniliformis*	T	G	A	C	C	A	G	C	C	G	T	G	G	C	G	C	**T**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A
CMW 10134 *C. moniliformis*	T	G	A	C	C	A	G	C	C	G	T	G	G	C	G	C	**T**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A
CMW 4114 *C. moniliformis*	T	G	A	C	C	A	G	C	C	G	T	G	G	C	G	C	**T**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A
CMW 9990 *C. moniliformis*	T	G	A	C	C	A	G	C	C	G	T	G	G	C	G	C	**T**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A
CMW 8240 *C. moniliformis*	T	G	A	C	C	A	G	C	C	G	T	G	G	C	G	C	**T**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A
CMW 8379 *C. moniliformis*	T	G	A	C	C	A	G	C	C	G	T	G	G	C	G	C	**T**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A
CMW 9986 *C. moniliformopsis*	T	G	A	C	C	A	G	C	C	G	T	G	G	C	G	C	**C**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A
CMW 10214 *C. moniliformopsis*	T	G	A	C	C	A	G	C	C	G	T	G	G	C	G	C	**C**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	T	G	T	C	A
CMW 3276 *C. virescens*	T	G	A	C	C	A	G	T	C	G	C	G	G	T	G	C	**T**	C	A	C	T	C	T	T	T	C	C	G	C	G	C	C	G	T	C	A




βt
βt
CMW 13011 C. tribiliformis
CMW 13012 C. tribiliformis
CMW 13013 C. tribiliformis
CMW 13015 C. tribiliformis
CMW 11048 C. calidophila
CMW 3800 C. calidophila
CMW 8242 C. bhutanensis
CMW 8217 C. bhutanensis
CMW 9590 C. moniliformis
CMW 10134 C. moniliformis
CMW 4114 C. moniliformis
CMW 9990 C. moniliformis
CMW 8240 C. moniliformis
CMW 8379 C. moniliformis
CMW 9986 C. moniliformopsis
CMW 10214 C. moniliformopsis
CMW 3276 C. virescens


βt
650
660
670
680
CMW 13011 C. tribiliformis
CMW 13012 C. tribiliformis
CMW 13013 C. tribiliformis
CMW 13015 C. tribiliformis
CMW 11048 C. calidophila
CMW 3800 C. calidophila
CMW 8242 C. bhutanensis
CMW 8217 C. bhutanensis
CMW 9590 C. moniliformis
CMW 10134 C. moniliformis
CMW 4114 C. moniliformis
CMW 9990 C. moniliformis
CMW 8240 C. moniliformis
CMW 8379 C. moniliformis
CMW 9986 C. moniliformopsis
CMW 10214 C. moniliformopsis
CMW 3276 C. virescens
βt
690
700
710
720
CMW 13011 C. tribiliformis
CMW 13012 C. tribiliformis
CMW 13013 C. tribiliformis
CMW 13015 C. tribiliformis
CMW 11048 C. calidophila
CMW 3800 C. calidophila
CMW 8242 C. bhutanensis
CMW 8217 C. bhutanensis
CMW 9590 C. moniliformis
CMW 10134 C. moniliformis
CMW 4114 C. moniliformis
CMW 9990 C. moniliformis
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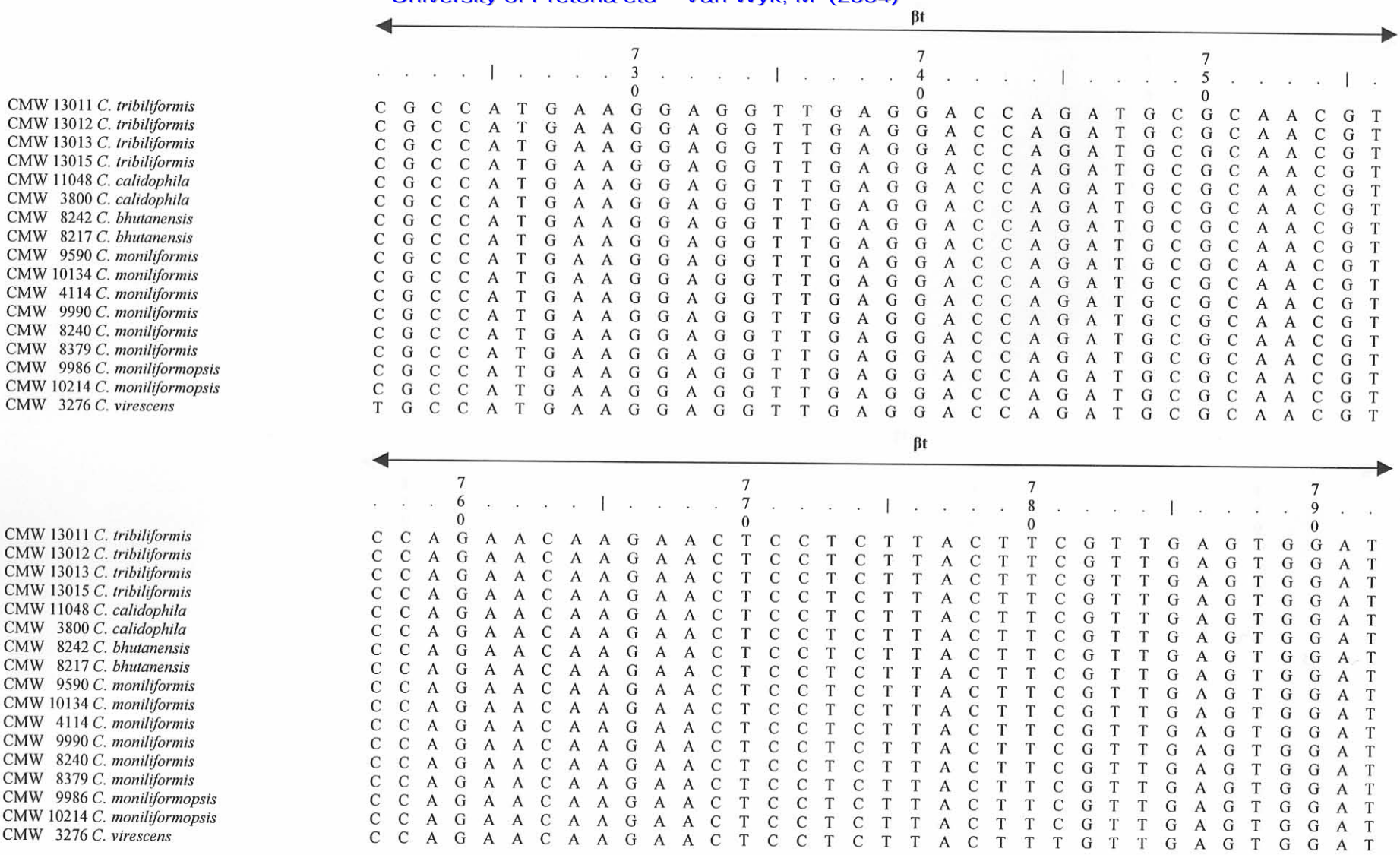
CMW 10214 C. moniliformopsis
CMW 3276 C. virescens

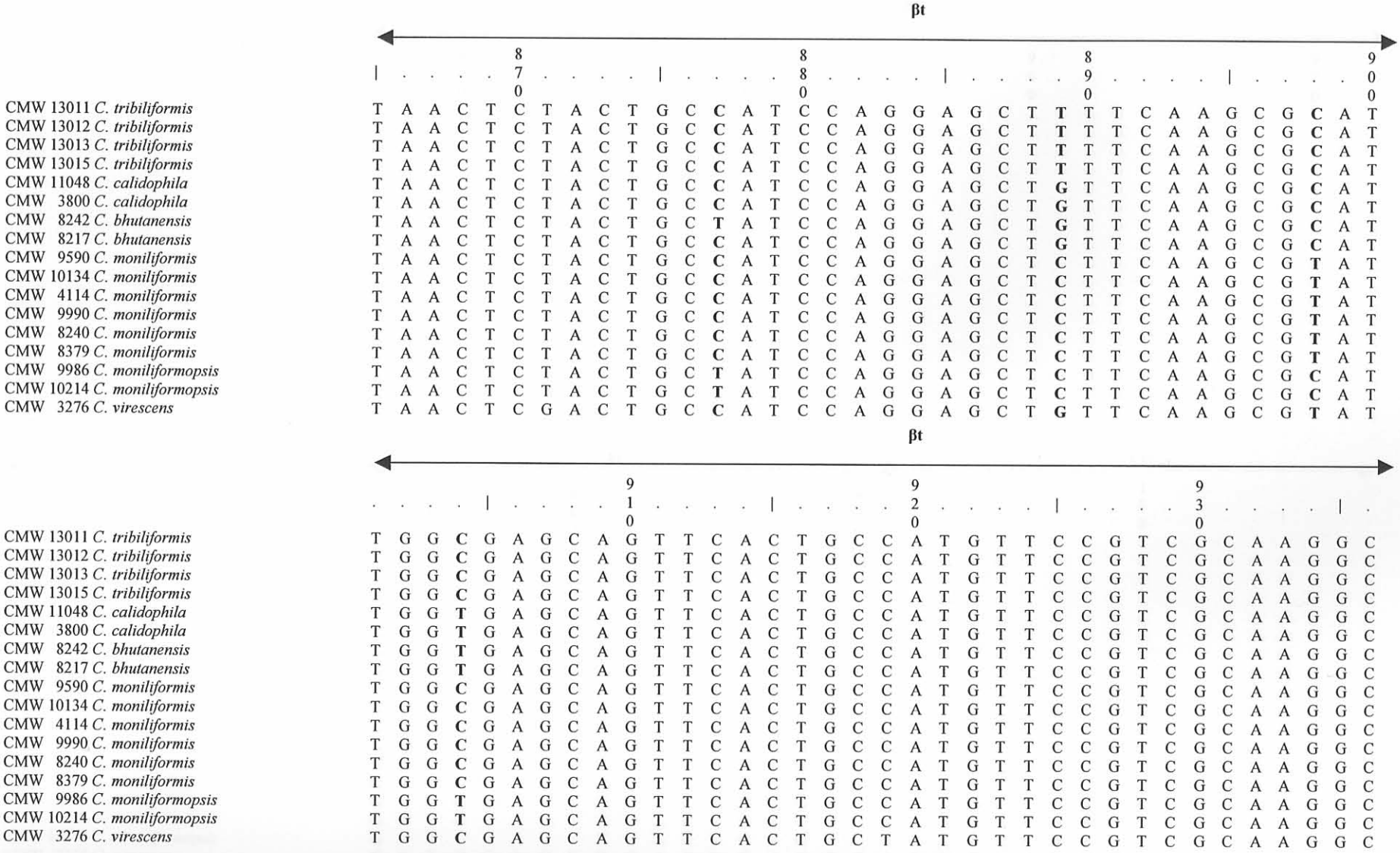


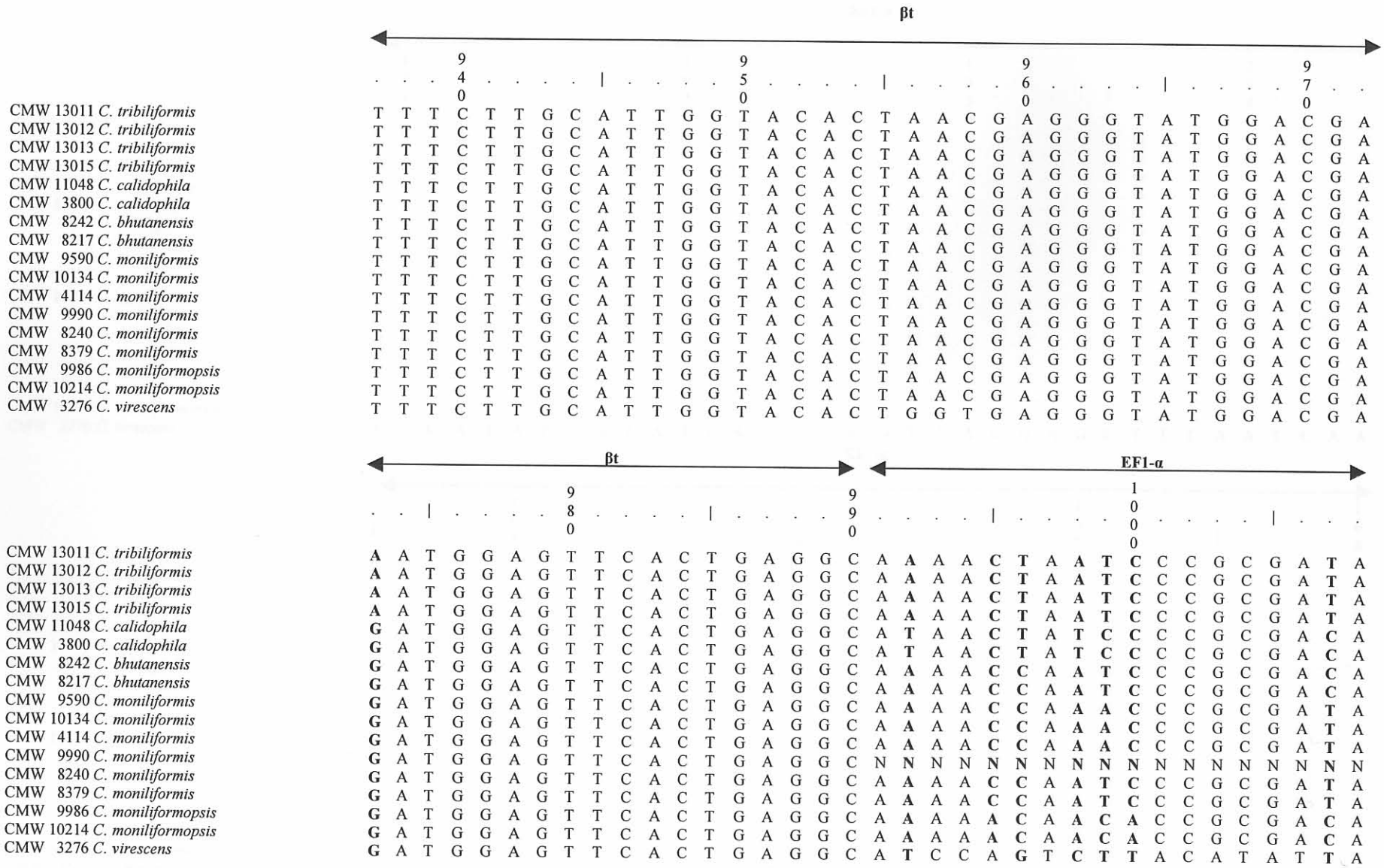
βt

								800										810										820								
CMW 13011 *C. tribiliformis*	C	C	C	C	A	A	C	A	A	C	G	T	C	C	A	G	A	C	C	G	C	T	C	T	T	T	G	C	T	C	C	A	T	T	C	C
CMW 13012 *C. tribiliformis*	C	C	C	C	A	A	C	A	A	C	G	T	C	C	A	G	A	C	C	G	C	T	C	T	T	T	G	C	T	C	C	A	T	T	C	C
CMW 13013 *C. tribiliformis*	C	C	C	C	A	A	C	A	A	C	G	T	C	C	A	G	A	C	C	G	C	T	C	T	T	T	G	C	T	C	C	A	T	T	C	C
CMW 13015 *C. tribiliformis*	C	C	C	C	A	A	C	A	A	C	G	T	C	C	A	G	A	C	C	G	C	T	C	T	T	T	G	C	T	C	C	A	T	T	C	C
CMW 11048 *C. calidophila*	C	C	C	C	A	A	C	A	A	C	G	T	C	C	A	G	A	C	C	G	C	C	C	T	G	T	G	C	T	C	C	A	T	T	C	C
CMW 3800 *C. calidophila*	C	C	C	C	A	A	C	A	A	C	G	T	C	C	A	G	A	C	C	G	C	C	C	T	G	T	G	C	T	C	C	A	T	T	C	C
CMW 8242 *C. bhutanensis*	C	C	C	C	A	A	C	A	A	C	G	T	C	C	A	G	A	C	C	G	C	C	C	T	G	T	G	C	T	C	C	A	T	T	C	C
CMW 8217 *C. bhutanensis*	C	C	C	C	A	A	C	A	A	C	G	T	C	C	A	G	A	C	C	G	C	C	C	T	G	T	G	C	T	C	C	A	T	T	C	C
CMW 9590 *C. moniliformis*	T	C	C	C	A	A	C	A	A	C	G	T	C	C	A	G	A	C	C	G	C	C	C	T	G	T	G	C	T	C	C	A	T	T	C	C
CMW 10134 *C. moniliformis*	T	C	C	C	A	A	C	A	A	C	G	T	C	C	A	G	A	C	C	G	C	T	C	T	T	T	G	C	T	C	C	A	T	T	C	C
CMW 4114 *C. moniliformis*	T	C	C	C	A	A	C	A	A	C	G	T	C	C	A	G	A	C	C	G	C	T	C	T	T	T	G	C	T	C	C	A	T	T	C	C
CMW 9990 *C. moniliformis*	T	C	C	C	A	A	C	A	A	C	G	T	C	C	A	G	A	C	C	G	C	T	C	T	T	T	G	C	T	C	C	A	T	T	C	C
CMW 8240 *C. moniliformis*	T	C	C	C	A	A	C	A	A	C	G	T	C	C	A	G	A	C	C	G	C	T	C	T	T	T	G	C	T	C	C	A	T	T	C	C
CMW 8379 *C. moniliformis*	T	C	C	C	A	A	C	A	A	C	G	T	C	C	A	G	A	C	C	G	C	T	C	T	T	T	G	C	T	C	C	A	T	T	C	C
CMW 9986 *C. moniliformopsis*	C	C	C	C	A	A	C	A	A	C	G	T	C	C	A	G	A	C	C	G	C	T	C	T	T	T	G	C	T	C	C	A	T	T	C	C
CMW 10214 *C. moniliformopsis*	C	C	C	C	A	A	C	A	A	C	G	T	C	C	A	G	A	C	C	G	C	T	C	T	C	T	G	C	T	C	C	A	T	T	C	C
CMW 3276 *C. virescens*	C	C	C	C	A	A	C	A	A	C	G	T	C	C	A	G	A	C	T	G	C	C	C	T	G	T	G	C	T	C	C	A	T	T	C	C

βt

		830										840										850										860				
CMW 13011 *C. tribiliformis*	T	C	C	C	C	G	T	G	G	C	C	T	C	A	A	A	A	T	G	T	C	T	T	C	G	A	C	C	T	T	C	G	T	C	G	G
CMW 13012 *C. tribiliformis*	T	C	C	C	C	G	T	G	G	C	C	T	C	A	A	A	A	T	G	T	C	T	T	C	G	A	C	C	T	T	C	G	T	C	G	G
CMW 13013 *C. tribiliformis*	T	C	C	C	C	G	T	G	G	C	C	T	C	A	A	A	A	T	G	T	C	T	T	C	G	A	C	C	T	T	C	G	T	C	G	G
CMW 13015 *C. tribiliformis*	T	C	C	C	C	G	T	G	G	C	C	T	C	A	A	A	A	T	G	T	C	T	T	C	G	A	C	C	T	T	C	G	T	C	G	G
CMW 11048 *C. calidophila*	T	C	C	C	C	G	T	G	G	C	C	T	C	A	A	G	A	T	G	T	C	T	T	C	G	A	C	C	T	T	C	G	T	C	G	G
CMW 3800 *C. calidophila*	T	C	C	C	C	G	T	G	G	C	C	T	C	A	A	G	A	T	G	T	C	T	T	C	G	A	C	C	T	T	C	G	T	T	G	G
CMW 8242 *C. bhutanensis*	T	C	C	C	C	G	T	G	G	C	C	T	C	A	A	G	A	T	G	T	C	T	T	C	G	A	C	C	T	T	C	G	T	T	G	G
CMW 8217 *C. bhutanensis*	T	C	C	C	C	G	T	G	G	C	C	T	C	A	A	G	A	T	G	T	C	T	T	C	G	A	C	A	T	T	C	G	T	T	G	G
CMW 9590 *C. moniliformis*	T	C	C	C	C	G	T	G	G	C	C	T	C	A	A	G	A	T	G	T	C	T	T	C	G	A	C	C	T	T	C	G	T	T	G	G
CMW 10134 *C. moniliformis*	T	C	C	C	C	G	T	G	G	C	C	T	C	A	A	G	A	T	G	T	C	T	T	C	G	A	C	C	T	T	C	G	T	C	G	G
CMW 4114 *C. moniliformis*	T	C	C	C	C	G	T	G	G	C	C	T	C	A	A	G	A	T	G	T	C	T	T	C	G	A	C	C	T	T	C	G	T	C	G	G
CMW 9990 *C. moniliformis*	T	C	C	C	C	G	T	G	G	C	C	T	C	A	A	G	A	T	G	T	C	T	T	C	G	A	C	C	T	T	C	G	T	C	G	G
CMW 8240 *C. moniliformis*	T	C	C	C	C	G	T	G	G	C	C	T	C	A	A	G	A	T	G	T	C	T	T	C	G	A	C	C	T	T	C	G	T	C	G	G
CMW 8379 *C. moniliformis*	T	C	C	C	C	G	T	G	G	C	C	T	C	A	A	G	A	T	G	T	C	T	T	C	G	A	C	C	T	T	C	G	T	C	G	G
CMW 9986 *C. moniliformopsis*	T	C	C	C	C	G	T	G	G	C	C	T	C	A	A	G	A	T	G	T	C	T	T	C	C	A	C	C	T	T	C	G	T	C	G	G
CMW 10214 *C. moniliformopsis*	T	C	C	C	C	G	T	G	G	C	C	T	C	A	A	G	A	T	G	T	C	T	T	C	C	A	C	C	T	T	C	G	T	C	G	G
CMW 3276 *C. virescens*	T	C	C	C	C	G	T	G	G	C	C	T	C	A	A	G	A	T	G	T	C	G	T	C	T	A	C	C	T	T	C	G	T	C	G	G




βt
EF1-α
CMW 13011 C. tribiliformis
CMW 13012 C. tribiliformis
CMW 13013 C. tribiliformis
CMW 13015 C. tribiliformis
CMW 11048 C. calidophila
CMW 3800 C. calidophila
CMW 8242 C. bhutanensis
CMW 8217 C. bhutanensis
CMW 9590 C. moniliformis
CMW 10134 C. moniliformis
CMW 4114 C. moniliformis
CMW 9990 C. moniliformis
CMW 8240 C. moniliformis
CMW 8379 C. moniliformis
CMW 9986 C. moniliformopsis
CMW 10214 C. moniliformopsis
CMW 3276 C. virescens

EF1-α

Isolate	.	1010	.	.	.	.	|	.	.	.	.	1020	.	.	.	.	|	.	.	.	.	1030	.	.	.	.	|	.	.	.	.	1040	.	.	.	.
CMW 13011 *C. tribiliformis*	C	C	A	A	T	C	G	A	A	C	C	A	A	A	C	A	A	G	A	T	A	C	C	G	A	G	A	G	G	T	T	A	A	A	T	G
CMW 13012 *C. tribiliformis*	C	C	A	A	T	C	G	A	A	C	C	A	A	A	C	A	A	G	A	T	A	C	C	G	A	G	A	G	G	T	T	A	A	A	T	G
CMW 13013 *C. tribiliformis*	C	C	A	A	T	C	G	A	A	C	C	A	A	A	C	A	A	G	A	T	A	C	C	G	A	G	A	G	G	T	T	A	A	A	T	G
CMW 13015 *C. tribiliformis*	C	C	A	A	T	C	G	A	A	C	C	A	A	A	C	A	A	G	A	T	A	C	C	G	A	G	A	G	G	T	T	A	A	A	T	G
CMW 11048 *C. calidophila*	T	C	A	A	C	C	G	A	G	C	C	G	A	A	C	A	A	G	A	T	A	C	A	G	A	T	A	G	G	A	T	G	T	G	T	T
CMW 3800 *C. calidophila*	T	C	A	A	C	C	G	A	G	C	C	G	A	A	C	A	A	G	A	T	A	C	A	G	A	T	A	G	G	A	T	G	T	G	T	T
CMW 8242 *C. bhutanensis*	T	C	A	A	T	C	G	A	A	C	C	G	A	A	C	A	A	G	A	T	A	C	C	G	A	T	A	G	G	A	T	G	T	G	T	T
CMW 8217 *C. bhutanensis*	T	C	A	A	T	C	G	A	A	C	C	G	A	A	C	A	A	G	A	T	A	C	C	G	A	T	A	G	G	A	T	G	T	G	T	T
CMW 9590 *C. moniliformis*	C	C	A	G	C	C	G	A	A	C	T	G	A	A	C	A	A	G	A	T	A	C	C	G	A	G	A	G	G	A	T	A	A	G	T	T
CMW 10134 *C. moniliformis*	C	C	A	G	C	C	G	A	A	C	T	G	A	A	C	A	A	G	A	T	A	C	C	G	A	G	A	G	G	A	T	A	A	G	T	T
CMW 4114 *C. moniliformis*	C	C	A	G	C	C	G	A	A	C	T	G	A	A	C	A	A	G	A	T	A	C	C	G	A	G	A	G	G	A	T	A	A	G	T	T
CMW 9990 *C. moniliformis*	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
CMW 8240 *C. moniliformis*	C	C	A	G	C	C	G	A	A	C	C	G	A	A	C	A	A	G	A	T	A	C	C	G	A	G	A	G	G	A	T	A	A	G	T	T
CMW 8379 *C. moniliformis*	C	C	A	G	C	C	G	A	A	C	C	G	A	A	C	A	A	G	A	T	A	C	C	G	A	G	A	G	G	A	T	A	A	G	T	T
CMW 9986 *C. moniliformopsis*	C	C	A	A	T	C	G	A	A	T	C	A	A	C	C	A	A	G	A	T	A	C	A	G	A	T	C	G	G	A	T	G	A	G	T	T
CMW 10214 *C. moniliformopsis*	C	C	A	A	T	C	G	A	A	T	C	A	A	C	C	A	A	G	A	T	A	C	A	G	A	T	C	G	G	A	T	G	A	G	T	T
CMW 3276 *C. virescens*	T	T	G	A	T	A	T	C	A	T	A	T	C	C	C	T	C	C	G	T	T	A	C	G	A	G	C	T	T	C	A	A	T	C	A	A

EF1-α

Isolate	|	.	.	.	.	1050	.	.	.	.	|	.	.	.	.	1060	.	.	.	.	|	.	.	.	.	1070	.	.	.	.	|	.	.	.	.	1080
CMW 13011 *C. tribiliformis*	G	C	T	G	G	T	G	G	T	G	C	G	T	T	T	T	T	C	-	A	C	G	G	A	G	C	G	T	T	C	A	G	T	C	T	T
CMW 13012 *C. tribiliformis*	G	C	T	G	G	T	G	G	T	G	C	G	T	T	T	T	T	C	-	A	C	G	G	A	G	C	G	T	T	C	A	G	T	C	T	T
CMW 13013 *C. tribiliformis*	G	C	T	G	G	T	G	G	T	G	C	G	T	T	T	T	T	C	-	A	C	G	G	A	G	C	G	T	T	C	A	G	T	C	T	T
CMW 13015 *C. tribiliformis*	G	C	T	G	G	T	G	G	T	G	C	G	T	T	T	T	T	C	-	A	C	G	G	A	G	C	G	T	T	C	A	G	T	C	T	T
CMW 11048 *C. calidophila*	G	C	T	G	G	T	G	G	T	G	C	G	T	T	T	T	T	C	-	A	C	G	C	A	G	C	G	T	T	C	A	G	C	C	T	T
CMW 3800 *C. calidophila*	G	C	T	G	G	T	G	G	T	G	C	G	T	T	T	T	T	C	-	A	C	G	C	A	G	C	G	T	T	C	A	G	C	C	T	T
CMW 8242 *C. bhutanensis*	G	C	T	A	G	T	G	A	T	G	C	G	T	T	T	T	-	C	T	A	C	G	C	A	G	C	G	T	T	C	A	G	T	C	T	T
CMW 8217 *C. bhutanensis*	G	C	T	A	G	T	G	A	T	G	C	G	T	T	T	T	-	C	T	A	C	G	C	A	G	C	G	T	T	C	A	G	T	C	T	T
CMW 9590 *C. moniliformis*	G	C	T	A	G	C	G	G	T	G	T	G	T	T	T	T	-	C	-	A	C	G	G	A	G	C	G	T	T	C	A	G	T	C	T	T
CMW 10134 *C. moniliformis*	G	C	T	A	G	C	G	G	T	G	T	G	T	T	T	T	-	C	-	A	C	G	G	A	G	C	G	T	T	C	A	G	T	C	T	T
CMW 4114 *C. moniliformis*	G	C	T	A	G	C	G	G	T	G	T	G	T	T	T	T	-	C	-	A	C	G	G	A	G	C	G	T	T	C	A	G	T	C	T	T
CMW 9990 *C. moniliformis*	N	N	N	N	N	N	N	N	N	N	N	N	C	T	T	T	-	C	-	A	C	G	G	A	G	C	G	T	T	C	A	G	T	C	T	T
CMW 8240 *C. moniliformis*	G	C	T	A	G	C	G	G	T	G	T	G	T	T	T	T	-	C	-	A	C	G	G	A	G	C	G	T	T	C	A	G	T	C	T	T
CMW 8379 *C. moniliformis*	G	C	T	A	G	C	G	G	T	G	T	G	T	T	T	T	-	C	-	A	C	G	G	A	G	C	G	T	T	C	A	G	T	C	T	T
CMW 9986 *C. moniliformopsis*	G	C	T	G	G	T	C	A	T	G	C	G	T	T	T	T	-	C	C	A	C	G	C	A	G	C	G	T	T	T	A	G	C	C	T	T
CMW 10214 *C. moniliformopsis*	G	C	T	G	G	T	C	A	T	G	C	G	T	T	T	T	-	C	C	A	C	G	C	A	G	C	G	T	T	T	A	G	C	C	T	T
CMW 3276 *C. virescens*	T	A	A	T	G	A	A	A	A	C	T	T	G	A	C	T	G	T	T	C	A	C	C	G	G	-	T	T	T	C	G	A	A	T	T	C

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Isolate	. . . . . | . . . . 1090 . . . . . | . . . . . 1100 . . . . . | . . . . . 1110 . . . . . | .
CMW 13011 *C. tribiliformis*	G T A T A G A G G T T G A G T T G T T T T T G A T G A G G C A A T A C T
CMW 13012 *C. tribiliformis*	G T A T A G A G G T T G A G T T G T T T T T G A T G A G G C A A T A C T
CMW 13013 *C. tribiliformis*	G T A T A G A G G T T G A G T T G T T T T T G A T G A G G C A A T A C T
CMW 13015 *C. tribiliformis*	G T A T A G A G G T T G A G T T G T T T T T G A T G A G G C A A T A C T
CMW 11048 *C. calidophila*	G T A T A G A G G T T G A G C T G A T T T T G A C G A G G C A G C C C T
CMW 3800 *C. calidophila*	G T A T A G A G G T T G A G C T G A T T T T G A C G A G G C A G C C C T
CMW 8242 *C. bhutanensis*	G T A T A A A G G T T G A G T T G A T T T T C A T G A G G C A G C C C T
CMW 8217 *C. bhutanensis*	G T A T A A A G G T T G A G T T G A T T T T C A T G A G G C A G C C C T
CMW 9590 *C. moniliformis*	G T A T G G A G G T T G A G T T G T T T T T G A T G A G G C A A T A C T
CMW 10134 *C. moniliformis*	G T A T G G A G G T T G A G T T G T T T T T G A T G A G G C A A T A C T
CMW 4114 *C. moniliformis*	G T A T G G A G G T T G A G T T G T T T T T G A T G A G G C A A T A C T
CMW 9990 *C. moniliformis*	G T A T G G A G G T T G A G T T G T T T T T G A T G A G G C A A T A C T
CMW 8240 *C. moniliformis*	G T A T A G A G G T T G A G T T G T T T T T G A T G A G G C A A T A C T
CMW 8379 *C. moniliformis*	G T A T G G A G G T T G A G T T G T T T T T G A T G A G G C A A T A C T
CMW 9986 *C. moniliformopsis*	G C A A A G A G G T C G A G T T G T T T T T C A T G A G G C A A C A C T
CMW 10214 *C. moniliformopsis*	G C A A A G A G G T C G A G T T G T T T T T C A T G A G G C A A C A C T
CMW 3276 *C. virescens*	G T T G T T G G A T C T G A G T C T T G T G T C C C T A C C C A C - C T

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Isolate	. . . 1120 . . . . . | . . . . . 1130 . . . . . | . . . . . 1140 . . . . . | . . . . . 1150 . .
CMW 13011 *C. tribiliformis*	G C C C - C G C C T G G C G G G G T A G T T C T G T G T T G T G A A A A
CMW 13012 *C. tribiliformis*	G C C C - C G C C T G G C G G G G T A G T T C T G T G T T G T G A A A A
CMW 13013 *C. tribiliformis*	G C C C - C G C C T G G C G G G G T A G T T C T G T G T T G T G A A A A
CMW 13015 *C. tribiliformis*	G C C C - C G C C T G G C G G G G T A G T T C T G T G T T G T G A A A A
CMW 11048 *C. calidophila*	A C C C - C G C C C G G C G G G G T A G T G T T G T G T T G T G A A A A
CMW 3800 *C. calidophila*	A C C C - C G C C C G G C G G G G T A G T G T T G T G T T G T G A A A A
CMW 8242 *C. bhutanensis*	A C C C - C G C C T G G C G G G G T A G T G T T G T G T T G T G A A A A
CMW 8217 *C. bhutanensis*	A C C C - C G C C T G G C G G G G T A G T G T T G T G T T G T G A A A A
CMW 9590 *C. moniliformis*	A C C C A C G C C T G G C G G G G T A G T T T T G T G T T A T G A A A A
CMW 10134 *C. moniliformis*	A C C C A C G C C T G G C G G G G T A G T T T T G T G T T A T G A A A A
CMW 4114 *C. moniliformis*	A C C C A C G C C T G G C G G G G T A G T T T T G T G T T A T G A A A A
CMW 9990 *C. moniliformis*	A C C C A C G C C T G G C G G G G T A G T T T T G T G T T A T G A A A A
CMW 8240 *C. moniliformis*	A C C C A C G C C T G G C G G G G T A G T T T T G T G T T A T G A A A A
CMW 8379 *C. moniliformis*	A C C C A C G C C T G G C G G G G T A G T T T T G T G T T A T G A A A A
CMW 9986 *C. moniliformopsis*	G C C C - C G C C T G G C G G G G T A G T T T T G T G T T G T G G A A A
CMW 10214 *C. moniliformopsis*	G C C C - C G C C T G G C G G G G T A G T T T T G T G T T G T G G A A A
CMW 3276 *C. virescens*	G - - - - - - - - - - - - - - - A A C A G C C A T C T C A A T - - A A A

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								1160										1170										1180								
CMW 13011 *C. tribiliformis*	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**	G	G	A	A	**G**	**G**	**C**	**A**	**C**	-	A	C	A	A	G	G
CMW 13012 *C. tribiliformis*	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**	G	G	A	A	**G**	**G**	**C**	**A**	**C**	-	A	C	A	A	G	G
CMW 13013 *C. tribiliformis*	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**	G	G	A	A	**G**	**G**	**C**	**A**	**C**	-	A	C	A	A	G	G
CMW 13015 *C. tribiliformis*	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**	G	G	A	A	**G**	**G**	**C**	**A**	**C**	-	A	C	A	A	G	G
CMW 11048 *C. calidophila*	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**	G	G	A	A	-	-	C	**A**	**T**	**A**	A	C	A	A	G	G
CMW 3800 *C. calidophila*	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**	G	G	A	A	-	-	C	**A**	**T**	**A**	A	C	A	A	G	G
CMW 8242 *C. bhutanensis*	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**A**	G	G	A	A	**G**	**A**	C	**A**	**T**	-	A	C	A	A	G	G
CMW 8217 *C. bhutanensis*	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**A**	G	G	A	A	**G**	**A**	C	**A**	**T**	-	A	C	A	A	G	G
CMW 9590 *C. moniliformis*	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**	G	G	A	A	**G**	**T**	C	**A**	**T**	-	A	C	A	A	G	G
CMW 10134 *C. moniliformis*	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**	G	G	A	A	**G**	**T**	C	**A**	**T**	-	A	C	A	A	G	G
CMW 4114 *C. moniliformis*	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**	G	G	A	A	**G**	**T**	C	**A**	**T**	-	A	C	A	A	G	G
CMW 9990 *C. moniliformis*	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**	G	G	A	A	**G**	**T**	C	**A**	**T**	-	A	C	A	A	G	G
CMW 8240 *C. moniliformis*	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**	G	G	A	A	**G**	**T**	C	**A**	**T**	-	A	C	A	A	G	G
CMW 8379 *C. moniliformis*	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**	G	G	A	A	**G**	**T**	C	**A**	**T**	-	A	C	A	A	G	G
CMW 9986 *C. moniliformopsis*	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**	G	G	A	A	**G**	**C**	**T**	**A**	**T**	-	A	C	A	A	G	G
CMW 10214 *C. moniliformopsis*	A	T	T	C	T	A	C	T	C	T	T	T	C	C	T	C	T	T	T	**G**	G	G	A	A	**G**	**C**	**T**	**A**	**T**	-	A	C	A	A	G	G
CMW 3276 *C. virescens*	A	T	C	C	C	A	-	T	G	C	T	T	G	-	-	-	**T**	T	-	-	C	G	A	**A**	**C**	**C**	**C**	**C**	-	A	C	T	A	G	C	C

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		1190										1200										1210										1220				
CMW 13011 *C. tribiliformis*	G	T	**A**	**C**	**A**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**T**	T	T	G	**T**	**C**	C	C	C	T	G	T	A	C	A	A	**G**	G
CMW 13012 *C. tribiliformis*	G	T	**A**	**C**	**A**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**T**	T	T	G	**T**	**C**	C	C	C	T	G	T	A	C	A	A	**G**	G
CMW 13013 *C. tribiliformis*	G	T	**A**	**C**	**A**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**T**	T	T	G	**T**	**C**	C	C	C	T	G	T	A	C	A	A	**G**	G
CMW 13015 *C. tribiliformis*	G	T	**A**	**C**	**A**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**T**	T	T	G	**T**	**C**	C	C	C	T	G	T	A	C	A	A	**G**	G
CMW 11048 *C. calidophila*	G	T	**A**	**T**	**A**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**T**	T	T	G	**T**	**A**	C	C	C	T	G	T	A	C	A	A	**G**	G
CMW 3800 *C. calidophila*	G	T	**A**	**T**	**A**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**T**	T	T	G	**T**	**A**	C	C	C	T	G	T	A	C	A	A	**G**	G
CMW 8242 *C. bhutanensis*	G	T	**A**	**T**	**A**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**C**	T	T	G	**T**	**A**	C	C	C	T	G	T	A	C	A	A	**G**	G
CMW 8217 *C. bhutanensis*	G	T	**A**	**T**	**A**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**C**	T	T	G	**T**	**A**	C	C	C	T	G	T	A	C	A	A	**G**	G
CMW 9590 *C. moniliformis*	G	T	**A**	**T**	**A**	A	T	T	A	C	C	C	C	G	C	T	**A**	T	**T**	T	T	G	**C**	**C**	C	C	C	T	G	T	A	C	A	A	**A**	G
CMW 10134 *C. moniliformis*	G	T	**A**	**C**	**A**	A	T	T	A	C	C	C	C	G	C	T	**A**	T	**T**	T	T	G	**C**	**C**	C	C	C	T	G	T	A	C	A	A	**A**	G
CMW 4114 *C. moniliformis*	G	T	**A**	**C**	**A**	A	T	T	A	C	C	C	C	G	C	T	**A**	T	**T**	T	T	G	**C**	**C**	C	C	C	T	G	T	A	C	A	A	**A**	G
CMW 9990 *C. moniliformis*	G	T	**A**	**C**	**A**	A	T	T	A	C	C	C	C	G	C	T	**A**	T	**T**	T	T	G	**C**	**C**	C	C	C	T	G	T	A	C	A	A	**A**	G
CMW 8240 *C. moniliformis*	G	T	**A**	**C**	**A**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**T**	T	T	G	**C**	**C**	C	C	C	T	G	T	A	C	A	A	**A**	G
CMW 8379 *C. moniliformis*	G	T	**A**	**C**	**A**	A	T	T	A	C	C	C	C	G	C	T	**A**	T	**T**	T	T	G	**C**	**C**	C	C	C	T	G	T	A	C	A	A	**A**	G
CMW 9986 *C. moniliformopsis*	G	T	**G**	**T**	**T**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**T**	T	T	G	**T**	**C**	C	C	C	T	G	T	A	C	A	A	**G**	G
CMW 10214 *C. moniliformopsis*	G	T	**G**	**T**	**T**	A	T	T	A	C	C	C	C	G	C	T	**G**	T	**T**	T	T	G	**T**	**C**	C	C	C	T	G	T	A	C	A	A	**G**	G
CMW 3276 *C. virescens*	T	A	**G**	**G**	-	A	T	T	G	A	A	C	C	C	C	T	**G**	-	-	-	-	-	-	-	-	-	-	-	G	C	A	C	-	-	-	-

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Isolate						1230										1240										1250										1260
CMW 13011 *C. tribiliformis*	A	C	C	C	G	C	T	T	T	G	T	G	G	G	A	A	G	A	C	A	C	C	C	T	G	G	T	C	A	C	A	C	A	-	-	T
CMW 13012 *C. tribiliformis*	A	C	C	C	G	C	T	T	T	G	T	G	G	G	A	A	G	A	C	A	C	C	C	T	G	G	T	C	A	C	A	C	A	-	-	T
CMW 13013 *C. tribiliformis*	A	C	C	C	G	C	T	T	T	G	T	G	G	G	A	A	G	A	C	A	C	C	C	T	G	G	T	C	A	C	A	C	A	-	-	T
CMW 13015 *C. tribiliformis*	A	C	C	C	G	C	T	T	T	G	T	G	G	G	A	A	G	A	C	A	C	C	C	T	G	G	T	C	A	C	A	C	A	-	-	T
CMW 11048 *C. calidophila*	A	C	C	C	G	C	T	G	G	G	T	G	G	G	A	A	G	G	T	A	C	C	C	T	G	G	T	C	A	C	A	C	A	C	A	-
CMW 3800 *C. calidophila*	A	C	C	C	G	C	T	G	G	G	T	G	G	G	A	A	G	G	T	A	C	C	C	T	G	G	T	C	A	C	A	C	A	C	A	-
CMW 8242 *C. bhutanensis*	A	C	C	C	G	C	T	G	T	G	T	G	G	G	A	A	G	G	T	A	C	C	C	T	G	G	T	C	A	C	A	C	A	C	A	T
CMW 8217 *C. bhutanensis*	A	C	C	C	G	C	T	G	T	G	T	G	G	G	A	A	G	G	T	A	C	C	C	T	G	G	T	C	A	C	A	C	A	C	A	T
CMW 9590 *C. moniliformis*	A	C	C	C	G	T	T	T	T	G	T	G	G	G	A	A	G	G	A	A	C	C	C	T	G	G	T	T	A	C	G	C	C	C	C	T
CMW 10134 *C. moniliformis*	A	C	C	C	G	T	T	T	T	G	T	G	G	G	A	A	G	G	A	A	C	C	C	T	G	G	T	T	A	C	G	C	C	C	C	T
CMW 4114 *C. moniliformis*	A	C	C	C	G	T	T	T	T	G	T	G	G	G	A	A	G	G	A	A	C	C	C	T	G	G	T	T	A	C	G	C	C	C	C	T
CMW 9990 *C. moniliformis*	A	C	C	C	G	T	T	T	T	G	T	G	G	G	A	A	G	G	A	A	C	C	C	T	G	G	T	T	A	C	G	C	C	C	C	T
CMW 8240 *C. moniliformis*	A	C	C	C	G	T	T	T	T	G	T	G	G	G	A	A	G	G	A	A	C	C	C	T	G	G	T	T	A	C	G	C	C	C	C	T
CMW 8379 *C. moniliformis*	A	C	C	C	G	T	T	T	T	G	T	G	G	G	A	A	G	G	A	A	C	C	C	T	G	G	T	T	A	C	G	C	C	C	C	T
CMW 9986 *C. moniliformopsis*	A	C	C	C	G	C	T	G	T	G	T	G	A	A	A	G	G	G	C	A	C	C	C	T	G	G	T	C	A	T	C	T	C	C	A	T
CMW 10214 *C. moniliformopsis*	A	C	C	C	G	C	T	G	T	G	T	G	A	A	A	G	G	G	C	A	C	C	C	T	G	G	T	C	A	T	C	T	C	C	A	T
CMW 3276 *C. virescens*	-	T	T	C	C	T	C	T	T	A	C	A	C	A	A	C	A	A	-	A	C	C	T	T	G	C	T	-	-	-	-	-	-	-	-	-

EF1-α

Isolate										1270										1280										1290						
CMW 13011 *C. tribiliformis*	C	C	A	A	T	C	C	A	T	G	T	A	T	G	C	T	C	T	T	T	C	-	G	T	A	T	G	G	T	G	G	T	A	T	C	T
CMW 13012 *C. tribiliformis*	C	C	A	A	T	C	C	A	T	G	T	A	T	G	C	T	C	T	T	T	C	-	G	T	A	T	G	G	T	G	G	T	A	T	C	T
CMW 13013 *C. tribiliformis*	C	C	A	A	T	C	C	A	T	G	T	A	T	G	C	T	C	T	T	T	C	-	G	T	A	T	G	G	T	G	G	T	A	T	C	T
CMW 13015 *C. tribiliformis*	C	C	A	A	T	C	C	A	T	G	T	A	T	G	C	T	C	T	T	T	C	-	G	T	A	T	G	G	T	G	G	T	A	T	C	T
CMW 11048 *C. calidophila*	-	C	A	G	T	C	C	A	T	G	C	A	T	G	C	T	C	T	T	-	C	G	G	C	A	T	G	A	G	A	G	T	G	T	C	T
CMW 3800 *C. calidophila*	-	C	A	G	T	C	C	A	T	G	C	A	T	G	C	T	C	T	T	-	C	G	G	C	A	T	G	A	G	A	G	T	G	T	C	T
CMW 8242 *C. bhutanensis*	C	C	A	G	T	C	C	A	T	G	C	A	T	G	C	T	C	T	T	-	C	G	G	C	A	T	G	A	T	A	G	T	G	T	C	T
CMW 8217 *C. bhutanensis*	C	C	A	G	T	C	C	A	T	G	C	A	T	G	C	T	C	T	T	-	C	G	G	C	A	T	G	A	T	A	G	T	G	T	C	T
CMW 9590 *C. moniliformis*	C	T	A	A	T	C	C	A	T	G	C	A	T	G	C	T	C	T	T	A	C	-	G	T	A	T	G	G	T	G	G	G	G	T	C	T
CMW 10134 *C. moniliformis*	C	T	A	A	T	C	C	A	T	G	C	A	T	G	C	T	C	T	T	A	C	-	G	T	A	T	G	G	T	G	G	G	G	T	C	T
CMW 4114 *C. moniliformis*	C	T	A	A	T	C	C	A	T	G	C	A	T	G	C	T	C	T	T	A	C	T	G	T	A	T	G	G	-	G	G	G	G	T	C	T
CMW 9990 *C. moniliformis*	C	T	A	A	T	C	C	A	T	G	C	A	T	G	C	T	C	T	T	A	C	-	G	T	A	T	G	G	T	G	G	G	G	T	C	T
CMW 8240 *C. moniliformis*	C	T	A	A	T	C	C	A	T	G	C	A	T	G	C	T	C	T	T	A	C	-	G	T	A	T	G	G	T	G	G	G	G	T	C	T
CMW 8379 *C. moniliformis*	C	T	A	A	T	C	C	A	T	G	C	A	T	G	C	T	C	T	T	A	C	-	G	T	A	T	G	G	T	G	G	G	G	T	C	T
CMW 9986 *C. moniliformopsis*	C	C	C	A	T	C	T	A	C	A	T	G	C	A	A	G	G	T	C	T	C	T	T	G	C	A	C	-	-	C	A	G	C	T	C	T
CMW 10214 *C. moniliformopsis*	C	C	C	A	T	C	T	A	C	A	T	G	C	A	A	G	G	T	C	T	C	T	T	G	C	A	C	-	-	C	A	G	C	T	C	T
CMW 3276 *C. virescens*	-	-	-	-	-	C	A	T	G	C	A	-	-	T	T	G	-	T	A	T	C	-	T	A	T	G	C	A	T	G	-	-	-	-	C	C

EF1-α

	1300							1310					
CMW 13011 *C. tribiliformis*	T	G	T	T	T	G	**G**	**T**	T	**G**	T	T	G	**T**	**T**
CMW 13012 *C. tribiliformis*	T	G	T	T	T	G	**G**	**T**	T	**G**	T	T	G	**T**	**T**
CMW 13013 *C. tribiliformis*	T	G	T	T	T	G	**G**	**T**	T	**G**	T	T	G	**T**	**T**
CMW 13015 *C. tribiliformis*	T	G	T	T	T	G	**G**	**T**	T	**G**	T	T	G	**T**	**T**
CMW 11048 *C. calidophila*	T	G	T	T	T	G	**G**	**G**	T	**G**	T	T	G	**G**	**C**
CMW 3800 *C. calidophila*	T	G	T	T	T	G	**G**	**G**	T	**G**	T	T	G	**G**	**C**
CMW 8242 *C. bhutanensis*	T	G	T	T	T	G	**G**	**G**	T	**G**	T	T	G	**G**	**T**
CMW 8217 *C. bhutanensis*	T	G	T	T	T	G	**G**	**G**	T	**G**	T	T	G	**G**	**T**
CMW 9590 *C. moniliformis*	T	G	T	T	T	G	**G**	**G**	T	**A**	T	T	G	**G**	**T**
CMW 10134 *C. moniliformis*	T	G	T	T	T	G	**G**	**G**	T	**G**	T	T	G	**G**	**T**
CMW 4114 *C. moniliformis*	T	G	T	T	T	G	**G**	**T**	T	**G**	T	T	G	**G**	**T**
CMW 9990 *C. moniliformis*	T	G	T	T	T	G	**G**	**T**	T	**G**	T	T	G	**G**	**T**
CMW 8240 *C. moniliformis*	T	G	T	T	T	G	**G**	**T**	T	**G**	T	T	G	**G**	**T**
CMW 8379 *C. moniliformis*	T	G	T	T	T	G	**G**	**T**	T	**G**	T	T	G	**G**	**T**
CMW 9986 *C. moniliformopsis*	T	G	T	T	T	G	**A**	**T**	T	**A**	T	T	G	**G**	**T**
CMW 10214 *C. moniliformopsis*	T	G	T	T	T	G	**A**	**T**	T	**A**	T	T	G	**G**	**T**
CMW 3276 *C. virescens*	A	T	T	T	A	C	**A**	**A**	T	**A**	A	C	T	**T**	**T**

SUMMARY

This dissertation represents a study on *Ceratocystis* spp. with hat-shaped ascospores. Previously only six species of *Ceratocystis* with this spore form were known. These include *C. fimbriata, C. pirilliformis, C. albofundus, C. moniliformis, C. moniliformopsis* and *C. acericola*. In this study, we have discovered and tentatively described three new species with hat-shaped ascospores. One of these group with the larger *C. fimbriata* clade, while the other two reside within the larger *C. coerulescens* clade.

Chapter one provides a concise summary of the literature pertaining to the genus *Ceratocystis*. The intention of the chapter is to introduce readers to this important genus of plant pathogens and to provide a background regarding their taxonomy, ecology, biochemistry and variation in species. Emphasis is placed on the distinction of *Ceratocystis* from *Ophiostoma*, as well as on those *Ceratocystis* spp. with hat-shaped ascospores that negatively impact upon plantation forestry species. This chapter shows how *Ceratocystis* spp. associated with hardwood species in commercial forestry plantations have increased in number and it provides the background for research presented in the following five chapters.

In the second chapter of this dissertation, a new species of *Ceratocystis* was discovered amongst isolates from the Himalayan mountain range of Bhutan. This fungus, in association with the bark beetle *Ips schmutzenhoferi*, is responsible for large-scale deaths of Himalayan spruce trees in Bhutan. The fungus is morphologically very similar to *C. moniliformis* and *C. moniliformopsis,* but differences in culture morphology, survival at different incubation temperatures and DNA sequence data based on three different gene regions supported the fact that this is a unique species. The fungus has thus been tentatively described as *Ceratocystis bhutanensis prov. nom.*

In chapter 3, I consider isolates of a *Ceratocystis* sp. recently discovered associated with dying clove trees in Northern Sulawesi, Indonesia. The fungus was found at a very high level of incidence, but was at first identified as *C. fimbriata*, based on morphological characteristics. Differences were observed in cultures of this fungus when they were compared with *C. fimbriata* especially in terms of colony colour and growth at different

temperatures. Morphological differences were also observed when the clove fungus was compared with *C. fimbriata* isolates. When three different DNA gene regions were sequenced and compared, it was clear that this fungus represents a new species. The fungus is, therefore, tentatively described as *Ceratocystis polychroma prov. nom.* in this dissertation.

Ceratocystis polychroma prov. nom. isolates obtained from cloves in Sulawesi displayed three distinctly different culture morphologies. In Chapter 4 of this dissertation we, used DNA sequence data and microsatellite markers to consider whether these differences could be observed at the molecular level. Comparisons of sequence data for the ITS region gave no distinction between any of the morphological groups. A total of 50 isolates were studied using microsatellites markers developed for *C. fimbriata.* No distinction could be obtained between isolates representing the three different culture morphological groups. The 50 isolates were subsequently treated as one population in further analyses. With the aid of the microsatellite markers, it was shown that that this population probably originated from Sulawesi and that it benefits form sexual outcrossing.

In chapter five of this dissertation, a study was undertaken to consider the taxonomic status of *C. moniliformis*. Considerable variation has been noted in different descriptions of this species. It also has a very wide host and geographic distribution raising speculation that *C. moniliformis* represents a species complex rather than a single taxon. Based on morphological and DNA sequence data from three gene regions, isolates from Sumatra were described as a new species, which we have tentatively named *C. tribiliformis prov. nom.* The other *C. moniliformis* isolates were all the same, despite the fact that they originated from a wide range of hosts and areas. The fungus correctly bearing the name *C. moniliformis, C. moniliformis sensu stricto*, therefore does not seem to represent a species complex. Species such as the closely related *C. tribiliformis prov. nom., C. bhutanensis prov. nom., C. omanensis prov. nom.* and *C. moniliformopsis* all belong to the larger *C. moniliformis sensu lato group*, and all have hat-shaped ascospores, conical spines on the ascomatal bases, disc-shaped bases to the ascomatal necks and are phylogenetically closely related to *C. moniliformis.*

Studies presented in this dissertation provide considerable new knowledge regarding various *Ceratocystis* spp. with hat-shaped ascospores. Three new species are described and I have also been able to show that *C. moniliformis sensu stricto* is monophyletic. Two of the species (*C.*

tribiliformis prov. nom. and *C. bhutanensis nom. prov*) group within the larger *C. coerulescens* clade while *C. polychroma prov. nom.* groups within the larger *C. fimbriata* clade. Studies in this dissertation have also improved our knowledge of the identity of several species previously incorrectly identified as either *C. moniliformis* or *C. fimbriata*. What has clearly emerged from this dissertation is the need for a monograph of *Ceratocystis* to include all new species and to thoroughly consider the population biology and ecology of all species