CHAPTER 1

A review of *C. fimbriata* and other species of *Ceratocystis*

INTRODUCTION

Ceratocystis sensu lato includes a group of ascomycetous fungi that are plant pathogens primarily associated with sap staining of wood, and which are distinctly adapted to insect dispersal. This broader group of fungi, more commonly known as the ophiostomatoid fungi, includes over 100 species in the genera *Ceratocystis* Ellis & Halst., *Ceratocystiopsis* Upad. & Kendr., *Ophiostoma* H. & P. Syd. (De Hoog & Scheffer 1984, Wingfield *et al.* 1993) and *Gondwanamyces* Marais & M. J. Wingf. (Marais *et al.* 1998).

The oldest genus of ophiostomatoid fungi is *Ceratocystis,* which was first established in the late eighteenth century when *Ceratocystis fimbriata* Ellis & Halst. was described as the cause of a black rot of sweet potato (*Ipomoea batatas* [L.] Lam.) (Halsted & Fairchild 1891). Members of *Ceratocystis* are typified by perithecia with globose bases and long necks, emitting ascospores in droplets from their apices (Halsted & Fairchild 1891, Hunt 1956, Upadhyay 1981).

The taxonomy of *Ceratocystis* spp. was controversial from an early stage. Confusion first emerged when the ascocarps or perithecia of the type species, *C. fimbriata*, were mistaken for pycnidia and similarly, the ascospores for conidia (Halsted & Fairchild 1891). Since then, the genus has been subjected to many name changes, as attempts have been made to resolve the confusion generated by this misinterpretation. Substantial confusion has also arisen due to the general morphological similarities of *Ophiostoma* and *Ceratocystis* spp., and the fact that species of these two genera have similar biologies (Upadhyay 1993, Wingfield *et al.* 1993). The taxonomic history of *Ceratocystis sensu lato* has been eloquently reviewed numerous times (De Hoog 1974, Upadhyay 1981, De Hoog & Scheffer 1984) and will, therefore, not be repeated here. An extract taken from the presentation of Seifert & Okada (1993), however, summarizes the name changes and taxonomic history of the type species of *Ceratocystis* and is repeated here as follows:

Ceratocystis (*ceratos* = horn and *cyst* = pouch or sac)
Ceratocystis fimbriata Ellis & Halsted, N.J. Agric. Exp. Sta. Bull. 76:14. 1890.
= *Sphaeronaema fimbriatum* (Ellis & Halsted) Sacc., Syll. Fung. 10:125. 1892.
= *Ceratostomella fimbriata* (Ellis & Halsted) Elliot, Phytopathology 13:56. 1923.

= *Ophiostoma fimbriataum* (Ellis & Halsted) Nannf., Svenska Skogsfor. Tidskr. 32:408. 1934.

= *Endoconidiophora fimbriata* (Ellis & Halsted) 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* (Davidson) Moreau, Rev. Mycol. (Paris) Suppl. Col. 17:22. 1952.

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

The taxonomy of *Ceratocystis* was substantially complicated when *Ophiostoma* was described by Sydow & Sydow in 1919. This important genus also has globose perithecia emitting ascospores at the apices of long tubular necks. This similarity in morphology with species of *Ceratocystis* and *Ophiostoma* (Upadhyay 1993, Wingfield *et al.* 1993), led to considerable confusion between these two genera and as a result, the two genera have tended to be treated collectively (Elliott 1925, Hunt 1956, Upadhyay 1981). During the course of the last three decades, many studies have clearly shown that significant differences, both morphologically and genetically, exist to warrant the separation of these two genera (De Hoog 1974, Harrington 1981, De Hoog & Scheffer 1984, Wingfield *et al.* 1993). A third ophiostomatoid genus, *Ceratocystiopsis* Upadh. & Kendr., was easily distinguished from these two genera on the basis of sheathed, elongated to flacate ascospores (Upadyhay 1981). Some authors, however, believe that *Ceratocystiopsis* should be reduced to synonymy with *Ophiostoma* (Hausner *et al.* 1993).

Morphologically, the most convincing characters separating *Ophiostoma* and *Ceratocystis* are elements of their associated anamorphs. *Ceratocystis* spp. have *Chalara* (Corda) Rabenh. anamorphs with long tubular conidiophores with chains of enteroblastically produced conidia (ring wall building development) (De Hoog 1974, Minter *et al.* 1983, Mouton *et al.* 1994). In contrast, *Ophiostoma* spp. have anamorphs in *Graphium* Corda, *Leptographium* Lag.. & Melin and *Sporothrix* Hekt. & Perkins. The conidia in these cases are produced holoblastically (apical wall building development) (De Hoog 1974, Wingfield *et al.* 1993). *Ceratocystis* spp. also lack cellulose and rhamnose in their cells walls, while species of *Ophiostoma* contain both these polysaccharides in their cell walls (Rosinski & Campana 1964, Spencer & Gorin 1971, Jewell 1974, Weijman & De Hoog 1975). Biochemically, species in *Ceratocystis* are sensitive to the antibiotic cycloheximide

in culture, while *Ophiostoma* spp., have the ability to tolerate high concentrations of this antibiotic (Harrington 1981, De Hoog & Scheffer 1984).

The use of molecular techniques has provided the most convincing evidence justifying the separation between *Ceratocystis* and *Ophiostoma*. Phylogenetic studies based on DNA sequence have indicated that *Ophiostoma* spp. are most closely related to species in the Diaporthales and *Ceratocystis* spp. to taxa belonging to the Hypocreales (Spatafora & Blackwell 1994, Wingfield *et al.* 1999). These phylogenetic data have reaffirmed the taxonomic value of various phenotypic characteristics thought to separate *Ceratocystis* and *Ophiostoma* (Hausner *et al.* 1993, Spatafora & Blackwell 1994). It is now generally accepted that *Ophiostoma* and *Ceratocystis* are distinct and phylogenetically unrelated genera (Hausner *et al.* 1993, Spatafora & Blackwell 1994.). Their respective type species are *Ophiostoma piliferum* (Fr.) H. & P. Syd. and *Ceratocystis fimbriata*.

The objective of this review is twofold. Firstly, details of the biology of *Ceratocystis* spp., with particular reference to the type species, *C. fimbriata* is considered. A brief summary of the morphology, distribution, host-range, pathogenicity and reproduction of the pathogen is given. Secondly, the review deals with the molecular aspects used to characterise fungal taxa and populations, especially *Ceratocystis* spp.

MORPHOLOGY

The primary and most basic taxonomic tool used for the identification of fungi is morphology (Hunt 1956, Upadhyay 1981). *C. fimbriata* has a relatively simple holomorph morphology consisting of teleomorph and anamorph stages. A general description of *C. fimbriata* is given below, although it should be noted that isolates from different geographic locations tend to differ in colony type, growth rate and conidial states (Webster & Butler 1967a, b). Morphological descriptions are mostly made from isolates grown on artificial media.

Cultural characteristics: Cultures are hyaline when young becoming light brown and darker with age. The superficial, aerial mycelium is normally lighter in colour than the submerged mycelium, which is brownish to dark greenish (Hunt 1956, Upadhyay 1981).

The reverse side of cultures, therefore, normally provides a better indication of colony colour. Submerged mycelium also darkens as perithecia develop and the margins of the cultures normally consist of fine, radiating fibrils. Colonies are generally described as slow growing (approx. 21 mm in 10 days) (Hunt 1956, Upadhyay 1981). A certain level of variability in culture characteristics between different isolates of *C. fimbriata*, has been reported (Webster & Butler 1967a, b).

Teleomorph: The bases of the perithecia of *C. fimbriata* are dark brown to black, and globose (Hunt 1956, Upadhyay 1981, Grylls & Seifert 1993). The perithecial bases are sometimes ornamented with hyphae. Perithecial necks are long and slender, black at the base but hyaline towards the apex. The necks also taper slightly towards the tip. Hyaline ostiolar hyphae extend from the outer layers of the neck cells and are mostly convergent (Hunt 1956, Upadhyay 1981). Perithecia are produced singly or in clusters, superficially or are partly embedded in agar (Hunt 1956, Upadhyay 1981, Grylls & Seifert 1993).

Ascospores are produced in great numbers in the perithecial bases. These are hyaline, single-celled, and are characteristically hat-shaped (Hunt 1956, Upadhyay 1981, Grylls & Seifert 1993). Asci are evanescent and, therefore, rarely seen. During the formation of the ascospores, the cells lining the interior of the perithecial cavity disintegrate forming a gelatinous mass, which envelops the ascospores. Hydrostatic pressure develops inside the perithecia as it absorbs water pushing the oily gelatinous masses of ascospores up the perithecial necks and out of the ostioles as a white to yellowish droplet (Hunt 1956, Upadhyay 1981).

Anamorph: *Ceratocystis fimbriata* has a *Chalara* anamorph producing two types of conidia. The hyphae making up the mycelium are hyaline to pale brown, smooth and septate. Most of the hyphae terminate as conidiophores. Conidiogenous cells, at the end of the conidiophores, produce conidia through ring wall building (Minter *et al.* 1983). These enteroblastically formed conidia are either barrel-shaped or cylindrical with truncate ends. Both types of conidia are smooth-walled, non-septate, hyaline and borne singly or in chains. Thick-walled, oval chlamydospores are also characteristic. These are olive-brown in colour, normally found embedded in the agar and are formed singly or in short chains (Pontis 1951, Hunt 1956, Upadhyay 1981, Grylls & Seifert 1993).

Physiological characteristics: *Ceratocystis fimbriata* grows well on artificial media, especially 2 % malt extract agar (MEA). To obtain a higher ratio of perithecial to mycelial growth, any media used should, however, be supplemented with thiamin (Barnett & Lilly 1947). Perithecial formation occurs only when the ratio of thiamine to other nutrients in the medium is relatively high (Barnett & Lilly 1947). Calcium has also been shown to be favourable for the production of perithecia, while excess nitrogen prevents perithecial formation in *C. fimbriata* (Campbell 1960). To keep cultures from deteriorating and loosing viability, masses of ascospores should be transferred. If only mycelium is repeatedly transferred, vitality can be lost, resulting in sterile cultures (Hunt 1956, Upadhyay 1981). Culture media appears to favour rapid mycelial growth at the expense of reproductive structures (Upadhyay 1981). The pH determined for optimal vegetative growth and formation of perithecia is between 5-7, with pH 6 the most optimal (Kile 1993, Upadhyay 1981). The optimum temperature for growth in culture is generally between the range 22-26 °C (Kile 1993) although with differing effects. For example, the conidial or vegetative growth forms abundantly at 18 °C, but with reduced perithecial growth. At 25 °C, however, more perithecial growth is observed (Barnett & Lilly 1947).

DISTRIBUTION AND HOST RANGE

Ceratocystis fimbriata was first discovered more than 100 years ago where it was causing severe rot of exotic *Ipomoea batatas* in New Jersey, U.S.A. (Halsted & Fairchild 1891). Since then, the fungus has been recorded from most parts of the world where it causes diseases on a wide range of plants (Kile 1993) (Table 1). *C. fimbriata* is thus an important plant pathogen, often resulting in large scale losses in agriculture and forestry (Kile 1993).

Most reports of *C. fimbriata* have been from Latin America (McCracken & Burkhardt 1977), where the fungus infects a wide range of native and exotic host plant species (Beeley 1929, Costa & Krug 1935, Sharples 1936, Iton 1959, Ribeiro *et al.* 1985) (Table 1). In areas such as Colombia, Venezuela and Brazil, *C. fimbriata* causes major losses in coffee (*Coffea arabica* L.) production (Pontis 1951). Other agricultural crops in these countries include *Citrus* spp., *Crotalaria* sp., *Inga* spp., *Mangifera indica* L. and *Theobroma cocao* L. (Rincon 1983, Nieto 1991, Buritica 1994, Mourichon 1994). Woody hosts which have been infected include *Acacia decurrens* (Wendl.) Willd. (Ribeiro *et al.*

1985), *Gmelina arborea* Roxb. (Muchovej *et al.* 1978) and *Hevea braziliensis* Muell.-Arg. (Sharples 1936).

Ceratocystis fimbriata is also present in Asia and Europe. At one stage, infections of *Ipomoea batatas* were a serious problem in Japan until effective control strategies were implemented to contain the disease (Clark & Moyer 1988). In Europe and U.S.A., the most important disease has been infection of *Platanus* spp. by *C. fimbriata* f.sp. *platani*. This pathogen causes severe cankers on trees (Walter *et al.* 1952, Panconesi 1981). Other hosts infected by *C. fimbriata* in these countries are listed in Table 1.

New reports of *C. fimbriata* infecting previously unknown hosts in new areas are continuously made. The presence of *C. fimbriata* in Australia was first recorded in 1987 (Walker *et al.* 1988) on rotting *Syngonium* spp. in nurseries. The pathogen is thought to have been introduced into the country via infected *Syngonium* material (Walker *et al.* 1988, Vogelzang & Scott 1991). The presence of *C. fimbriata* on the African continent has only recently been recognised (Roux *et al.* 1999) when severe wilting of *Eucalyptus* clones in plantations in the Republic of Congo were caused by this pathogen. The pathogen has subsequently also been discovered infecting *E. grandis* in Uganda (Roux *et al.* 2001a).

DISEASE DEVELOPMENT

Ceratocystis fimbriata has been found on a variety of substrates including root and fruit crops, ornamentals and forest trees (Halsted & Fairchild 1891, Pontis 1951, Walter *et al.* 1952, Gremmen & De Kam 1977, Kile 1993, Panconesi 1999, Roux *et al.* 2001a,) (Table 1). The disease symptoms produced by this pathogen vary according to the type of host it infects. Three main categories of symptoms are recognized. These include vascular stains, cankers, root and stem rots (Table 1).

Vascular stains normally occur on hardwood species (Kile 1993). After infection, for which a wound is required, mycelium grows in the medullary rays and axial parenchyma (Olchowecki & Reid 1973). The movement of *C. fimbriata* in the vascular system is, however, partially restricted to the area of infection. This is mainly due to plant host defense reactions against infection, such as the production of tyloses, gels and vessel wall

thickening (Clerivet & El Modafar 1994). The fungus then stains the sapwood and causes extensive discolouration. When the bark of the tree is removed, dark brown radial patches or streaks are usually observed (Pontis 1951, Kile 1993). This disease can be severe and has the potential to kill vigorously growing trees within a few months (Gibbs 1993).

The most common symptoms of infection by *C. fimbriata* are cankers (Table 1). Symptoms first appear as circular, necrotic areas surrounding pruning wounds or other mechanical injuries (Pontis 1951). Discolouration and death of the bark and wood occurs, resulting in stem or branch deformation or death of the affected plant part (Wood & French 1963, Kile 1993). Penetration of the wood by the fungus is slight. Infection, and therefore staining, is restricted to areas of the cambium and inner bark tissue, resulting in various types of lesions (Kile 1993, Hinds 1972). These cankers are formed by the mycelium continually invading the cambium during the dormant season, killing new zones of tissue. Continuous concentric layers of callus can be formed, producing cankers that appear target-shaped (Hinds 1972, Gremmen & De Kam 1977). Ceratocystis cankers are perennial and enlarge slowly with vertical growth exceeding horizontal growth (Pontis 1951, Hinds 1972). The development of cankers is, however, dependent on the virulence of the isolates, fungal-host interaction and the location of infection (Kile 1993).

Root and stem rots normally occur on agricultural crops. *C. fimbriata* has been reported to cause black rot of sweet potato, characterised by dark greenish circular spots on the tubers (Halsted & Fairchild 1891). It also causes mouldy rot of tapping panels on rubber (*Hevea* sp.) and rot disease of *Crotalaria* sp. (Pontis 1951) (Table 1). *C. fimbriata* infects the non-lignified or lightly lignified tissues and results in the blackening or browning of the infected areas of the host, causing chlorosis or wilting and stunting (Kile 1993).

DISSEMINATION

Many species of *Ceratocystis* are well-known for their association with certain insect genera (Crone & Bachelder 1961, Moller & DeVay 1968, Upadhyay 1981). In general, *Ceratocystis* spp. are vectored non-specifically by casual insects such as flies (Diptera) and beetles (Coleoptera: Nitidulidae) (Crone & Bachelder 1961, Moller & DeVay 1968, Hinds 1972, Juzwik & French 1983). There are, however, a small number of *Ceratocystis* spp.

such as *C. polonica* (Siemaszko) C. Moreau, *C. laricicola* Redfern & Minter, and *C. rufipenni* Wingfield, Harrington & Solheim, that are specifically associated with bark beetles that infest conifers (Siemaszko 1939, Jewell 1956, Redfern *et al.* 1987, Wingfield *et al.* 1997).

Ceratocystis fimbriata does not have close associations with any specific insect and is not reliant on insects for survival. Insects do, however, play an important role in the dispersal of this fungus (Moller & DeVay 1968, Upadhyay 1981). In an experiment to determine possible vectors for *C. fimbriata*, Hinds (1972), baited wounds on *Populus tremuloides* Michx. trees in Colorado with *C. fimbriata*. After catching insects visiting these sights, it was observed that *C. fimbriata* was not selective to a particular vector, but was carried by several orders and families including beetles, flies, aphids and moths. The primary vectors were beetles of the families Nitidulidae, Staphylinidae and Rhizophagidae. Crone & Bacheldor (1961), performed insect transmission studies on susceptible *Platanus* spp., and found that *Coleopterous* insects, especially *Nitidulids* were the main vectors distributing *C. fimbriata*. Eight different insect species and mites have also been reported to be vectors of *C. fimbriata* on stone fruits (Moller & DeVay 1968).

As with other *Ceratocystis* species, *C. fimbriata* is morphologically adapted for dispersal by insects. It has a long perithecial neck from which sticky ascospores are released. The characteristic fragrant, fruity aroma emitted from *C. fimbriata* serves as an additional attraction for insects (Moller & DeVay 1968, Kile 1993). When the insects forage on the fungi, the sticky masses of ascospores adhere to the body of the insects. As the insects move to neighbouring trees, the fungus is also transmitted to these new trees (Crone & Bacheldor 1961). *C. fimbriata*, however, requires fresh wounds to infect its hosts (Moller & DeVay 1968, Hinds 1972, Teviotdale & Harper 1991, Kile 1993). These wounds can be caused by insect damage or by mechanical or environmental factors such as hail and wind breakage (Leather 1966, Moller & DeVay 1968).

The principle factor responsible for the spread of *C. fimbriata* is man. There has been no indication that major economic losses on crops have occurred when the fungus was dependent on natural agencies for dispersal (Walter *et al.* 1952). It seems, especially on woody hosts, that the primary means of fungal transmission is mechanical, by way of infected pruning tools and related equipment (Walter *et al.* 1952, DeVay *et al.* 1968,

Teviotdale & Harper 1991). Walter *et al.* (1952) noted that in numerous small outbreaks of canker stain of *Platanus* sp., a very high proportion of the primary infections started at pruning wounds. Pontis (1951), also noted that most of the cankers observed on coffee trees were at sites where pruning had taken place.

PATHOGENICITY

Many studies pertaining to the pathogenicity of *C. fimbriata* have confirmed that different strains of the pathogen have developed strict host specificity (DeVay *et al.* 1963, Leather 1966, Kojima & Uritani 1974, Kile 1993, Kojima 1993). Although morphologically indistinguishable from the type species of *C. fimbriata* from sweet potato (*Ipomoea batatas*), most strains differ in their pathogenicity to different hosts. For example, coffee trees inoculated with a sweet potato strain showed no signs of infection, although severe cankers were produced from inoculations with the coffee (*Coffea* sp.) strain (Pontis 1951). Similarly, inoculations of sweet potato, cacao (*Theobroma cacao*)and coffee with an isolate of *C. fimbriata* from pimento (*Pimenta officinalis*) gave negative results (Leather 1966). Vogelzang & Scott (1991), carried out studies using an isolate of *C. fimbriata* from a goose foot plant (*Syngonium*) to determine the extent of its pathogenicity. They found that the isolate was not pathogenic on plum (*Prunus domestica* L.), almond (*P. dulcis* [Miller] D. Webb), apricot (*P. armeniaca* L.) or mango (*M. indica* L.) and only slightly pathogenic on taro (*Colocasia esculenta* [L.] Schott). Although the taro plants initially showed signs of infection, they were able to recover over time (Vogelzang & Scott 1991). Olson & Martin (1949), also found that isolates from rubber (*Hevea*) in Mexico were not pathogenic to sweet potato and likewise, isolates from sweet potato (U.S.A.), were not pathogenic to rubber plants.

In hybridisation studies, Olson & Martin (1949), showed that, although rubber tree isolates and sweet potato isolates were not pathogenic on their respective hosts, hybridisation between the two isolates was possible. Similarly, Feazell & Martin (1950), showed that sweet potato and sycamore isolates, although not pathogenic on their respective hosts, were able to hybridise with each other and produce fertile cultures. Webster & Butler (1967a), in similar but more detailed studies, identified that differences exist among *C. fimbriata* isolates from different hosts and origins. The ability of different strains of *C. fimbriata* to

hybridise and form fertile progeny increases the potential for new host-pathogen combinations to emerge (Kile 1993). Such hybridisations also give the fungus a greater ability to rapidly evolve and adapt to new hosts when introduced to a new environment (Brasier 1993).

Several studies based on pathogen-host interactions have identified certain factors within host plants that are linked to host specificity in certain strains of *C. fimbriata* (Kojima & Uritani 1974, Kojima & Uritani 1978, Kojima *et al.* 1982, Kawakita & Kojima 1983, El Modafar *et al.* 1995). In studies carried out by Kojima *et al.* (1993), potato roots and taro tubers were used as hosts, and seven strains of *C. fimbriata* isolated from sweet potato, coffee, prune, cacao, oak, taro and almond as the infection agents. Cross inoculations of these hosts with the different strains showed a variety of host-interactions and demonstrated that certain phytoalexins, spore agglutinating factors, divalent cations (Ca^{2+} and Mg^{2+}), and inhibitory proteins on germinated spores are all involved in determining host-pathogen specificity. These studies have provided much support for the hypothesis that *C. fimbriata* represents a species complex rather than a discrete taxon.

ORIGIN

A form of *C. fimbriata* similar to that infecting sweet potato, but different in host specificity, is considered native to North America (Walter *et al.* 1952, Anonymous 1986). This fungus, *Ceratocystis fimbriata* f.sp. *platani*, was found in low incidence infecting native *Platanus* sp. in areas not exposed to human activity (Walter *et al.* 1952). Spread of this fungus to other *Platanus* spp. and areas of the U.S.A. is believed to have started in New Jersey as early as 1929 (Jackson & Sleeth 1935). Subsequently, *C. fimbriata* f.sp. *platani* was reported causing wide-spread and severe canker stain disease to ornamental *Platanus* spp. throughout Europe, in countries such as Italy, France and Switzerland (Walter *et al.* 1952, Vigouroux 1979, Panconesi 1981). The pathogen is thought to have spread from the U.S.A. into Europe at several southern European ports at the end of the second world war (Anonymous 1986).

REPRODUCTION

Chalara, which relies solely on the asexual reproduction and production of conidia via mitosis, is the anamorph state of *Ceratocystis*. *Ceratocystis*, on the other hand, reproduces ascospores sexually via meiosis. Within *Ceratocystis*, species are either heterothallic such as *C. paradoxa* Dade, *C. radicicola* Bliss, *C. fagacearum* (Bretz) Hunt and *C. eucalypti* Yuan & Kile (Hepting *et al.* 1952, Kile *et al.* 1996) or homothallic as in *C. fimbriata*, *C pinicola* and *C. virescens* (Davids.) C. Moreau (Olson 1949, Webster & Butler 1967a, b, c, Harrington & McNew 1997).

Two opposite mating type genes, MAT-1 (self-sterile strains) and MAT-2 (self-fertile strains), are involved in the sexual reproduction in *Ceratocystis* spp. In heterothallic *C. eucalypti,* both mating type genes are required for sexual reproduction. Single ascospore progeny of crosses are then either of the MAT-1 or MAT-2 mating type. In homothallic *C. fimbriata,* sexual reproduction occurs by means of selfing. Single ascospore transfers from a selfing event (i.e. MAT-2 strain) produces an equal ratio of self-sterile and self-fertile progeny (Hansen & Snyder 1952, Webster & Butler 1967b, c). This phenomenon, whereby the MAT-2 gene is deleted, allowing for expression of the MAT-1 gene, has been attributed to uni-directional mating type switching (Harrington & McNew 1997, Witthuhn *et al.* 2000a). *C. fimbriata* has the ability, therefore, to outcross in nature with the potential to form new genotypes. The frequency and occurrence of this event, however, has not been determined.

MOLECULAR BIOLOGY

Since the advent of the polymerase chain reaction (PCR), molecular methods have become invaluable in studies of fungal phylogeny and population biology (Foster *et al.* 1993, Glass & Donaldson 1995). The ability to differentiate between fungi at the DNA level has provided additional characters with which to classify species. This has been particularly valuable where traditional phenotypic characteristics are limiting. Molecular techniques including studies of various molecules such as DNA, RNA, enzymes and other proteins have many applications. They can be used for taxonomic purposes to classify genera, species, and strains of fungi, to delimit individuals or species based on their genealogical or

phylogenetic relationships, to determine anamorph and teleomorph connections and to identify individuals or clones within fungal populations (Michelmore & Hulbert 1987, Foster *et al.* 1993, Maclean *et al.* 1993, Taylor *et al.* 1999a, b).

Sequences with useful phylogenetic inference come from genes that have the same function in all taxa, evolve at approximately the same rate, are present only once in a genome or behave like a single copy region (Glass & Donaldson 1995). One of the sets of genes that fulfils these criteria is the ribosomal RNA genes from the nuclear and mitochondrial genomes (Bruns *et al.* 1991). Fungal taxonomy and phylogenetic inferences have, therefore, been largely based on molecular sequence data obtained from the ribosomal gene cluster. It comprises three structural regions coding for the 5.8S, 18S (small subunit) and 28S (large subunit) ribosomal RNA genes (Mitchell *et al.* 1995). Interspersed between the genes are highly variable internal transcribed spacer regions ITS1 and ITS2 (Mitchell *et al.* 1995).

Sequences coding for the structural genes in the ribosomal operon are conserved due to their functional nature (Jukes 1987, Raue *et al.* 1988). There is limited sequence divergence within species making their use in the characterisation of organisms above the species level favourable. The ITS regions, on the other hand, are highly variable (Nazar *et al.* 1987, Mitchell *et al.* 1995). These regions evolve at different rates among fungi and thus, because of their high resolution, can be used to discriminate between taxa at different levels (Bruns *et al.* 1991, Messner *et al.* 1995, Mitchell *et al.* 1995). The ITS regions are routinely used to study closely related species and populations of fungi.

MOLECULAR GENETICS DEFINING *CERATOCYSTIS*

The ophiostomatoid fungi are thought to have similar morphologies due to convergent evolution (Wingfield *et al.* 1993). The use of morphological data alone has, therefore, proven insufficient to categorise taxa into distinct lineages. Orders and genera within the ascomycetes, including those belonging to the Ophiostomatales have, however, been successfully classified using ribosomal DNA sequences of the small subunit (18s) rDNA (Berbee & Taylor 1992, Spatafora & Blackwell 1993, 1994, Okada *et al.* 1997, 1998).

Various genes and enzymes have been used to address questions pertaining to the taxonomy of the species in the genus *Ceratocystis*. Paulin & Harrington (2000), used portions of the 18S and 28S rDNA domains to determine which *Chalara* anamorphs have phylogenetic affinities and, therefore, associations with teleomorphs belonging to the genus *Ceratocystis*. Wingfield *et al.* (1996), with the aid of sequence data from the ITS regions, showed that *Ceratocystis* isolates, causing severe disease on *Acacia mearnsii* in South Africa, and morphologically similar to *C. fimbriata*, represented a distinct, new species now named *C. albofundus* Wingfield, De Beer & Morris. Hausner *et al.* (1993), demonstrated the effectiveness of using restriction enzyme analysis on ribosomal and mitochondrial DNA to group species and strains within *Ceratocystis*. An easy and reliable identification method whereby species of *Ceratocystis* could clearly be distinguished using PCR-based restriction fragment length polymorphisms (RFLP), was developed by Witthuhn *et al.* (1999). In the same study, phylogenetic relationships between various *Ceratocystis* species were determined with the use of sequence data obtained from the large subunit of the rRNA gene.

Comprehensive studies on the *C. coerulescens* (Münch) Bakshi, complex using electrophoretic phenotypes based on isozyme analysis, showed that *C. coerulescens* represents a complex of five different species (Harrington *et al.* 1996). These morphologically indistinguishable variants were previously identified based solely on their different conifer hosts and insect vectors. DNA sequence data using ITS1, ITS2 and MAT-2 confirmed the delineation of the complex into five species and indicated that a strong monophylectic group exists for all *Ceratocystis* species occurring primarily on conifers (Witthuhn *et al.* 1998, Witthuhn *et al.* 2000b). The complex now consists of eleven species, highlighting the importance of host range in the evolution of *Ceratocystis* species.

In some cases, instead of showing divergence of taxa into different species, molecular tools have shown conspecificity of taxa. For example, in the *C. coerulescens* complex, *C. polonica* (Siemaszko) Moreau, and *C. laricicola* Redfern & Minter are morphologically identical but differ in their respective hosts and associated vectors. *C. polonica* infects spruce and is associated with *Ips typographus* L. (Siemaszko 1939) and *C. laricicola* attacks larch and is associated with the bark beetle *Ips cembrae* (Redfern *et al.* 1987). These two species differ in only one isozyme locus and it was suggested that they should be synonymized (Harrington *et al.* 1996). This suggestion was supported by identical ITS

sequences (Witthuhn *et al.* 1999). Further investigations using sequence data from the MAT-2 HMG box, however, showed that *C. laricicola* and *C. polonica,* could be differentiated from each other although separation was relatively weak (Witthuhn *et al.* 2000b). This is a clear example where analysis of multiple genealogical sequence data could be used to resolve an important taxonomic question.

Limited studies have been carried out to investigate the phylogenetic relationships between different host specific strains of *C. fimbriata.* After finding a virulent strain of this fungus causing severe wilting in *Eucalyptus* trees in the Republic of Congo, Roux *et al.* (1999), performed initial analysis to determine the possible origin of the strain. Phylogenetic data based on sequence from the ITS region, grouped the *C. fimbriata* isolates from Congo closest to those from South America and South Africa, but separate from isolates originating in North America. Further studies using ITS and MAT-2 sequence data on various isolates of *C. fimbriata* showed that they cluster into geographic and host-associated lineages (Baker & Harrington 2000, Baker *et al.* 2001). Isolates were separated into a North American clade, Latin American clade and an Asian clade (Baker & Harrington 2000). Within the Latin American clade, three specific host lineages, namely sweet potato, sycamore and cacao, were recognized. Results of the above studies suggest host specialization to be a major factor driving the speciation of specific lineages (Baker & Harrington 2000). They also indicate that *C. fimbriata* represents a complex of host specific strains or species, with a wide geographic distribution.

POPULATION GENETICS

Populations of pathogens and not individual strains generally cause disease. It is thus necessary for plant pathologists to study the genetics of pathogens at the population level. Population genetics, specifically in plant pathogenic fungi, is a rapidly emerging field of study providing valuable information for the development of management strategies to reduce the impact of pathogens (McDermott & McDonald 1993, Wolf & McDermott 1994 Brygoo *et al.* 1998). By studying levels of genetic diversity amongst and within populations, deductions can be made regarding the possible origin, structure, migration, mode of reproduction and evolutionary relationships of pathogens. Populations with higher diversities normally have an increased capacity to adapt to environmental changes via

natural selection. They are, therefore, less affected by introduced management strategies than populations with no or little diversity. If the origin, and subsequently, migration patterns of the pathogens are known, efficient quarantine strategies can be implemented to either contain a pathogen within a country or minimise introduction into other countries.

***Genetic diversity studies in* Ceratocystis**

Using RFLP fingerprinting studies, Harrington *et al.* (1998), found a correlation between the type of reproduction of a fungus and the resulting genetic diversity that is observed within a native population. Radioactively labelled microsatellite probes $(CAT)_5$ and $(CAC)_5$ were hybridised to total genomic DNA digested with *Pst*I and the nuclear DNA diversity was determined. Similarly, the mitochondrial DNA diversity was calculated from *Hae*III-restricted mitochondrial DNA. *C. eucalypti* Yuan & Kile, an obligately outcrossing fungus, had a higher genetic diversity compared to *C. virescens* (Davids.) C. Moreau, which is homothallic. The asexual fungus, *Chalara australis* Walker & Kile was the least diverse (Harrington *et al.* 1998). Roux *et al.* (2001b), used the same techniques to determine the genetic diversity within a population of *C. albofundus* from South Africa. A high level of both nuclear and mitochondrial gene diversity was observed for this homothallic fungus (Roux *et al.* 2001b). This high gene diversity, its association with native *Protea* spp. and its limited geographic distribution, led the authors to hypothesise that *C. albofundus* is native to South Africa.

There has only been a single population study conducted on *C. fimbriata*. In an attempt to determine the genetic diversity and possible origin of *C. fimbriata* f.sp. *platani* in Europe, Santini & Capretti (2000), used RAPD and direct amplification of minisatellite-region DNA (DAMD) PCR on a population from Italy and isolates of *C. fimbriata* on different hosts from North America. Their investigation supported the view that *C. fimbriata* f.sp. *platani* was introduced into Europe from North America. The population in Italy was clonal, sharing an identical genotype with some of the isolates from North America.

Microsatellites as population genetic tools

Molecular methods are increasingly used to study populations of organisms and a variety of techniques have been developed to facilitate such studies. These include Amplified Fragment Length Polymorphisms (AFLP) (Majer *et al.* 1996), Randomly Amplified Polymorphic DNA (RAPDs) (Santini & Capretti 2000), Randomly Amplified

Microsatellites (RAMS) (Hantula *et al.* 1998), RFLP's (Kuninaga & Yokosawa 1992) and nuclear DNA fingerprinting markers using $(CAT)_5$ (DeScenzo & Harrington 1994, Roux *et al.* 2001b). Co-dominant markers such as microsatellites are, however, prefered for population studies as they can identify different alleles within a single locus in individuals.

The increased use of microsatellite markers for population studies is based on a number of factors. Microsatellites consist of runs of short repeating sequences that can be highly variable in number between individuals (Jarne & Lagoda 1996). This feature provides an attractive source of polymorphisms between isolates, which can easily be studied and compared between individuals by designing DNA primers flanking these repeating regions (Schlötterer & Pemberton 1998). Furthermore, microsatellites are co-dominant and inherited in a mendelian fashion, abundant in eucaryotic genomes, easy to use and score, and they are selectively neutral with a high mutation rate (Tautz 1989, Rafalski *et al.* 1996). Excellent reviews on design, function and applications of microsatellites can be found in '*Microsatellites, Evolution and Applications*', edited by Goldstein & Schlötterer (1998).

The success of using microsatellites, also known as simple sequence repeats (SSR) (Tautz 1989) or short tandem repeats (STRs) (Edwards *et al.* 1991), is evident in the number of population studies that have recently been carried out on fungi. For example, microsatellites have been used to determine gene flow between two ectomycorrhizal populations (Zhou *et al.* 2001), for the detection, quantification, and diversity of grass endophytes (Groppe *et al.* 1995, Moon *et al.* 1999) and host and pathogen population variations (Delmotte *et al.* 1999), population diversity studies (Longato & Bonfante 1997, Meng & Chen 2001), population structure studies (Burgess *et al.* 2001, Burcheli *et al.* 2001) and phylogenetic studies (Fisher *et al.* 2000).

CONCLUSIONS

The taxonomy of the genus *Ceratocystis* has been reviewed many times, and its placement within the Ascomycetes is well established. Within the genus, the phylogenetic placement of the different species has recently received much attention and continues to be investigated. Studies of different variants and forms found within species are, however,

seriously lacking. This is with the exception of *C. coerulescence*, where investigations on the different variants resulted in the description of five new species.

Ceratocystis fimbriata is thought to consist of a complex of morphologically similar species. This hypothesis has emerged from evidence based on host specialisation, pathogenicity and molecular differences observed between isolates from different hosts and different geographical locations. The phylogenetic relationships between the different strains within *C. fimbriata* are, however, poorly understood. Although the biology of this species has been well documented, there is also a general lack of knowledge regarding the origin, genetics, population dynamics and movement of isolates comprising this species.

Detailed studies on different populations of *C. fimbriata* from different hosts and countries is likely to yield valuable information pertaining to population diversities and structure of this pathogen. Since microsatellite markers are associated with the highest level of polymorphism, due to the high evolution rate at these loci, they are potentially the most informative class of markers. This makes them the prime choice for the use in studying populations. Data obtained using microsatellite markers will not only be valuable in determining the area of origin of *C. fimbriata*, but will also aid in the development of management and quarantine strategies against certain strains of *C. fimbriata*. Studies to collect such data form an important part of this thesis.

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Table 1: Geographical distribution, host ranges and some of the diseases caused by *Ceratocystis fimbriata* reported in literature.

Disease	**Host**	***Country***	***Reference***
Cankers	*Populus* spp.	Europe, Canada, U.S.A.	Wood & French 1963, Zalasky 1965, Manion & French 1967, Hinds 1972, Gremmen & De Kam 1977
	Pimenta officinalis	Jamaica	Leather 1966
	Hevea braziliensis	Brazil	Beeley 1929, Sharples 1936
	Prunus spp.	U.S.A.	DeVay 1968, Teviotdale & Harper 1991
	Citrus spp	Colombia	Mourichon 1994
	Colocasia esculenta	Western Samoa	Walker *et al.* 1988
	Xanthosoma sagittifolium	Fiji	Walker *et al.* 1988
	Acacia decurrens	Brazil	Ribeiro 1985
Wilts and canker stain	*Gmelina arborea*	Brazil	Muchovej *et al.* 1978
	Eucalyptus spp.	Brazil	Laia *et al.* 1999
	Platanus spp.	USA, France, Italy, Spain, Switzerland,	Walter *et al.* 1952, Vigouroux 1979, Panconesi 1981
	Eucalyptus hybrids	Republic of Congo	Roux *et al.* 1999
	Eucalyptus spp.	Uganda	Roux *et al.* 2001a
	Coffea arabica	Central and South America, i.e. Indonesia, Colombia, Venezuela, Brazil	Zimmermann 1900, Pontis 1951, Szkolnik 1951, Echandi 1955
	Theobroma cacao	Central and South America	Iton 1959
	Mangifera indica	Brazil, Colombia	Batista 1960
Root and stem rot	*Crotolaria juncea*	Brazil, Colombia, Taiwan	Costa 1935, ChunYee *et al.* 1997
	Syngonium cultivars	Australia	Walker *et al.* 1988
	Ipomoea batatas	U.S.A., South East Asia, Oceania, Papua New Guinea, World-wide	Halsted 1980, Clark & Moyer 1988, Taubenhaus 1913

CHAPTER 2

Microsatellite markers reflect intra-specific relationships between isolates of the vascular wilt and canker pathogen, *Ceratocystis fimbriata*

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ABSTRACT

Ceratocystis fimbriata is a serious wilt and canker stain pathogen of many economically important agricultural and forestry crops. It has a wide geographic distribution and host range that includes both woody and herbaceous plants. Previous studies using hybridisation, have shown that differences exist in isolates of *C. fimbriata* from different hosts and origins. These isolates also differ in colony morphology, pathogenicity, growth rate as well as conidial states. Therefore, it has been suggested that distinct strains or special forms are encompassed in *C. fimbriata,* and that these could be linked to host or geographical origin. The aim of this study was to develop PCR based microsatellite markers for population studies on *C. fimbriata.* ISSR-PCR was used to target specific microsatellite regions of genomic DNA from two isolates of *C. fimbriata.* These amplified products were cloned and sequenced. Primer pairs were designed from these sequences to flank the microsatellite regions. From 24 primer pairs, 11 polymorphic primers were selected. These primers were tested on a number of *C. fimbriata* isolates representing a wide host and geographic range. Cluster analyses of the results indicate that these microsatellite markers clearly distinguish between different geographical as well as host specific populations of *C. fimbriata.* The results are concordant with sequence data from the internal transcribed spacer (ITS) region of the rDNA operon of the same isolates. These markers will be useful in future studies of population structures and diversity in *C. fimbriata.*

INTRODUCTION

The fungal pathogen, *Ceratocystis fimbriata* Ell. & Halst., causes serious wilt and canker-stain diseases on a wide range of plants world-wide. Some of the economically important agricultural and tree crops damaged by this pathogen include sweet potato (Halsted & Fairchild 1891), coffee (Pontis 1951), cacao (Kile 1993), stone fruit trees (DeVay *et al.* 1963), poplar (Gremmen & De Kam 1977), rubber (Olsen & Martin 1949) as well as *Eucalyptus* spp. (Roux *et al.* 1998). Although *C. fimbriata* has predominantly been reported from Central and South America (McCracken & Burkhardt 1977), its occurrence is geographically widespread in temperate and tropical regions of the world.

Identification of *C. fimbriata* in disease reports and general taxonomic treatments has been based, for the most part, on morphological and cultural characteristics (Upadhyay 1981). Isolates from different hosts and geographic areas, however, have been shown to differ not only in colony morphology but also in growth rate and conidial states (Webster & Butler 1967). Pathogenicity tests have shown that some isolates tend to be host specific. For example, inoculation studies carried out on cacao, coffee and sweet potato with an isolate of *C. fimbriata* from pimento gave negative results (Leather 1966). Pontis (1951), inoculated coffee trees with coffee and sweet potato isolates and showed that only the coffee isolate could infect coffee.

The name, *C. fimbriata* f.sp. *platani,* has been assigned to *C. fimbriata* isolates that specifically infect plane trees (*Platanus* spp.). Although morphologically indistinguishable from the type species of *C. fimbriata* from sweet potato, the plane fungus is specifically pathogenic to its hosts. Furthermore, Webster & Butler (1967), in hybridisation studies, showed that differences exist in *C. fimbriata* isolates from different hosts and origins. Based on this finding, they suggested that *C. fimbriata* is comprised of distinct host specific strains. Host specialisation in morphologically indistinguishable isolates illustrates the taxonomic limits of morphology. It also emphasises the advantages of contemporary molecular techniques that might facilitate differentiation between strains or cryptic forms within fungal species (Fisher *et al.* 2000).

Many different DNA fingerprinting techniques have been used to define fungal populations, including Randomly Amplified Polymorphic DNA (RAPDs) (Santini &

Capretti 2000), Randomly Amplified Microsatellites (RAMS) (Hantula *et al.* 1998), Restriction Fragment Length Polymorphims (RFLP's) (Kuninaga & Yokosawa 1992), nuclear DNA fingerprinting markers using $(CAT)_5$ (Roux *et al.* 2001) and Amplified Fragment Length Polymorphisms (AFLP) (Majer *et al.* 1996). There is, however, an increasing interest in using co-dominant markers in population studies. These markers allow for the detection and characterisation of multiple alleles at a given locus. Microsatellite regions provide an attractive source of polymorphisms between isolates and many properties favour their use as genetic markers. They are abundant in Eucaryotic genomes, have a co-dominant inheritance, are highly polymorphic, easy to use and score, and they are selectively neutral with a high mutation rate (Tautz 1989, Rafalski *et al.* 1996). Various studies employing microsatellite markers have made it possible to define population relationships, parentage and kinship, gene mapping and forensics (Queller *et al.* 1993, Engel *et al.* 1996, Jarne & Lagoda 1996).

In this study, we report on the development and characterisation of PCR based microsatellite markers for *C. fimbriata.* These primers were tested on isolates collected from a wide geographic and host range. Results based on microsatellites were also compared with those from sequence data obtained from the internal transcribed spacer region (ITS), and phylogenetic relationships between isolates were considered.

MATERIALS AND METHODS

Fungal isolates

Ceratocystis fimbriata isolates were selected to represent a wide variety of hosts and geographical origins (Table 1). Isolates were grown on potato dextrose agar (PDA, Biolab) containing chloramphenicol (100 mg/liter, Centaur Laboratories). A drop of ascospores from a single perithecium of each isolate was suspended in 50 µl mineral oil, spread onto PDA plates and incubated at 25 °C. Hyphal tips from germinating ascospores were isolated and incubated for 2 weeks at 25 °C in Erlenmeyer flasks containing 50 ml malt extract broth (2 %, Biolab). The mycelium was collected by filtration (Whatman No. 1) and lyophilized. The freeze-dried cultures were pulverized using liquid nitrogen. DNA was extracted for each isolate using the method previously described by Barnes *et al.* (2001).

ISSR- PCR

DNA from isolates CMW 4822 and CMW 4835 was randomly amplified using ISSR primers 5' DHB$(CGA)_5$, 5'HV$(GT)_5$G, 5' DDB$(CCA)_5$, 5'DBD$(CAC)_5$, 5' HVH$(GTG)_5$, 5'NDB$(CA)_7$C and 5'NDV$(CT)_8$ (Lieckfeldt *et al.* 1993, Buscot *et al.* 1996). The polymerase chain reaction (PCR) mixture consisted of a final concentration of 2 ng DNA, 200 µM of each dNTP, 600 nM primer, 3.5 U *Taq* DNA polymerase and 1x buffer with $MgCl_2$ (Roche Molecular Biochemicals, Alameda, CA). The reaction volume was adjusted to 50 µl using Sabax water. PCR conditions consisted of a 2 min denaturation step at 95 °C followed by 40 cycles of 30 s at 95 °C, 45 s at 48 °C and 2 min at 72 °C. A final step at 72 °C for 10 min ensured completion of the elongation process. Products were visualised on a 1.5% agarose gel containing ethidium bromide, under UV illumination and purified using the Magic PCR Preps Purification System (Promega Corp., Madison, WI) before cloning.

Cloning

The PCR products produced from each ISSR primer were cloned using the pGEM®-T Easy Vector System (Promega Corporation, Madison, Wisconsin, USA) according to the manufacturers instructions. Bacterial colonies containing recombinant plasmids were selected and grown in test tubes containing 2 ml Luria-Bertani (LB) broth supplemented with 100 µg/ml ampicillin (Sigma Chemicals Co, USA). The cells were incubated at 37 °C for 8 hours after which plasmid DNA was recovered using alkaline lysis (Sambrook *et al.* 1989). This DNA was re-suspended in 20 µl water containing RNase (10 mg/ml, Roche Molecular Biochemicals). Restriction digests with *Eco*RI (Roche Molecular Biochemicals) were performed on the plasmid DNA. The sizes of the cloned fragments were determined using Agarose gel electrophoreses.

Primer design

For each ISSR reaction, cloned fragments of different sizes were sequenced using an ABI PRISM™ 377 Autosequencer (Perkin-Elmer Applied Biosystems, Inc., Forster City, Calif). Sequence reactions were carried out with universal plasmid primers T7 and SP6 using an ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Allplied Biosystems). Sequence electropherograms were analyzed using Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems). The fragments sequenced were screened for microsatellite regions. Primer pairs were designed to flank these microsatellite regions and to amplify a DNA fragment in the range of between 150 to

500 bp so that each primer pair produced a different sized fragment to facilitate multiplexing. Primers were also designed to have a length of between 18 to 22 bases, annealing temperatures ranging from 58 °C to 64 °C and a GC content of around 50% (Table 2). Genome walking was performed as described by Burgess *et al.* (2001) and Siebert *et al.* (1995), on sequences that contained microsatellite regions at either the beginning or end of the sequence. The DNA from isolate CMW 4829 was used in the restriction digestions with enzymes *Eco*RV, *Sca*I, *Hae*III and *Rsa*I (Roche Molecular Biochemicals). Specific primers were designed for each of these sequences and used in subsequent reactions involved in genome walking.

Primer Testing

All the primers designed were used in PCR reactions to test for amplification. DNA from five isolates (CMW 2218, CMW 2242, CMW 4791, CMW 4829, and CMW 5328) with different geographic locations was selected for this purpose. PCR reactions were performed in 25 µl reaction volumes, consisting of 1 ng DNA, 200 µM of each dNTP, 300 nM of each primer, 0.35 U Expand High Fidelity enzyme (Roche Molecular Biochemicals) and 1.2 x Expand HF buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme). The PCR programme consisted of a 2 min denaturation step at 96 °C followed by 10 cycles of 20 s at 94 °C, 48 s at the specified annealing temperature for each primer and 45 s at 72 °C. A further 25 cycles were carried out with a 5 s extension after each cycle with the annealing time altered to 40 s. A final elongation step was carried out for 10 min at 72 °C. Samples were separated on a PAGE gel (6% acrylamide in 50 mM Tris-borate-EDTA buffer, 7 h at 140V) and subsequently silver stained (Blum 1987) to visualise amplification products. One primer from each primer pair that yielded a single polymorphic PCR product for all isolates, was re-synthesised and labelled with either a TET or FAM phosphoramidite fluorescent dye (MWG, Ebersberg, Germany) (Table 3). The labelled primers were then used in subsequent PCR reactions to amplify all *C. fimbriata* isolates in this study (Table 1).

Polymorphisms of loci

Fluorescent-labeled PCR products (0.5 µl containing 1.5 ng DNA) were combined with 0.5 µl of the internal standard GENESCAN –TAMRA (Perkin-Elmer Corp) and 1.5 µl of loading buffer. The samples were fractionated by PAGE (4.25 %) on an ABI Prism 377 DNA sequencer. The size of DNA fragments was determined using a combination of the

GeneScan® 2.1 analysis software (Perkin Elmer Corp.) and Genotyper® 3.0 (Perkin Elmer Corp.). A few isolates were sequenced to determine whether the observed polymorphisms resulted from mutations specifically within the microsatellite regions. Sequence reactions were carried out with an ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Allplied Biosystems) with protocols recommended by the manufacturers. Sequence electropherograms were analyzed with Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems).

ITS PCR and sequencing

Primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White *et al.* 1990) were used to sequence the ITS1, 5.8S and ITS2 ribosomal RNA operon regions. The PCR reaction mix consisted of 2–10 ng DNA, Expand High Fidelity PCR System enzyme mix (1.75 U) (Roche Molecular Biochemicals), 1 x Expand HF buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme), 200 nM of each primer and 200 µM of each dNTP. The same PCR programme was used as described above for the microsatellites. The annealing temperature was set to 55 °C. PCR amplicons were purified using the Magic PCR Preps Purification System (Promega, Madison, WI) and sequenced in both directions. Sequence electropherograms were analyzed using Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems).

Data analysis

For each isolate, the presence or absence of an allele at a specific locus was scored for each microsatellite primer and the data compiled in a matrix. Both the microsatellite matrix and the ITS sequences were analyzed using PAUP (Phylogenetic Analysis Using Parsimony [*and other methods]) (Swofford, 1998). The heuristic search option (based on parsimony) with random stepwise addition and tree bisection reconnection (TBR) was used. All characters were given equal weight and the Mulpar option was in effect. Length distribution of 1000 trees and confidence intervals using 1000 bootstrap replicates were calculated. *Ceratocystis albofundus* de Beer, Wingfield & Morris, (CMW 2475, accession number AF043605), was used as the outgroup for the ITS sequence data set and treated as a monophyletic sister group with respect to the ingroup. All trees generated from the microsatellite matrix were rooted using the Midpoint rooting option. The microsatellite

data matrix was also analyzed using cluster analysis of Bray-Curtis coefficient of similarity and Euclidean distance using the program PRIMER (Clarke & Warwick 1994).

RESULTS

ISSR- PCR

All the RAMS primers produced between 7 to 10 bands, each ranging in size from 200 to 600 bp. For each primer, only the clones containing different sized inserts with lengths smaller than 1000 bp were selected for sequencing.

Primer design

A total of 52 cloned fragments were sequenced. From these, 18 primer pairs were designed to flank microsatellite regions. Six sequences from the cloned fragments had microsatellite regions at the beginning of the sequence. Genome walking was performed on these sequences to obtain the full microsatellite sequence with flanking regions suitable for primer design (Table 2). The microsatellite regions, around which primers were designed, included those rich in di-, tri-, and tetra-nucleotide repeats as well as regions rich in thymine repeats.

Primer testing

All the designed primers were tested on a number of *C. fimbriata* isolates to ensure effective amplification. Four primer pairs failed to amplify in PCR reactions at different annealing temperatures and one primer pair produced multiple bands. These five primers were discarded. Fractionation on PAGE gels revealed that of the 19 remaining primer pairs, eight were monomorphic and 11 polymorphic. The 11 polymorphic primer pairs were amplified at different annealing temperatures in order to obtain the highest optimal conditions to produce single, clean bands (Table 2). The eight putative monomorphic primers were further tested on a number of different *C. fimbriata* isolates in order to confirm their monomorphic nature.

Polymorphisms of loci

The 11 polymorphic primers successfully amplified all 21 isolates of *C. fimbriata* selected for testing. The only exception was primer CF 13/14 that produced null alleles for isolates

CMW 1547, CMW 2901, CMW 2911, CMW 2913 and CMW 3264. Genescan analysis showed that the polymorphic markers produced a total of 65 alleles across the 11 loci analyzed (Table 3). The smallest and largest number of alleles per locus was 3 and 10 respectively.

Alleles ranged from 154 to 415 bp in length. Of the 11 loci, two or more alleles from 6 loci were sequenced to determine the source of polymorphisms. Within the locus amplified by primer AG 17/18, the polymorphisms observed between the different alleles were due to indels of CTT repeats. The differences in alleles observed from the loci amplified by primers CF 5/6, CF 15/15 and CF 17/18 were due to indels of repeating units of CTG, CT and AG respectively. The polymorphisms observed in the alleles from primer CF 21/22 were due to a difference in the number of T repeats (Figure 2). There were a number of transitions and transversions between the different sequences within the same locus. This, however, did not alter the size of the allele. Although most of the polymorphisms observed were due to differences in the microsatellite regions, there were some insertions and deletions found within the flanking sequences.

Some alleles obtained in the 11 loci were unique to certain isolates or groups of isolates. Primers AG 7/8, AG 17/18, CF 11/12, CF 13/14, CF 15/16, CF 17/18 and CF 21/22 all amplified unique alleles for some of the isolates. Primer AG 7/8, a $(TC)_{21}$ repeat, produced the greatest number of unique alleles (5), CF 17/18, a $(CA)_{15}$ repeat, produced three unique alleles, while the others produced only one or two unique alleles from the 21 isolates studied.

Data analysis

A total of 65 aligned characters were obtained from analysis of the 11 microsatellite loci. The heuristic search option based on parsimony showed one of these characters to be constant, 23 parsimony un-informative and 41 parsimony informative. A total of four most parsimonious trees were generated with tree lengths of 96. The consistency index (CI), retention index (RI) and g1 values were 0.67, 0.74 and –0.73 respectively for all trees. The dendograms produced from the analysis using the Bray-Curtis coefficient of similarity and the Euclidean distance were similar to the phylogenetic trees produced using PAUP. The dendogram based on similarity analysis was selected for presentation (Figure 1) and bootstrap values based on parsimony were included.

The manual alignment of the ITS sequences produced a total of 555 characters. Of these, 319 were constant, 89 parsimony un-informative and 147 parsimony informative. A total of four trees were produced with a length of 389. The CI, RI and g1 values were 0.82, 0.85 and –1.57 respectively. One tree (Figure 1), with similar topology and minor differences in branch lengths from the other 11 trees was chosen for presentation.

The phylogenetic trees generated from the microsatellite and ITS data showed that isolates of *C. fimbriata* included in this study, reside in several sub-groups supported by high bootstrap values. All the *Platanus* isolates from France, Switzerland and Italy grouped together, forming a European sub-group with strong (100 %) bootstrap in both the microsatellite and the ITS tree. *C. fimbriata* isolates from Congo and Uganda grouped together with 62 % confidence in the microsatellite and 51 % in the ITS tree. African isolates were all collected from diseased *Eucalyptus* species and formed a separate central African clade. Another distinct sub-group encompassed all isolates from North America. These included isolates from Canada and the USA, with a bootstrap value of 70 % in the microsatellite and 100 % in the ITS tree. The Colombian isolates from *Coffea* sp. and *Citrus* sp. reside in two distinct sub-groups. Bootstrap values for both these groups were high. For the one group, these were 98 % in the microsatellite and 100 % in the ITS tree. Bootstrap values for the other sub-group were 72 % and 100 % respectively for the same trees.

DISCUSSION

In this study, 11 microsatellite primers that display strong polymorphisms in a wide variety of *C. fimbriata* isolates were produced. The markers clearly elucidate relationships between the different isolates based on host and geographic origin. These relationships were supported by analysis of sequence data from the ITS region of the rDNA operon. The results also support those of previous studies based on hybridisation (Webster & Butler 1967) and pathogenicity (Pontis 1951, Walter *et al.* 1952, Leather 1966), showing that *C. fimbriata* encompasses a wide variety of strains.

The phylogenetic and similarity trees generated from the microsatellite and ITS data clearly resolved isolates of *C. fimbriata* into distinct sub-groups based on hosts. Within

these host-based sub-groups, it was also possible to recognise further groupings linked to geographical origin of isolates. For example, all isolates from *Eucalyptus* grouped collectively in a Central African sub-group, but this could be further sub-divided to separate isolates from Congo and Uganda. The same was true for the isolates from North America, where those infecting *Populus* spp. were distinct from those from *Prunus*. Within the *Populus* sub-group, isolates from Canada and the USA were distinct. An intriguing observation was that isolates from Colombia reside in two strongly resolved clades, although most are found on a single host. We believe that the Colombian groups are genetically isolated and further studies using the microsatellite primers and a greater number of isolates are needed to test this hypothesis.

Santini & Capretti (2000), used RAPD and direct amplification of minisatellite-region DNA (DAMD) PCR to examine the genetic variability in a population of *C. fimbriata* f.sp. *platani* from Italy and some isolates of *C. fimbriata* from North America. Their study showed that all Italian isolates have the same genotype, thus representing a clone. Although they suggested the entire European population might be clonal and the result of a single introduction, the results of our study based on microsatellites show otherwise. Isolates from Switzerland, France and Italy had the same genotype, but differed from a second isolate from France. Although the numbers of isolates from Europe were few in our study, there is evidence for at least two different genotypes in Europe. We believe that this difference is due to the fact that Santini & Capretti (2000), used dominant markers, which have lower resolution than the co-dominant markers used in the present study.

We have shown that isolates from *Populus* in North America reside in a group that is distinct from the *Prunus* isolate. Similarly, our isolate from sweet potato (*Ipomoea batatas*) was different from all the other isolates. These results are similar to those of Santini & Capretti (2000), and strongly support the view that isolates of *C. fimbriata* can be divided into groups based on host. These data also support those from hybridisation experiments (Webster & Butler 1967), host specificity (Kojima *et al.* 1982), and pathogenicity assays (Pontis 1951, Walter *et al.* 1952, Leather 1966), suggesting the host represents an important factor separating isolates of *C. fimbriata*. Furthermore, the hypothesis that host range is an important factor in the evolution of *Ceratocystis* spp. (Witthuhn *et al.* 1998) is supported by the results of this study.

The two most common methods to develop microsatellite markers are through screening genomic DNA libraries for microsatellite- rich regions and through searching public DNA databases (Yu *et al.* 1999). The production of DNA libraries and their subsequent screening is time consuming and expensive. Although computer databases have refined the process of selecting microsatellite regions for primer design, large numbers of DNA sequences are required. These are not available for most fungi and therefore, we have followed the approach of Burgess *et al.* (2001) in choosing ISSR-PCR or RAMS- PCR to develop microsatellite markers for *C. fimbriata*. Of 52 clones that were sequenced, 22 primers were designed, and of these, 11 were polymorphic. The success rate using this method was 21 % for clones sequenced. Burgess *et al.* 2001, who also used this method, had a success rate of 35 %. This is compared to the less than 10 % success rate that is more commonly attained when using genomic libraries (Brady *et al.* 1996).

Sequences of PCR products amplified using the designed microsatellite primers revealed that the main source of polymorphisms was from mutations within the repetitive microsatellite regions. Although it has been suggested that most microsatellites in fungi represent AT repeats (Groppe *et al.* 1995), many of those in this study were of a CT repeat. Insertions and deletions also contributed to the polymorphisms observed between isolates. For example, the sequence data generated from the PCR amplicon using primer CF15/16 in isolate CMW 2901 from Canada, showed there was an insertion of 12 bp which was absent in all the other alleles sequenced. However, elsewhere in the allele sequence of this isolate, there was a deletion of 13 bp. Thus, a size difference of only one base pair was observed, despite the fact that the polymorphism was considerably larger. This indicates that microsatellite data might underestimate genetic diversity.

Some alleles present in the 11 loci were unique to certain isolates or groups of isolates. Goodwin *et al.* (1992) termed these types of alleles 'private alleles'. In the North American sub-group for example, three alleles in different loci were unique to this group and not found in any other isolate. The same was true with the Central African sub-group that had one of the Colombian sub-groups five unique alleles, not shared by any other isolates. Private alleles provide evidence for reproductive isolation over a long period of time (Fisher *et al.* 2000), and the fact that these groups had unique alleles further defines the sub-groups.

Detailed studies of the frequencies of specific alleles, particularly private alleles, in populations of *C. fimbriata* from different geographically diverse origins will make it possible to determine gene-flow between populations, as well as random mating mechanisms within the populations. Allelic differences at these polymorphic loci can also be used to determine evolutionary history and relationships between populations of *C. fimbriata* from different countries. Determining the area of origin of *C. fimbriata* will also aid in the development of management and quarantine strategies against certain strains of *C. fimbriata*.

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Table 1: Isolates of *Ceratocystis fimbriata* used for the development and testing of microsatellite markers and their respective microsatellite profile.

Isolate no[1]	Host	Country	Collector	Microsatellite profile[2]	ITS Genbank Accession No.
CMW 1547	*Ipomoea batatas*	Papua New Guinea (PNG)	E.H.C McKenzie	4 1 225306211	AF264904
CMW 1894	*Platanus* sp.	Switzerland	O. Petrini	4 7 217673513	AF395682
CMW 1896	"	"	"	4 7 217673513	AF395681
CMW 2218	"	France	C. Grosclaude	4 8 217663513	AF395680
CMW 2219	"	"	"	4 7 217673513	AF395679
CMW 2242	"	Italy	A. Panconesi	4 7 217673513	AF264903
CMW 2901	*Populus tremuloides*	Canada	G. Smalley	3 2 251101233	AF395696
CMW 3264	"	"	"	3 4 342401823	No sequence
CMW 2911	*Prunus* sp.	U.S.A	R. Bostock	2 2 252201133	AF395693
CMW 2913	*Populus tremuloides*	"	T. Hinds	3 2 252201233	AF395694
CMW 4791	*Eucalyptus* clone	Rep. of Congo	J. Roux	2 5 213242312	AF395685
CMW 4793	"	"	"	3 2 216432513	AF395684
CMW 4829	*Citrus* sp.	Colombia	B. L. Castro	3 2 216432513	AF395688
CMW 4835	*Coffea* sp.	"	"	2(10)239553724	AF395689
CMW 4824	"	"	"	2(10)332553724	AF395692
CMW 4844	"	"	"	1 9 216225312	AF395691
CMW 4845	"	"	"	1 4 214274312	AF395690
CMW 4903	*Eucalyptus* sp.	Brazil	A. Alfenas	1 6 214215312	AF395683
CMW 5312	*Eucalyptus grandis*	Uganda	J. Roux	3 2 117332513	AF395687
CMW 5328	"	"	"	3 3 218432413	AF395686

[1] All isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

[2] The microsatellite profile represents the multilocus genotype observed for each isolate. Each different allele in a loci is allocated a number. All eleven loci are scored in this manner and when combined, a profile is created for each isolate. Isolates with the same microsatellite profile have the same genotype.

Table 2: Primers designed from inserts of plasmid clones.

Primer	Primer sequence	Tm[1]	Ta[2]	Banding pattern
AG1	CGG GGA TGC TGT TGT CTC G	58.8	58	Polymorphic
AG2	GTT TCT CGA CTT CCA GGC CC	61.4		
AG3	CGT CGC ACG CTC ACA TCT AC	61.4	58	No amplification
AG4	GAC AGA GTC TCT GGA CAG	61		
AG5	CCT CGT TGA CTA GCA CGT CC	61.4	58	No amplification
AG6	CAC CTC AGC GTA ACG CGA G	59.4		
AG7	GGG GCG GTG GTG CAA TTG TC	61	56	Polymorphic
AG8	CGA GAC AGC AAC ACA AGC CC	61.4		
AG9	GAG CAG TTA AGC GGC GAT TC	59.4	55	Stutter bands
AG10	CGT CCG ATA GTA ACA GTC GTG	59.8		
AG11	GCA TCT CTT TGC CAA GCA AGC	59.8	55	Monomorphic
AG12	CCA TTA ATG CCA GCG GCA AC	59.4		
AG13	GGA GCA GCA ACT GGA GCA AC	61.4	58	Monomorphic
AG14	CCC CCT AGA CAG CAT ATC C	58.8		
AG15	GGA TAG CAG CGA CAA GGA CC	61.4	62	Polymorphic
AG16	CTT GAC CGA CCT GCC GAT TG	61.4		
AG17	CGG CCC TGC CAA CGG ATG	62.8	62	Polymorphic
AG18	GTC GGT GGT GGA GAC GGT C	63.1		
AG19	GGC GGC GCG CAA GAT CGC	65.1	60	Monomorphic
AG20	GCA GCC CGG GCC ACT TGC	65.1		
AG21	CGT GGC CGG AGT AAA TGA CTG	61.8	56	No amplification
AG22	GTC GGA AAC GGG TCA GTC AG	61.4		
AG23	GCT GAC ATG TCC GCA CTG C	61	56	Monomorphic
AG24	GCA TTC TGG CGC GTT GAG G	61		
AG27	CTA TTG CGG TCA CGT TCA CG	59.4	58	Monomorphic
AG28	CTC ATG GAA GGA AAG ACC GTG	59.8		
CF1	GGT GCA TGC GTG TGC ATA TGC	61.8	56	No amplification
CF2	CGC CAA GCT ATT TAG GTG ACA C	60.3		
CF5	CAT GGG CAT GCC TAG CCT TG	61.4	62	Polymorphic
CF6	GAC CAA AGA TGG TGG CGA GC	61.4		
CF7	CCG TGT AGG TCA CTA GGG TC	59.4	55	Monomorphic
CF8	CTA GCA AGC TGA CTG ACT GG	59.4		
CF9	GAT GTC GGT TAG GAC GGG	58.2	52	Monomorphic
CF10	CCC ACG GGA TCA ACT TGG	58.2		
CF11*	GGG ACG AAA CTG GAG CGT CT	61.4	60	Polymorphic
CF12	CTC CCA AAC TCC ATG CTC TTG	59.8		
CF13*	GAT CGA TCG GCA GAC CGA TAC	61.8	64	Polymorphic
CF14	GGA ACT CTG ATG CCT CCA GTG	61.8		
CF15*	CAG GGA CTA GGG TCT GCC AG	63.5	60	Polymorphic
CF16	CGT TTG CAA GGC AAG GCA GC	61.4		
CF17*	CGA GCC AAG ACG TTC ATT GAA G	60.3	64	Polymorphic
CF18	GAA ACC GAG AGT CAT CGT CC	59.4		
CF19*	GCA GCT CTA GTC GTC AGA TAC	59.8	55	Monomorphic
CF20	CTA GAG GCC AGC TCT TGT GC	61.4		
CF21*	GCA CTA CGA GAA TAG AAT GCA G	58.4	60	Polymorphic
CF22	GCG TTG AAA GAT GTG GCG TG	59.4		
CF23	CAG GGA ATT CCC GAT GGC AG	61.4	60	Polymorphic
CF24	CAT GAT CGA CAA GGG CGC TG	61.4		

[1] Tm (° C) = melting temperature

[2] Ta (° C) = annealing temperature

* Primers designed from genome walking.

Table 3: Core sequences and allelic properties of polymorphic primers designed for *C. fimbriata*.

Primer	Floures-cent label	Core sequence	Allelic range	No of alleles	Alleles
AG 1/2	FAM	$(T)_7C(T)_2CGC(T)_4(CTTT)_2GC(T)_4C(T)_3C(T)_2G(T)_4(CTT)_2$	255 - 266	4	255,263,265,266
AG 7/8	TET	$(TC)_{21}(TTC)_2$	284 - 304	10	284,285,286,288,289,290, 298,299,301,304
AG 15/16	FAM	Regions rich in A interrupted by C and G	272 - 276	3	272,274,276
AG 17/18	FAM	$(T)_5(C)_2(CT)_2T(CTT)_6(T)_2(C)_3TC(T)_3$	304 - 309	5	304,305,307,308,309
CF 5/6	TET	$(TGC)_{11}$	359 - 385	9	359,365,366,368,369,371, 377,380,385
CF 11/12	TET	$CA(AC)_7GC(AC)_2(N)_x(G)_8$	216 - 230	6	216,217,218,219,222,230
CF 13/14	TET	$(T)_5(N)_x(A)_7(N)_x(C)_{11}(N)_x(AGCAC)_5$	402 - 415	7	402,403,405,406,410,414, 415
CF 15/16	FAM	$(CT)_5(N)_x(CT)_3(N)_x(CT)_3$ sequence rich in T	218 - 267	6	218,240,248,250,254,267
CF 17/18	TET	$(CA)_{15}$ sequence rich in GT and T	266 - 292	8	266,267,268,271,272,277, 279,292
CF 21/22	TET	$(T)_8(N)_x(T)_6(N)_x(C)_2(T)_3C(CT)_2(CCTT)_2C(T)_3C(T)_2C(T)_4$	250 - 259	3	250,255,259
CF 23/24	TET	$TGCA(TG)_{15}$	154 - 168	4	154,156,160,168

Figure 1: Comparison of a dendogram for *Ceratocystis fimbriata* isolates produced using 11 polymorphic microsatellite loci with a phylogram produced from the ITS sequences of the same isolates. The dendogram was produced using cluster analysis of Bray-Curtis coefficient of similarity in PRIMER and the phylogram using the Heuristic search option with parsimony in PAUP 4. Bootstrap values are indicated below the branches while the number of base substitutions is indicated above the branches.

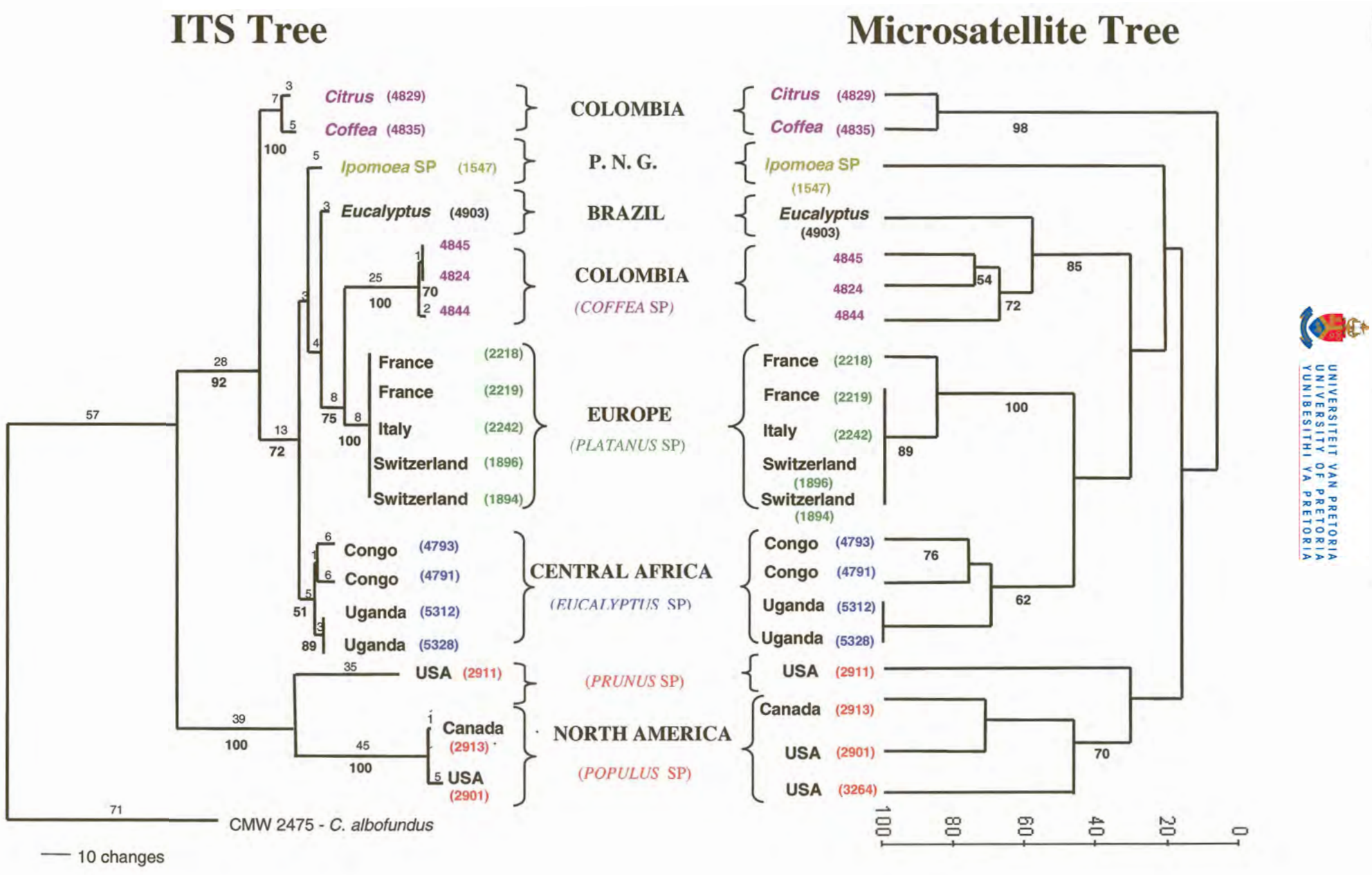

Figure 2: Sequences of *Ceratocystis fimbriata* isolates produced from primers AG 7/8, AG 17/18, CF 5/6, CF 15/16, CF 17/18 and CF 21/22.

PRIMER AG 7/8

```
CMW4829  *A*T*TAAAT*CCGGTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTTCTTCCC--TCCCG*
CMW1896  *A*G*TAAAC*CCGGTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC--------TTCTTCCCCCTCCCG*
CMW5312  *G*G*TAAAT*CCGGTCTCTCTCTCTCTCTCTCTC----------------------TTCTTCCCCCTCCCG*
CMW5328  *G*G*TAAAT*CCGGTCTCTCTCTCTCTCTCTCTC----------------------TTCTTCCCCCTCCCG*
```

PRIMER AG 17/18

```
CMW4829  *GTTTTTCCCTCTTCTTCTTCTTCTTCTTTTCCCTCTTTAAAA*G*T*
CMW4835  *GTTTTTCCCTCTTCTTCTTCTTCTTCTTTTCCCTCTTTAAAA*G*T*
CMW5312  *GTTTTTCCCTCTTCTTCTTCTTCTT---TTCCCTCTTTAAAA*A*T*
CMW1894  *GTTTTTCCCTCTTCTTCTTCTTCTT---TTCCCTCTTTAAAA*G*T*
CMW4903  *GTTTTTCCCTCTTCTTCTTCTTCTT---TTCCCTCTTTAAAA*G*A*
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PRIMER CF 5/6

```
CMW4903  *TTTT-*G*G*GTGCTGCTGCTGCTG----------------*A*CCTTCTTC------TTTC*G*
CMW5383  *TTTT-*A*A*GTGCTGCTGCTGCTGCTG---------------*A*TCGGCCCTCTTATTTTTC*G*
CMW1896  *TTTTT*A*G*GTGCTGCTGCTGCTGCTGCTGCTG---------*G*TCGGCCCTCTTATTTTTC*C*
CMW4829  *TTTTC*A*G*GTGCTGCTGCTGCTGCTGCTGCTGCTGCTG*A*TCGGCCCTCTTATTTTTC*C*
```

PRIMER CF 15/16

```
CMW4829  *C*-----------*T*A*A*CC*CTCGCTCTTG*TCTCTCTCTCT------CCCA*T*T*CCCAGTCTCTCTCG*CTCTCCCCACCTA*T*A*T*
CMW2901  *C*CCGTGAGTATTT*C*G*A*AC*----------*TCTCTCTCTCTC---------*-*G*C-----------A*-------------*C*G*C*
CMW2219  *A*-----------*T*G*G*CT*CTCGCTCTTG*TCTCTCTCTCTCT----CCCA*T*T*CCCAGTCTCTCTCG*CTCTCCCCACCTA*T*A*C*
CMW4795  *C*-----------*T*G*G*CC*CTCGCTCTTG*TCTCTCTCTCTCTCTCTCCCA*T*T*C-----------G*CTCTCCCCACCTA*T*A*T*
CMW5312  *C*-----------*T*G*G*CC*CTCGCTCTTG*TCTCTCTCTCTCTCTCTCCCA*T*T*C-----------G*CTCTCCCCACCTA*T*A*T*
```

PRIMER CF 17/18

```
CMW5328  *C*T*GAAACACACACACACACA----------T*A*A*A*
CMW4793  *C*C*CACACACACACACACACA----------T*A*A*A*
CMW1896  *C*C*CACACACACACACACACA----------T*A*G*A*
CMW2911  *A*C*CACACACACACACACACA----------T*A*A*A*
CMW4829  *C*C*CACACACACACACACACACACACACACACACAT*A*G*G*
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PRIMER CF 21/22

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CMW4822  *A*C*G*G*TAAAAG*T*A*TCCCT*GA*G*AATTAAA----------GCCTTTTTTTT-GTCCC--*TTC*C*T*G*A*G*CCT*C*C-*CT*T*T*A*A*C*
CMW4791  *A*C*G*G*TAAAAG*T*A*TCCCT*GA*G*AATCAAA----------GCCTTTT-----GTCCC--*TTC*T*C*G*A*G*CCT*C*TC*CT*T*T*A*G*C*
CMW4903  *A*C*G*G*TAAAAT*T*A*TCCCT*GA*G*AATCAAA----------GCCTTTT-----GTCCC--*TTC*T*C*G*A*G*CCT*C*CC*CT*T*T*A*G*C*
CMW4835  *A*C*G*G*TAAAAG*T*A*TCCCT*GA*G*AATCAAA----------GCCTTTTTTTTTGTCCC--*TTC*T*T*G*A*G*CCT*C*CC*CT*T*T*A*A*C*
CMW2901  *G*T*C*A*AGAAGG*C*G*TCC-T*AG*A*TATAAAGAAAAGAAAAAAAGCCTTTT-----G-CCCTG*TTC*T*C*A*G*A*C--*T*CC*GC*G*C*G*A*T*
```

* Indicates varying lengths of homologous sequence between isolates
\- Indicates absence of nucleotides within a sequence.

Figure 3: Sequence alignments (555bp) of the ITS regions of *Ceratocystis fimbriata* isolates used in this study. Dots represent bases identical to *C. fimbriata* from Papua New Guinea (CMW 1547), N indicates unknown bases and dashes represent gaps introduced to maximise alignment.

```
                                  10         20         30         40         50         60         70
Papua New Guinea  (CMW 1547)   CCATGTGTGA ACGTACC-TA TCTT-GTAGT GA-GAT-GAA T-GCTGTTTT GGTGGT---- AGGG------
France            (CMW 2219)   .......... .......-.. ....-..... ..-...-... .-........ ......---- ....------
France            (CMW 2218)   .......... .......-.. ....-..... ..-...-... .-........ ......---- ....------
Italy             (CMW 2242)   .......... .......-.. ....-..... ..-...-... .-........ ......---- ....------
Switzerland       (CMW 1896)   .......... .......-.. ....-..... ..-...-... .-........ ......---- ....------
Switzerland       (CMW 1894)   .......... .......-.. ....-..... ..-...-... .-........ ......---- ....------
Brazil            (CMW 4903)   .......... .......C.. ....-..... ..-...-... .-........ ......---- ....------
Congo             (CMW 4793)   .......... .......-.. ....-..... ..-...-... .-........ ......---- ....------
Congo             (CMW 4791)   .......... ..A....-.. ....T..... ..-...-... .-........ ......---- ....------
Uganda            (CMW 5328)   .......... .......-.. ....-..... ..-...-... .-........ ......---- ....------
Uganda            (CMW 5312)   .......... .......-.. ....-..... ..-...-... .-........ ......---- ....------
Colombia          (CMW 4829)   .......... ..A....C.. ....-..... ..-...-... .-........ ......T--- ....------
Colombia          (CMW 4835)   .......... ..A.T..C.. ....-..... ..A...-... .-........ ......T--- ....------
Colombia          (CMW 4845)   .......... .......-.. ....-..... ..-...-... .-........ ......---- ....------
Colombia          (CMW 4844)   .......... .......-.. ....-..... ..-...-... .-........ ......---- ....------
Colombia          (CMW 4824)   .......... .......-.. ....-..... ..-...-... .-........ ......---- ....------
U.S.A             (CMW 2911)   .......... ..A....CAT ....-C..A. A.-...-... A-....C... ......TTT- ....GGAA--
U.S.A.            (CMW 2913)   NNNNNNNNNN NNNNNNNN.- ....-C..TA -.-..-C... -C....C... ......TTTG ..--------
Canada            (CMW 2901)   .......... ..A....C.- ....-C..TA -.-..-C... -C....C... ......TTTG ..--------

                                  80         90         100        110        120        130        140
Papua New Guinea  (CMW 1547)   CCC--TTCT- -----GAAGG GAGGG----- --------CA CC--GCTGCC AGCAG--TAT TAGTCT-CAC
France            (CMW 2219)   ...--....- -----..... .--------- --------.. ..--...... ......--.- ......-.G.
France            (CMW 2218)   ...--....- -----..... .--------- --------.. ..--...... ......--.- ......-.G.
Italy             (CMW 2242)   ...--....- -----..... .--------- --------.. ..--...... ......--.- ......-.G.
Switzerland       (CMW 1896)   ...--....- -----..... .--------- --------.. ..--...... ......--.- ......-.G.
Switzerland       (CMW 1894)   ...--....- -----..... .--------- --------.. ..--...... ......--.- ......-.G.
Brazil            (CMW 4903)   ...--....- -----....A .....----- --------.. ..--...... ......--... ......T.G.
Congo             (CMW 4793)   ...--....- -----....A .....----- --------.. ..--...... ......--.- ......-.G.
Congo             (CMW 4791)   ...--....- -----....A .....----- --------.. ..--...... ......--.- ......-...
Uganda            (CMW 5328)   ...--....- -----....A .....----- --------.. ..--...... ......--... ......T.G.
Uganda            (CMW 5312)   ...--....- -----....A .....----- --------.. ..--...... ......--... ......T.G.
Colombia          (CMW 4829)   ...--....- -----...A. .G...G---- --------.. ..--...... ......--... A.....T...
Colombia          (CMW 4835)   ...--....- -----...A. .G...G---- --------.. ..--...... ......--... A.....T...
Colombia          (CMW 4845)   ...--....- -----..... .--------- --------.. ..--...... G.....--.- ......-.G.
Colombia          (CMW 4844)   ...--....- -----..... .T-------- --------A. ..--...... ......--.- ......-.G.
Colombia          (CMW 4824)   ...--....- -----..... .--------- --------.. ..--...... G.....--.- ......-.G.
U.S.A             (CMW 2911)   ..---A--.C CTAT-A..AT .GA..A---- ----CTTT.T --AA...A.. .A...--..- ......T-..
U.S.A.            (CMW 2913)   ...CG..-.C CTGTTA..AA .G.AAGAAGG GGGGCAAA.T .TAA...... G....CA--- ......T-..
Canada            (CMW 2901)   ...CG..-.C CTGTTA..AA .G.AAGAAGG GGGGCAAA.T .TAA...... G....CA--- ......T-..
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                                  150        160        170        180        190        200        210
Papua New Guinea  (CMW 1547)   CACTATAAA- ----CTCTTT T--ATTATTT T---CTAGA- -TTTT----- ----CATT-G CTGAGTGGCA
France            (CMW 2219)   ....G....- ----.....- ---..-.... .T--.C...- -.....T--- ----....-. ..........
France            (CMW 2218)   ....G....- ----.....- ---..-.... .T--.C...- -.....T--- ----....-. ..........
Italy             (CMW 2242)   ....G....- ----.....- ---..-.... .T--.C...- -.....T--- ----....-. ..........
Switzerland       (CMW 1896)   ....G....- ----.....- ---..-.... .T--.C...- -.....T--- ----....-. ..........
Switzerland       (CMW 1894)   ....G....- ----.....- ---..-.... .T--.C...- -.....T--- ----....-. ..........
Brazil            (CMW 4903)   ....G....- ----..... .T-..... .---.....- -.....---- ----....-. ..........
Congo             (CMW 4793)   ....G....A AAAA..... .--....C.. .---.....- -.....---- ----....-. ..........
Congo             (CMW 4791)   .........- ----..... .T-..... .---.....- -.....---- ----....-. ..........
Uganda            (CMW 5328)   ....G....A ----..... .T-..... .---.....- -.....---- ----....-. ..........
Uganda            (CMW 5312)   ....G....A ----..... .T-..... .---.....- -.....---- ----....-. ..........
Colombia          (CMW 4829)   ....G....A ----..... .T-..AT... ----..TA.- -.....---- ----....-. ..........
Colombia          (CMW 4835)   ....GG...A ----..... .TT..AT... ----..... -.....---- ----....-. ..........
Colombia          (CMW 4845)   ....G....- ----..... .T-------- ----.....--- -....CTTAA TTTC....T. ..........
Colombia          (CMW 4844)   ....G....- ----..... .T-------- ----.....--- -....CTTAA TTTC....T. ..........
Colombia          (CMW 4824)   ....G....- ----..... .T-------- ----.....--- -....CTTAA TTTC....T. ..........
U.S.A             (CMW 2911)   T..--....T T---..T..- ---....T... .TTA.....A A...----GA TTT-....-. ..........
U.S.A.            (CMW 2913)   ...--....A ----..... .--...T... .T-A.....A A...----GA TTT-....-. ..........
Canada            (CMW 2901)   ...--....A ----..... .--...T... .TTA.....A A...----GA TTT-....-. ..........

                                  220        230        240        250        260        270        280
Papua New Guinea  (CMW 1547)   T-AACTATAA AAAAA--GTT AAAACTTTCA ACAACGGATC TCTTGGCTCT AGCATCGATG AAGAACGCAG
France            (CMW 2219)   .-........ ....---... .......... .......... .......... .......... ..........
France            (CMW 2218)   .-........ ....---... .......... .......... .......... .......... ..........
Italy             (CMW 2242)   .-........ ....---... .......... .......... .......... .......... ..........
Switzerland       (CMW 1896)   .-........ ....---... .......... .......... .......... .......... ..........
Switzerland       (CMW 1894)   .-........ ....---... .......... .......... .......... .......... ..........
Brazil            (CMW 4903)   .-........ .....--... .......... .......... .......... .......... ..........
Congo             (CMW 4793)   .-........ .....--... .......... .......... .......... .......... ..........
Congo             (CMW 4791)   .-........ .....--... .......... .......... .......... .......... ..........
Uganda            (CMW 5328)   .-........ ....---... .......... .......... .......... .......... ..........
Uganda            (CMW 5312)   .-........ ....---... .......... .......... .......... .......... ..........
Colombia          (CMW 4829)   .-........ .....A-... .......... .......... .......... .......... ..........
Colombia          (CMW 4835)   .-........ .....AA... .......... .......... .......... .......... ..........
Colombia          (CMW 4845)   .-........ .....A-... .......... .......... .......... .......... ..........
Colombia          (CMW 4844)   .-........ .....A-... .......... .......... .......... .......... ..........
Colombia          (CMW 4824)   .-........ .....A-... .......... .......... .......... .......... ..........
U.S.A             (CMW 2911)   .-........ ...----... .......... .......... .......... .......... ..........
U.S.A.            (CMW 2913)   .A........ ...----... .......... .......... .......... .......... ..........
Canada            (CMW 2901)   .A........ ...----... .......... .......... .......... .......... ..........
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                                290       300       310       320       330       340       350
Papua New Guinea    (CMW 1547)  CGAAATGCGA TAAGTAATGT GAATTGCAGA ATTCAGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCT
France              (CMW 2219)  .......... .......... .......... .......... .......... .......... ..........
France              (CMW 2218)  .......... .......... .......... .......... .......... .......... ..........
Italy               (CMW 2242)  .......... .......... .......... .......... .......... .......... ..........
Switzerland         (CMW 1896)  .......... .......... .......... .......... .......... .......... ..........
Switzerland         (CMW 1894)  .......... .......... .......... .......... .......... .......... ..........
Brazil              (CMW 4903)  .......... .......... .......... .......... .......... .......... ..........
Congo               (CMW 4793)  .......... .......... .......... .......... .......... .......... ..........
Congo               (CMW 4791)  .......... .......... .......... .......... .......... .......... ..........
Uganda              (CMW 5328)  .......... .......... .......... .......... .......... .......... ..........
Uganda              (CMW 5312)  .......... .......... .......... .......... .......... .......... ..........
Colombia            (CMW 4829)  .......... .......... .......... .......... .......... .......... ..........
Colombia            (CMW 4835)  .......... .......... .......... .......... .......... .......... ..........
Colombia            (CMW 4845)  .......... .......... .......... .......... .......... .......... ..........
Colombia            (CMW 4844)  .......... .......... .......... .......... .......... .......... ..........
Colombia            (CMW 4824)  .......... .......... .......... .......... .......... .......... ..........
U.S.A               (CMW 2911)  .......... .......... .......... .......... .......... .......... ..........
U.S.A.              (CMW 2913)  .......... .......... .......... .......... .......... .......... ..........
Canada              (CMW 2901)  .......... .......... .......... .......... .......... .......... ..........

                                360       370       380       390       400       410       420
Papua New Guinea    (CMW 1547)  GGCAGTATTC TGCCAGGCAT GCCTGTCCGA GCGTCATTTC ACCACTCAA- GA-TT---AT TCT-------
France              (CMW 2219)  .......... .......... .......... .......... .........G ..C.CC---. .T--------
France              (CMW 2218)  .......... .......... .......... .......... .........G ..C.CC---. .T--------
Italy               (CMW 2242)  .......... .......... .......... .......... .........G ..C.CC---. .T--------
Switzerland         (CMW 1896)  .......... .......... .......... .......... .........G ..C.CC---. .T--------
Switzerland         (CMW 1894)  .......... .......... .......... .......... .........G ..C.CC---. .T--------
Brazil              (CMW 4903)  .......... .......... .......... .......... .........- ..C.C----. .T.-------
Congo               (CMW 4793)  .......... .......... .......... .......... .........- ..-..---.. ...TT-----
Congo               (CMW 4791)  .......... .......... .......... .......... .........- ..-..---.. ...TT-----
Uganda              (CMW 5328)  .......... .......... .......... .......... .........- ..-..---.. ...TT-----
Uganda              (CMW 5312)  .......... .......... .......... .......... .........- ..-..---.. ...TT-----
Colombia            (CMW 4829)  .......... .......... .......... .......... .........- ..--CC---- ...TTT----
Colombia            (CMW 4835)  .......... .......... .......... .......... .........- ..--CC---- ...TTT----
Colombia            (CMW 4845)  .......... .......... .......... .......... .........- ..C.CC---- -..TT-----
Colombia            (CMW 4844)  .......... .......... .......... .......... .........- ..C.CC---- -..TT-----
Colombia            (CMW 4824)  .......... .......... .......... .......... .........- ..C.CC---- -..TT-----
U.S.A               (CMW 2911)  .......... .......... .......... .......... .........- ..--.AAC-- .T.TT-----
U.S.A.              (CMW 2913)  .......... .......... .......... .......... .........- ..AA.-AC-- .T.TTTTTTT
Canada              (CMW 2901)  .......... .......... .......... .......... .........- ..AA.-AC-- .T.TTATTTT
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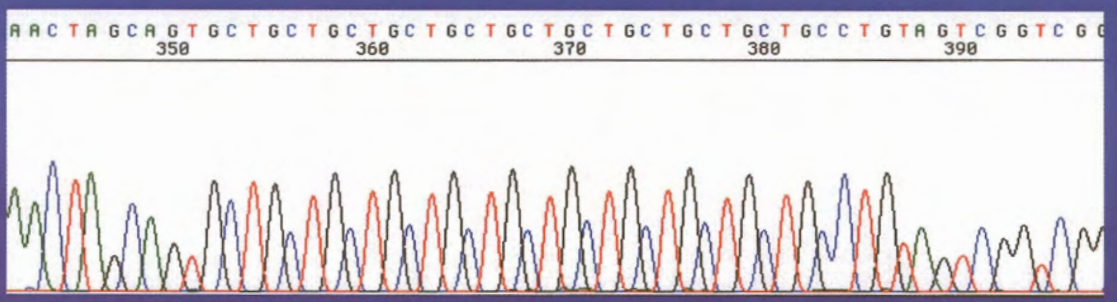
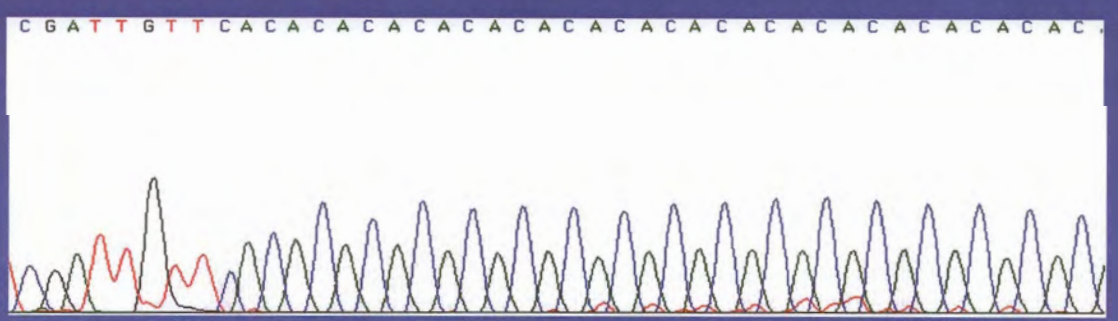
                                  430        440        450        460        470        480        490
Papua New Guinea  (CMW 1547)   --TTCTTGGC GTTGGAGGTC CTGTTCT--- --------CC CCTGAACAGG CCGCCGAAAT GCATCGGCTG
France            (CMW 2219)   -G..... ..... ..... .....--- --------.. ..... ..... ..... .T.....
France            (CMW 2218)   -G..... ..... ..... .....--- --------.. ..... ..... ..... .T.....
Italy             (CMW 2242)   -G..... ..... ..... .....--- --------.. ..... ..... ..... .T.....
Switzerland       (CMW 1896)   -G..... ..... ..... .....--- --------.. ..... ..... ..... .T.....
Switzerland       (CMW 1894)   -G..... ..... ..... .....--- --------.. ..... ..... ..... .T.....
Brazil            (CMW 4903)   -G..... ..... ..... .....--- --------.. ..... ..... ..... .T.....
Congo             (CMW 4793)   -C..---... ..... ..... .....--- --------.. ..... ..... ..... .T.....
Congo             (CMW 4791)   -C..---... ..... ..... .....--- --------.. ..... ..... ..... .T.....
Uganda            (CMW 5328)   -C..---... ..... ..... .....--- --------.. ..... ..... ..... .....
Uganda            (CMW 5312)   -C..---... ..... ..... .....--- --------.. ..... ..... ..... .....
Colombia          (CMW 4829)   -G..... ..... ..... .....--- --------.. ..... ..... ..... .T.....
Colombia          (CMW 4835)   -G..... ..... ..... .....--- --------.. ..... ..... ..... .T.....
Colombia          (CMW 4845)   -G..... ..... ..... .....--- -------C.. ..... ..... ..... .T.....
Colombia          (CMW 4844)   -G..... ..... ..... .....--- -------C.. ..... ..... ..... .T.....
Colombia          (CMW 4824)   -G..... ..... ..... .....--- -------C.. ..... ..... ..... .T.....
U.S.A             (CMW 2911)   --C.T--..T ..... ..... .....GTT TTTTTTTTAT A..... ..... ..... .T.....
U.S.A.            (CMW 2913)   TTC.T--..T ..... ..... .....TTA ---------- -....G.... ..... ..... .T.....
Canada            (CMW 2901)   TTC.T--..T ..... ..... .....TTA ---------- -....G.... ..... ..... .T.....

                                  500  510        520        530        540        550
Papua New Guinea  (CMW 1547)   TTA----TAC TTGCC-AACT CCCCTGTGTA GTATAAAA-T TTCT-AATT TTTACACTTT  GAAGT
France            (CMW 2219)   ...----... .....-.... ..........  .........-. ....--.... ..........  .....
France            (CMW 2218)   ...----... .....-.... ..........  .........-. ....--.... ..........  .....
Italy             (CMW 2242)   ...----... .....-.... ..........  .........-. ....--.... ..........  .....
Switzerland       (CMW 1896)   ...----... .....-.... ..........  .........-. ....--.... ..........  .....
Switzerland       (CMW 1894)   ...----... .....-.... ..........  .........-. ....--.... ..........  .....
Brazil            (CMW 4903)   ...----... .....-.... ..........  .........-. ....--.... ..........  .....
Congo             (CMW 4793)   ...----... .....-.... ..........  .........-. ....--.... ..........  .....
Congo             (CMW 4791)   ...----... .....-.... ..........  .........-. ....--.... ..........  .....
Uganda            (CMW 5328)   ...----... .....-.... ..........  .........-. ....--.... ..........  .....
Uganda            (CMW 5312)   ...----... .....-.... ..........  .........-. ....--.... ..........  .....
Colombia          (CMW 4829)   ...----... .....C.... ..........  .........-. ....--.... ..........  .....
Colombia          (CMW 4835)   ...----... .....C.... ..........  .........-. ....--.... ..........  .....
Colombia          (CMW 4845)   ...----... .....-.... ..........  .........-. ....--.... ..........  .....
Colombia          (CMW 4844)   ...----... .....-.... ..........  .........-. ....--.... ..........  .....
Colombia          (CMW 4824)   ...----... .....-.... ..........  .........-. ....--.... ..........  .....
U.S.A             (CMW 2911)   ...----... .....-.... ..........  ....T...A.  ..A.TT--.  ..-C.......  .....
U.S.A.            (CMW 2913)   ...----... .....-.... ..........  ...CT...A.  ..A.TT--.  ..-C.......  .....
Canada            (CMW 2901)   ...----... .....-.... ..........  ...CT...A.  ..A.TTTT.  ---C.......  .....
```

CHAPTER 3



Population structure of *Ceratocystis fimbriata* from Congo, Colombia and Uruguay determined using microsatellite markers



ABSTRACT

Ceratocystis fimbriata is a haploid ascomycete, pathogenic on many different hosts. The fungus has a worldwide distribution, including all continents except Antarctica. Although *C. fimbriata* has been known for over a century, very little is known regarding the population biology or origin of this important pathogen. In this study, we used 11 polymorphic PCR-based microsatellite markers to examine the population structure and genetic diversity for three different populations of *C. fimbriata*. Populations consisting of 32 isolates from each of Congo and Colombia and 22 from Uruguay were studied. High genetic diversities for all populations were observed. Colombia had the most diverse population including 82 % of all alleles and 53 % of the unique alleles. Genetic differentiation between the three populations was great (G_{ST} = 0.39) and minimal gene flow was observed (Nm = 0.77). I_A , PTLPT, and linkage disequilibrium tests to determine mode of reproduction, showed little evidence for recombination within populations. *C. fimbriata* appears to reproduce primarily without outcrossing. UPGMA dendrograms based on microsatellite distance showed that the Colombian population was more distantly related to the Congo and Uruguay populations than the latter were to each other. Some of the groups in the Colombian and Uruguay populations were genetically similar to those for isolates from the Congo. Results suggest that the African isolates of *C. fimbriata* originated in Latin America. Moreover, evidence indicates that Latin America is most likely a center of origin for *C. fimbriata*.

INTRODUCTION

Ceratocytis fimbriata Ellis & Halst., is an ascomycete that is an important pathogen on a wide range of plants, often resulting in large scale losses due to disease (Kile 1993). Hosts include agricultural crops such as sweet potato tubers (*Ipomoea batatas*) and woody plants including *Eucalyptus* and *Acacia* trees (Halsted & Fairchild 1891, Ribeiro 1985, Roux *et al.* 2001). In Colombia, for example, this pathogen causes major losses to the production of coffee (*Coffea arabica*) (Pontis 1951). Other agricultural crops in this country such as *Citrus* spp., *Crotalaria* sp., *Inga* spp., *Mangifera indica* and *Theobroma cocao* are also severely affected by *C. fimbriata* (Rincon 1983, Nieto 1991, Buritica 1994, Mourichon 1994). The disease symptoms produced by *C. fimbriata* vary, depending on host, and include canker-stain, vascular wilts and root/tuber rots (Pontis 1951, Hinds 1972, Kile 1993).

Although *C. fimbriata* has been known for more than a century (Halsted & Fairchild 1891), very little is known regarding the population biology or origin of this pathogen. *Ceratocystis fimbriata* f.sp. *platani* is thought to be native to North America (Walter *et al.* 1952) and to have spread to Europe during the Second World War (Anonymous 1986) causing serious outbreaks of canker stain disease on *Platanus* spp. (Panconesi 1999). The presence of *C. fimbriata* in Asia was first noted in 1900 when the fungus caused a serious disease of coffee, in Java (Zimmerman 1900). Subsequently, the same disease of coffee was reported from Colombia in 1932 (Muchovej 1978, Pontis 1951). Despite its worldwide distribution, *C. fimbriata* has predominantly been reported from Central and South America (McCracken & Burkhardt 1977) and is especially common on a wide variety of hosts in different parts of Latin America (Beeley 1929, Costa 1935, Sharples 1936, Iton 1959, Ribeiro 1985, Barnes Chapter 5). The pathogen was discovered in Australia for the first time in 1987 on rotting *Syngonium* spp. in nurseries but is not recognized as a serious pathogen in that country (Walker *et al.* 1988, Vogelzang & Scott 1990). *C. fimbriata* was not reported from Africa until 1998, when it was discovered causing severe wilt disease on *Eucalyptus* clones in the Republic of Congo (Roux *et al.* 1999). Since then, it has also been reported from Uganda on *E. grandis* (Roux *et al.* 2001).

Although *C. fimbriata* was first described from infected sweet potato in the United States of America (U.S.A.) (Halsted & Fairchild 1891), it is not known whether the pathogen is a

native to the U.S.A. Since its occurrence is so widespread, especially in Latin America, and on hosts not present in the U.S.A, Latin America appears as a more likely area of origin. Studying migration patterns and levels of diversities within *C. fimbriata* populations, it would be possible to gain insight regarding the probable areas of origin and spread of this important pathogen.

Molecular markers, especially microsatellites, or simple sequence repeats, represent ideal tools for studying population genetics of fungi (Tenzer *et al.* 1999, Santini & Capretti 2000, Bucheli *et al.* 2001, Burgess *et al.* 2001). These co-dominant markers allow for the identification of multiple polymorphic alleles at any given locus. By comparing multilocus genotypes, identification of individuals within a morphologically identical population is possible. The ability to accurately identify genotypes thus, makes it possible to study relationships between different populations of the same species.

A set of PCR-based microsatellite markers, specifically designed for population studies, has recently been developed for *C. fimbriata* (Barnes *et al.* 2001a, Chapter 2). The aim of the present investigation was to compare genetic diversities in three different populations of *C. fimbriata* from the Republic of Congo (Africa), Colombia and Uruguay (Latin America), using these microsatellite markers. The genetic diversities, mode of reproduction, population structure, gene flow, as well as the relationships between the populations were thus considered.

MATERIALS AND METHODS

Fungal isolates

In this study we utilised populations of *C. fimbriata* from three different countries. The isolates representing the Colombian population were collected from infected soil and coffee plants in coffee plantations. An additional two isolates were from citrus plants surrounding these coffee plantations (Table 1). The Republic of Congo population was comprised of isolates from individual trees in two different *Eucalyptus* plantations. Fifteen isolates were collected from diseased coppice stems of hybrid *Eucalyptus urophylla* S.T. Blake X *E. pellita* F. Muell. (UP) trees in the Kissoko plantation and seventeen from *E. grandis* Hill ex. Maid. X *E. territicornis* Sm. (GT) trees in the Tchittanga plantation (Roux

et al. 1999) (Table 1). The Uruguay population was comprised of 22 isolates collected from individual diseased *E. grandis*, in a single plantation, near the city of Rivera (Chapter 5) (Table 1).

All isolates were grown on 2 % malt extract agar (MEA, Biolab) supplemented with thiamine (100 mg/liter) (Centaur Laboratories). For DNA extraction, an ascospore mass from a single perithecium of each culture was incubated for 2 weeks at 25 °C in an Erlenmeyer flask containing 50 ml malt extract broth (2 %, Biolab). After sufficient growth, the mycelium was collected by filtration, freeze-dried, crushed and the DNA was extracted using the method previously described by Barnes *et al.* (2001b).

Microsatellite PCR amplification

Eleven microsatellite primers, designed for *C. fimbriata* (Barnes *et al.* 2001a, Chapter 2), were used in PCR reactions, for all isolates. PCR reaction mixtures, and primer conditions for these isolates were the same as those described previously (Barnes *et al.* 2001a, Chapter 2). The reverse primer, for primer pair CF 15/16, was replaced with a second primer, CF16.b 5'(CGT TTG CAA GGC AAG GCA GC) 3'. The expected size of the PCR amplicon, therefore, changed from 250 to 480 bp. This was done in order to increase the efficiency of multiplexing, during genescan analysis (Table 2). PCR products were not purified.

Genescan analysis

To facilitate multiplexing of the genescan analysis, PCR products were arranged according to the approximate size of the PCR product and type of fluorescent label attached to the primer (Table 2). Samples were prepared and analysed in the same manner as previously described (Chapter 2) using GeneScan® 2.1 analysis software (Perkin Elmer Corp.) and Genotyper® 3.0 (Perkin Elmer Corp.). Samples that showed ambiguity in results were re-amplified and re-analysed.

Population analysis

Genetic variation

Two measures of genetic variation were calculated. Allele frequencies (Table 3), were determined for each locus and subsequently used to determine the gene diversity of the

whole population according to the formulae, $H = 1 - \sum_k x_k^2$, where x_k is the frequency of the k^{th} genotype (Nei 1973) (Table 5). Values tending towards 1 indicate diverse populations and 0, uniform populations. Each allele at a locus was assigned an alphabetical character according to its size (Table 3). The alphabetical character for each of the eleven loci was combined to produce the multilocus genotype (Table 4). The multilocus genotype was used to calculate the genotypic diversity of the populations using the formulae $G = 1/\sum[fx\ (x/n)^2]$, where n is the sample size, and f_x is the number of genotypes occurring x times in the sample (Stoddart & Taylor 1988). To compare the genotypic diversities between populations, the maximum percentage of diversity, obtained in each population was calculated $\hat{G} = G/N*100$ where N is the population size (MacDonald *et al.* 1994). A t-test, with a significance level of $P \geq 0.05$, was used to determine if the difference between the percentages of maximum diversity between populations was significant (Chen *et al.* 1994).

Genetic distance and population structure

The genetic distance of the three populations was calculated using total nucleotide length for each allele. A distance matrix, using the measure $\delta\mu^2$ (Goldstein *et al.* 1995), was constructed in the program MICROSAT (http://human.stanford.edu/microsat). A UPGMA (Unweighted Pair-Group Method with Arithmetic mean) dendrogram based on the distance matrix was constructed in MEGA version 2.1 (Kumar *et al.* 2001). Each individual population was analyzed using all the available isolates. Two dendrograms for the combined populations were constructed. The first of these was to show genetic distance between all the isolates studied. In this case, all the duplicate genotypes were removed. The second dendrogram was constructed to reflect the genetic distance between the three populations.

The amount of genetic differentiation (G_{ST}) (Nei 1973), between populations was calculated in POPGENE version 1.31 (http://www.ualberta.ca/~fyeh/fyeh). This measure reflects the amount of sub-structuring occurring between populations. In general, a value in the range of 0 to 0.05 indicates little, 0.05 to 0.15 moderate, 0.15 to 0.25 great, and above 0.25 indicates very great, genetic differentiation (Wright 1978).

The amount of gene flow (Nm) between the populations was calculated from the estimate of G_{ST} (Slatkin & Barton 1989) where $Nm = 0.5(1 - Gst)/Gst$ (McDermott & McDonald

1993). Populations that are completely genetically isolated will have Nm values of zero, and G_{ST} values of one (Hartl & Clark 1989).

Reproduction

The mode of reproduction for each population was determined using the index of association (I_A) (Taylor *et al.* 1999a) and the parsimony tree length permutation test (PTLPT) (Burt *et al.* 1996). For each isolate, the output data obtained from the genescan analysis were organised into a spreadsheet according to the presence or absence of each allele at all eleven loci. The resulting matrix, including multilocus genotypes, was used in further computations. The PTLPT was calculated from PAUP version 4 (Phylogenetic Analysis Using Parsimony [*and other methods]) (Swofford 1998), and the I_A using Multilocus (http://www.bio.ic.ac.uk/evolve/software/multilocus/). Each population was analysed separately. For these analyses, data for 1000 randomly recombining populations for the I_A test, and 500 for the PTLPT test, was simulated and a distribution range for these recombining populations, produced. The value of the observed data (actual population being studied), was compared to this distribution range to determine whether recombination is occurring (Taylor *et al.* 1999a).

The amount of non-random association amongst polymorphic loci or the gametic disequilibrium was also calculated (Hartl & Clark 1989). The two-locus LD option in POPGENE was used to calculate the estimated gametic equilibria between all pairs of polymorphic loci (Weir 1979) (Table 5). Probability values were set at $P < 0.05$.

RESULTS

Genescan analysis

The eleven microsatellite markers produced a total of 58 alleles across all 86 isolates in the three populations studied. Individually, Colombia had the greatest number of alleles (47) followed by Congo (33) and Uruguay (22). Most alleles present in the Congo and Uruguay population (82 %), were also present in the Colombian population (Table 3). Monomorphic alleles were only present in the Uruguay population at locus A15/16, allele size 247 and at locus C21/22, allele size 250 (Table 3). Unique, or "private", alleles were found in all three

populations. Congo had 2 unique alleles over 2 loci, Colombia had 9 over 4 loci, and Uruguay had 6 unique alleles over 5 loci (Table 4).

Population analysis

Genetic variation

In most cases, one allele dominated in frequency compared to the other alleles at the same locus in the populations studied. Of these, Congo and Uruguay shared the same dominant allele at a specific locus, 27 % of the time. Congo and Colombia shared the same dominant allele in only 9 % of the cases (Table 3). Based on allele frequencies, all three populations had a high level of gene diversity. Colombia had the highest gene diversity (0.48), followed by Congo (0.33) and Uruguay (0.31) (Table 5). From a total of 86 isolates used in this study, there were 51 different genotypes for *C. fimbriata*. Although there were isolates with the same genotype (clones) within a population, none of the same genotypes were found between populations. The level of genotypic diversity amongst the populations was relatively high (Table 5), with the Colombian population having the highest value. However, if this was corrected for sample size, Uruguay had the greatest maximum level of genotypic diversity at 47.83 % while Colombia had 44 % and Congo, 23.88 % (Table 5).

Genetic distance and population structure

UPGMA dendrograms based on microsatellite distance separated the Congo populations into two lineages. These lineages were clearly defined by means of geographical location and host range. All the isolates collected from the Tchittanga plantation were distinct from the isolates collected at Kissoko plantation (Figure 1). The Colombian population could also be separated into two groups (Group 1 and Group 2), with substantial genetic distance between them (Figure 2). The same was true for the Uruguay population, which was highly structured with isolates residing in three groups (Figure 3). G_{ST} values confirmed this high genetic differentiation with values as high as 0.4 between Colombia and Uruguay (Table 5).

When all genotypes of all the isolates of *C. fimbriata* were pooled and analyzed collectively, the dendrogram was structured into two major groups. Most of the groups formed in the individual population analysis were retained in the combined analysis

(Figure 4). The most obvious retention of isolates was Group 1 of the Colombian population. This group was unique and comprised essentially the entire second group within the dendrogram, with the exception of one isolate from Congo. The second Colombian group (Group 2), was mainly interspersed amongst the isolates from Congo, with very little genetic difference. Group 1 of the Uruguay population was also separated from all other isolates. Group 2 and 3, although distinct, were not as strongly isolated and had closest associations with isolates from Congo (Figure 4).

The *C. fimbriata* isolates considered in this study had a high level of substructure with a G_{ST} value of 0.39 (Table 5). When only the distances between populations were analysed, Congo and Uruguay were most similar, and distantly separated from the Colombian population (Figure 5). The amount of gene flow (Nm), between populations (overall Nm = 0.77), was relatively low (Table 5).

Reproduction

In both the I_A and PTLPT tests, all the observed values for the Congo, Colombian and Uruguay populations fell outside the distribution range of a recombining population ($P < 0.001$ for I_A tests; $P = 0.002$ for PTLPT tests) (Figure 6). The hypothesis that the fungus is predominantly clonal is thus supported in the case of *C. fimbriata*. This observation is validated from results obtained from the linkage disequilibrium analysis. The amount of fixation of polymorphic loci (LD) in the populations ranged from 20 to 27 %, which is relatively high. A much lower level of linkage disequilibrium is expected for populations that are randomly mating.

The Colombian population had the greatest level of linkage disequilibrium, yet it also had the highest gene diversity. The I_A and LD tests were, therefore, calculated separately for each of Group 1 and Group 2 in the Colombian population. Results provided evidence for recombination in both smaller groups in I_A analyses (Figure 7) and in the amount of linkage disequilibrium (Group 1 = 2.22 %, Group 2 = 3.4 %). The G_{ST} value between the sub-populations was high (0.5), indicating a large amount of genetic differentiation between the two populations. The populations are also genetically isolated with little or no gene flow between them (Nm = 0.48)

DISCUSSION

To the best of our knowledge, this study is the first to consider population diversity in *C. fimbriata* using co-dominant DNA-based markers. High levels of genetic diversity were observed within populations of *C. fimbriata* from the three countries studied. Furthermore, the populations were highly structured with little evidence of gene flow.

Our results showed that the Colombian population of *C. fimbriata* was the most genetically diverse. This could be attributed to the population containing almost all the alleles present in the other populations and also containing the highest number of genotypes. The two very distinct groups observed within this population using microsatellites, were previously observed in analyses of sequence data from the ITS region of the ribosomal DNA operon (Barnes *et al.* 2001a). These two groups appear to be reproductively isolated with little, or no gene flow between them. There was, however, evidence for recombination within the two groups based on I_A and linkage disequilibrium analyses. Based on large genetic distances and reproductive isolation, we believe that these two distinct lineages of *C. fimbriata* from Colombia, constitute discrete populations that have undergone recent speciation or are, at best 'sibling species'. In his definition of the term "sibling species", Brasier (1993), defined these species as "more likely to be identified by population, genetical and molecular methods than by traditional morphological taxonomy". In most cases, these species show no morphological difference, but exhibit strong reproductive isolation.

Recent studies with other fungi have shown that cryptic species, which are not morphologically distinguishable, can be differentiated using multiple gene genealogies (Geiser *et al.* 1998, Taylor *et al.* 1999a, Taylor *et al.* 1999b, Koufopanou *et al.* 2001). A population study of *Aspergillus flavus* from Australia, for example, signified the existence of a species barrier in the population (Geiser *et al.* 1998). The use of five genes in a population of *Coccidiodes immitis* (Koufopanou *et al.* 2001) provided evidence for outcrossing in what is believed to be a clonal pathogen and showed subdivision of the population into two reproductively isolated groups. Although we have strong evidence to support the view that the two populations of *C. fimbriata* from Colombia probably represent discrete taxa, formal descriptions of these taxa awaits completion of multiple gene genealogies to confirm this hypothesis.

Of the three populations of *C. fimbriata* studied, the Uruguay population had the second highest number of unique alleles. The genotypic diversity value adjusted for sample size (maximum genotypic diversity), however, indicated this population to have a higher level of genetic diversity than the Colombian population. It is possible that the level of genetic diversity within this population was underestimated due to lower sample sizes compared to the other populations. Although the population was highly structured into three clonal lineages, a larger sample of isolates would be required to determine whether these groups are reproductively isolated.

Very little differentiation was found within the Republic of Congo population of *C. fimbriata*. The sensitivity of the markers, however, made it possible to distinguish between isolates collected from the two plantations sampled, dividing the population into two lineages. Genetic distance analysis showed close relationships between some of the Congo isolates with those from both Colombia and Uruguay. This suggests a Latin American origin for *C. fimbriata* isolates in Africa. A similar study on *Fusarium cicinatum* Nirenberg & O'Donnell indicated a southeastern U.S.A. population as the source of origin for the populations in California and Japan based on common genotypes producing patterns of relatedness (Milgroom 1994, Wikler *et al.* 2000). A single introduction of a large number of genotypes or multiple introductions of genotypes into the Republic of Congo, might account for the high level of genetic diversity within this essentially clonal population. The level of gene flow (Nm), between the Congo population and those in South and Central America was, however, relatively low (0.77), but is not surprising considering the geographical barrier between populations.

Host specialization appears to be an important factor influencing the evolution of *C. fimbriata*. This view emerges from the fact that the Congo and Uruguay populations from *Eucalyptus* spp., were genetically more similar to each other than they were to the Colombia population from *Coffea* sp. This is despite the fact that Colombia and Uruguay are geographically closer to each other than they are to Congo. Host specialization in *C. fimbriata* has been suggested in a number of previous studies based on pathogenicity (Pontis 1951, Walter *et al.* 1952, Webster & Butler 1967, Leather 1966, Kojima *et al.* 1982) and phylogenetic relationships (Baker *et al.* 2000, Santini & Capretti 2000, Barnes *et al.* 2001a). Our findings add substantially to the view that *C. fimbriata* probably represents a number of taxa, and that host specialisation is a major factor defining these units.

Results of this study show that all the populations of *C. fimbriata* have high levels of gene and genotypic diversity, but simultaneously have many loci that are in gametic disequilibrium. These high levels of genetic diversity in the populations indicate that sexual outcrossing is occurring within them. In contrast, large numbers of loci in gametic disequilibrium suggests clonal reproduction in the populations. This result might appear contradictory but has also emerged from studies with other fungal populations. For example, a population of *Erysiphe graminis* f.sp. *hordei* (Egh) from France, was shown to be highly diverse (65 - 83 % maximum diversity) while 20 % of the loci were in linkage disequilibrium, indicating that the population is clonal (Brygoo *et al.* 1998). *C. fimbriata* is a homothallic fungus, thus capable of producing progeny, after meiosis, that are genetically identical to parent strains. It is also possible that asexual propagules contribute to spread, although the relative importance of sexual versus asexual reproduction in nature is not known. It is, therefore, not surprising that most tests in this study show evidence of clonality. A limited level of recombination was, however, observed within populations, particularly from Colombia. New genotypes are formed in this way, apparently increasing the overall genetic diversity of the pathogen population.

In a study of the population diversity of *C. fimbriata* f.sp. *platani* from Italy, Santini & Capretti (2000) showed that the fungus was clonal, sharing identical genotypes with isolates from the USA. This led the authors to conclude that *C. fimbriata* f.sp. *platani* in Europe originated in the USA and has subsequently reproduced clonally. Results of the present study indicate a Latin American origin for the African (Congo) population of *C. fimbriata*. Moreover, the high levels of genetic diversity for Latin American populations of the fungus suggest that the center of origin for *C. fimbriata* might be in Latin America. In this regard, it would be interesting to compare allele frequencies and diversities for a USA population of *C. fimbriata* with those from Latin America. This is especially since Walter *et al.* (1952) suggested that *C. fimbriata* f.sp. *platani* is native to the USA.

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Table 1: Isolates of *Ceratocystis fimbriata* from Congo, Colombia and Uruguay used in this study.

Isolate numbers*	Country of origin	Host
CMW 4748	Congo	*Eucalyptus* (UP)**
CMW 4781 - 4782	"	"
CMW 4785 - 4788	"	"
CMW 4794 - 4810	"	"
CMW 4769	"	*Eucalyptus* (GT)***
CMW 4783 - 4784	"	"
CMW 4789 - 4793	"	"
CMW 4811 - 4713	Colombia	*Coffea arabica*
CMW 4817 - 4818	"	"
CMW 4820	"	"
CMW 4822	"	"
CMW 4824 - 4825	"	"
CMW 4827 - 4828	"	"
CMW 4830 - 4836	"	"
CMW 4838 - 4841	"	"
CMW 4843	"	"
CMW 4821	"	Coffee soil
CMW 4823	"	"
CMW 4837	"	"
CMW 4842	"	"
CMW 4844 - 4846	"	"
CMW 4826	"	*Citrus*
CMW 4829	"	"
CMW 7381	Uruguay	*Eucalyptus grandis*
CMW 7383	"	"
CMW 7385	"	"
CMW 7387	"	"
CMW 7389	"	"
CMW 7508	"	"
CMW 7510	"	"
CMW 7512	"	"
CMW 7514	"	"
CMW 7516	"	"
CMW 7518	"	"
CMW 7520	"	"
CMW 7522	"	"
CMW 7524	"	"
CMW 7764 - 7771	"	"

* Isolates of *C. fimbriata* are maintaind in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
** *Eucalyptus urophylla* X *E. pellita* (UP) from Kissoko plantation
*** *E. grandis* X *E. territicornis* (GT) from Tchittanga plantation

Table 2: Organization of PCR products for each isolate amplified with eleven microsatellite makers for Genescan analysis. Three lanes on a polyacrylamide gel were used for each individual isolate screened in the population.

	Fluorescent label	Allele size (bp)
Lane 1		
CF 21/22	TET	250 - 259
AG 17/18	FAM	304 - 309
CF 5/6	TET	359 - 385
Lane 2		
CF 23/24	TET	154 - 168
CF 11/12	TET	216 - 230
CF 17/18	TET	304 - 309
CF 13/14	TET	402 - 415
Lane 3		
AG 1/2	FAM	255 - 266
AG 15/16	FAM	272 - 276
AG 7/8	TET	284 - 304
CF 15/16b	FAM	480 - 500

Table 3: Alleles, genotype configuration for each allele and allele frequencies for each isolate present at eleven microsatellite loci.

Locus	*Allele	**Genotype configuration	***Allele frequencies Congo	Colombia	Uruguay
A1/2	255	A	-	0.13	-
	263	B	0.53	0.81	0.05
	264	C	0.13	-	0.91
	266	D	0.03	-	-
	265	E	0.31	0.06	-
	268	F	-	-	0.05
A7/8	285	A	0.25	0.03	-
	288	B	-	0.03	0.41
	289	C	0.19	0.03	-
	290	D	0.53	0.09	0.32
	300	E	-	0.31	-
	301	F	-	0.06	-
	304	G	-	0.41	-
	305	H	0.03	0.03	-
	287	I	-	-	0.27
A15/16	274	A	0.97	0.53	1.00
	276	B	0.03	0.47	-
A17/18	304	A	0.71	0.25	0.82
	305	B	0.19	0.03	-
	307	C	0.03	0.69	0.14
	308	D	-	0.03	-
	311	E	0.03	-	0.05
	313	F	0.03	-	-
CF 5/6	365	A	0.72	0.13	-
	368	B	-	0.06	0.77
	369	C	-	0.03	-
	370	D	-	0.03	-
	371	E	0.25	0.03	-
	382	F	-	0.41	-
	384	G	-	0.03	-
	385	H	0.03	0.28	-
	367	I	-	-	0.23
C11/12	217	A	0.72	0.22	0.68
	219	B	0.25	0.03	-
	222	C	0.03	0.75	-
	220	D	-	-	0.32
C13/14	402	A	-	0.03	0.18
	403	B	-	0.06	-
	405	C	0.97	0.13	-
	410	D	0.03	0.53	-
	413	E	-	0.09	-
	414	F	-	0.06	-
	415	G	-	0.09	-
	400	H	-	-	0.77
	407	I	-	-	0.05
C15.2	477	A	0.94	0.13	0.14
	480	B	0.06	0.78	-
	487	C	-	0.09	0.86
C17/18	268	A	0.72	0.22	0.36
	271	B	0.25	0.03	0.64
	279	C	0.03	0.75	-
C21/22	250	A	0.97	0.25	1.00
	255	B	0.03	0.72	-
	256	C	-	0.03	-
C23/24	156	A	0.72	0.22	0.36
	160	B	0.25	0.03	0.64
	168	C	0.03	0.75	-

* Alleles observed at each locus for all three populations of *C. fimbriata* from Congo, Colombia and Uruguay.
** Each allele in the locus is scored with an alphabetical character. The combination of these characters for each isolate across all loci will give the multilocus genotype (Table 4).
*** Allele frequencies were calculated by taking the number of times the allele was present in the population and dividing it by the total number of isolates in the population.

Table 4: Multilocus genotypes and unique alleles present in *C. fimbriata* populations from Congo, Colombia and Uruguay.

Congo population		Colombian population		Uruguay population	
Isolates	Multilocus genotype	Isolates	Multilocus genotype	Isolates	Multilocus genotype
4748	B D A A A A C A A A A	4811	B F A C F C E B C B C	7381	C I A A B A H C B A B
4769	E A A A E B C A B A B	4812	B E B C F C D B C B C	7383	C I A A B A H C B A B
4781	E D A E A A C A A A A	4813	B E B C F C D B C B C	7385	F B A A B A H C B A B
4782	B D A B A A C A A A A	4817	B G B C H C D B C B C	7387	C I A A B A H C B A B
4783	E A A A E B C A B A B	4818	B D A A A A C A A A A	7389	C I A A B A H C B A B
4784	E A A A E B C A B A B	4820	B G B C H C D B C B C	7508	B I A A B A I C B A B
4785	B D A A A A C B A A A	4821	B G B C F C D B C B C	7510	C D A A B A H C A A A
4786	B D A B A A C A A A A	4822	B G B C H C D B C B C	7512	C B A C B D A C B A B
4787	B C A A A A C A A A A	4823	B G B C F C D B C B C	7514	C B A C B D A C B A B
4788	B C A A A A C A A A A	4824	A F A A E A B C A A A	7516	C B A A B D H C B A B
4789	E A A A E B C A B A B	4825	B G A B H C D B C B C	7518	C B A A B D H C B A B
4790	E A A A E B C A B A B	4826	B G A C H C D B C B C	7520	C B A A B D H C B A B
4791	E A A A E B C A B A B	4827	B E A C F C G B C B C	7522	C D A A B A H C A A A
4792	E A A B E B C A B A B	4828	E D A A A A C A A A A	7524	C B A E B D A C B A B
4793	E A A A E B C A B A B	4829	B G A C H C D B C B C	7764	C B A A B A H C B A B
4794	C C A A A A C A A A A	4830	B G A C H C D B C B C	7765	C B A A B A H C A A A
4795	B D A B A A C A A A A	4831	A C A A A A C A A A A	7766	C D A A I A H A A A A
4796	B D A A A A C A A A A	4832	B E A A C A B C A A A	7767	C D A C I D H C A A A
4797	B D A A A A C A A A A	4833	B G A C F C G B C B C	7768	C D A A I A H A A A A
4798	B D A A A A C A A A A	4834	B G A C H C D B C B C	7769	C D A A I A H C A A A
4799	B D A A A A C A A A A	4835	B G A C A C D B C B C	7770	C I A A I A A C B A B
4800	B D A A A A C A A A A	4836	B E A C F C F B C B C	7771	C D A A B A H A A A A
4801	B D A A A A C A A A A	4837	B E A C F C F B C B C		
4802	B H B C A A C A A A A	4838	B H A D F C D B C C C		
4803	C D A B A A C A A A A	4839	B E A C F C D B C B C		
4804	C D A A A A C A A . A	4840	B E A C F C E B C B C		
4805	B D A A H C D B C B C	4841	B G A C H C D B C B C		
4806	B C A A A A C A A A A	4842	B E A C F C E B C B C		
4807	B C A A A A C A A A A	4843	B E A C G C D B C B C		
4808	C C A B A A C A A A A	4844	A B A A B A G B A A A		
4809	E D A . A A C A A A A	4845	A D A A B A A C A A A		
4810	D D A F A A C A A A A	4846	E A A A D B C A B A B		

(.) Dots represent isolates that failed to amplify with a specific primer. The shaded areas represent alleles that are unique to a particular population. In the Congo population three unique alleles were observed in three different loci. The multilocus genotype for each isolate is the combination of the alphabetical letters across all eleven loci. For example, the multilocus genotype for the Congo isolate CMW 4748 is BDAAAACAAAA.

Table 5: Population diversity parameters calculated for *C. fimbriata* isolates from Congo, Colombia and Uruguay.

	Congo	Colombia	Uruguay	Total
Population size	32	32	22	86
Number of alleles	33	47	24	58
No of unique alleles	2	9	6	17
Gene diversity[a]	0.33	0.48	0.31	0.63
Genotypic diversity[b]	7.64	14.22	10.52	
Max. genotypic diversity[c]	23.88%	44%	47.83%	
Linkage disequilibrium[d]	20,4 %	27%	19.56%	
	Congo/Colombia	**Colombia/Uruguay**	**Congo/Uruguay**	
Gst[e]	0.307	0.359	0.307	0.390
Nm[f]	1.128	0.892	1.126	0.770

[a] Nei's (1973) formula, $H = 1 - \sum_k x_k^2$, where x_k is the frequency of the k^{th} genotype.

[b] $G = 1/\sum[fx\ (x/n)^2]$, where n is the sample size, and f_x is the number of genotypes occurring x times in the sample (Stoddart & Taylor 1988).

[c] $\hat{G}$ To calculate maximum diversity, G is divided by the sample size.

[d] The two-locus LD option in POPGENE estimates gametic equilibria between pairs of loci (Weir 1979). The number of significant linkage disequilibria obtained (Congo-108, Colombia-298, Uruguay-54) is divided by [x (x-1)/2], where x = the number of alleles. This value is multiplied by 100 to obtain percentages.

[e] G_{ST} = Genetic differentiation coefficient (Nei 1973)

[f] Nm = geneflow, calculated from the estimate of G_{ST} E.g., $Nm = 0.5(1 - G_{ST})/ G_{ST}$; (McDermott & McDonald 1993, Slatkin & Barton 1989).

Figure 1: UPGMA dendrogram of the Congo population based on genetic distance calculated using Delta mu squared ($\delta\mu^2$) on total nucleotide length. The 32 isolates making up this population are sub-structured into two groups. The GT *Eucalyptus* clones from Tchittanga plantation are clearly differentiated from the UP *Eucalyptus* clones collected from Kissoko plantation.

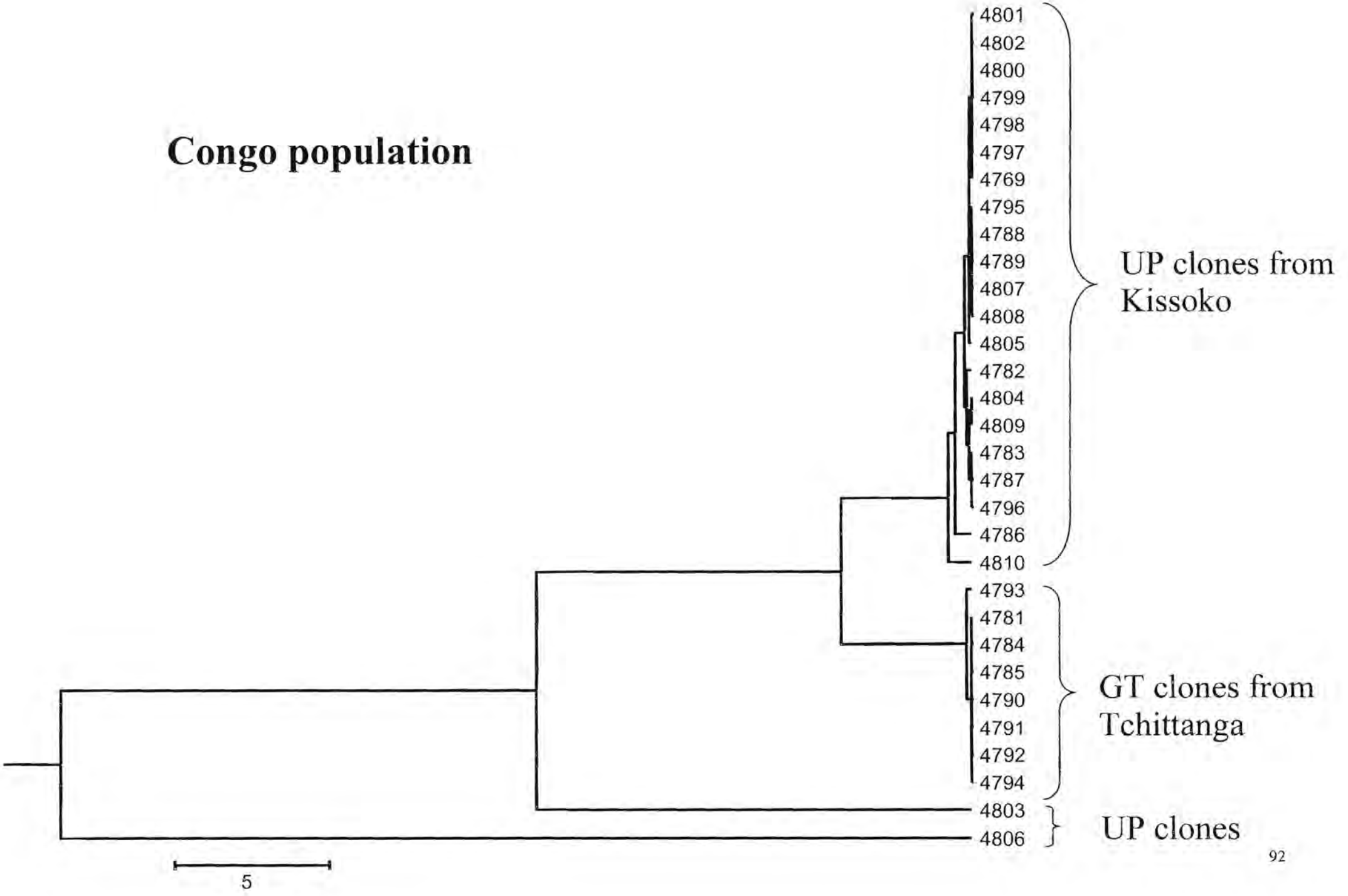

Congo population
4801
4802
4800
4799
4798
4797
4769
4795
4788
4789
4807
4808
4805
4782
4804
4809
4783
4787
4796
4786
4810
4793
4781
4784
4785
4790
4791
4792
4794
4803
4806
UP clones from Kissoko
GT clones from Tchittanga
UP clones
5

Figure 2: UPGMA dendrogram of the Colombian population. Genetic distance was based on total nucleotide length of each allele using the algorithm, $\delta\mu^2$. The 32 isolates making up this population are sub-structured into two main groups.

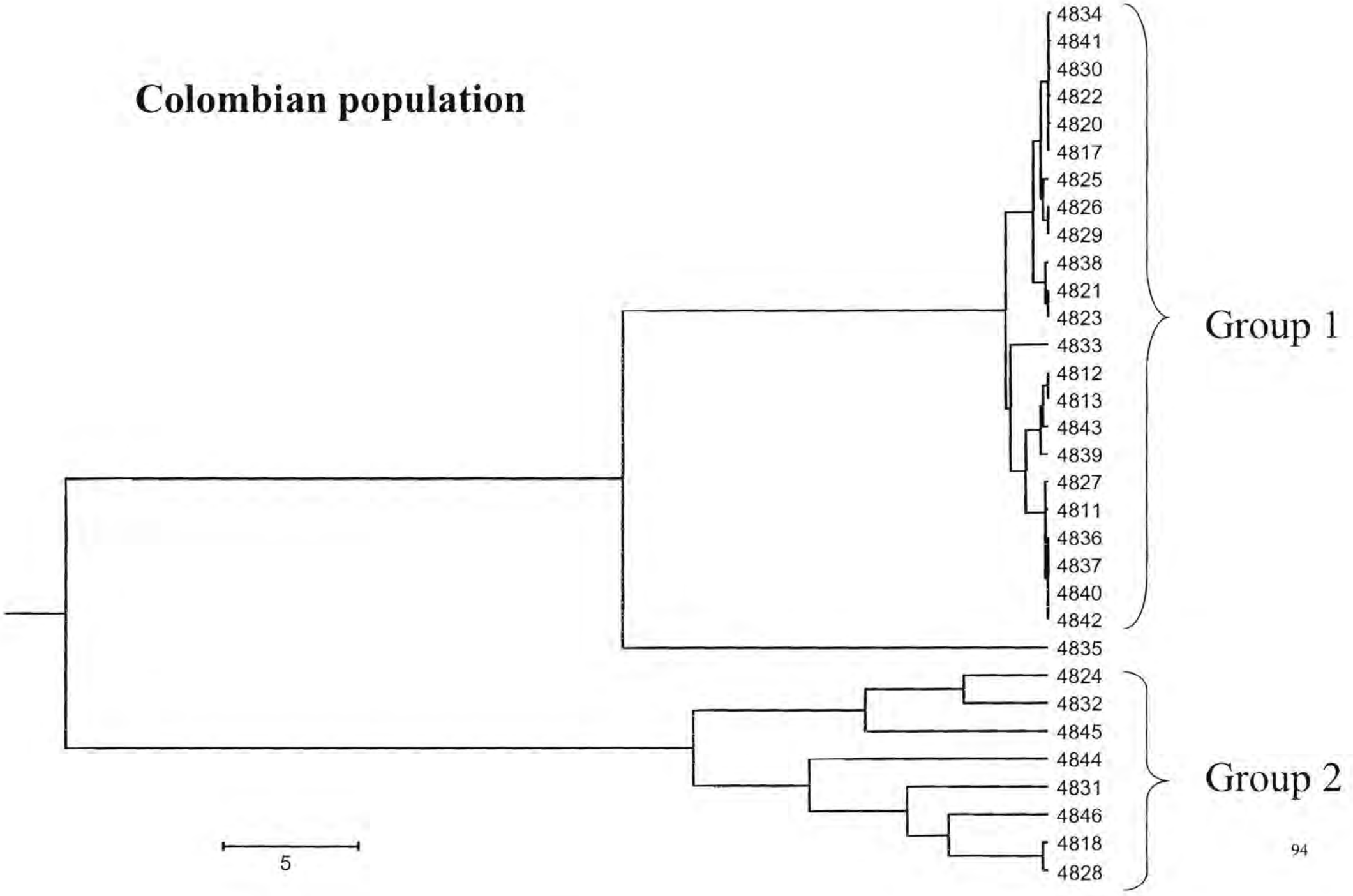

Colombian population
4834
4841
4830
4822
4820
4817
4825
4826
4829
4838
4821
4823
4833
4812
4813
4843
4839
4827
4811
4836
4837
4840
4842
4835
4824
4832
4845
4844
4831
4846
4818
4828
Group 1
Group 2
5

Figure 3: UPGMA dendrogram of the Uruguay population based on genetic distance calculated using Delta mu squared ($\delta\mu^2$) on total nucleotide length. The population, consisting of 22 isolates, is separated into three different lineages.

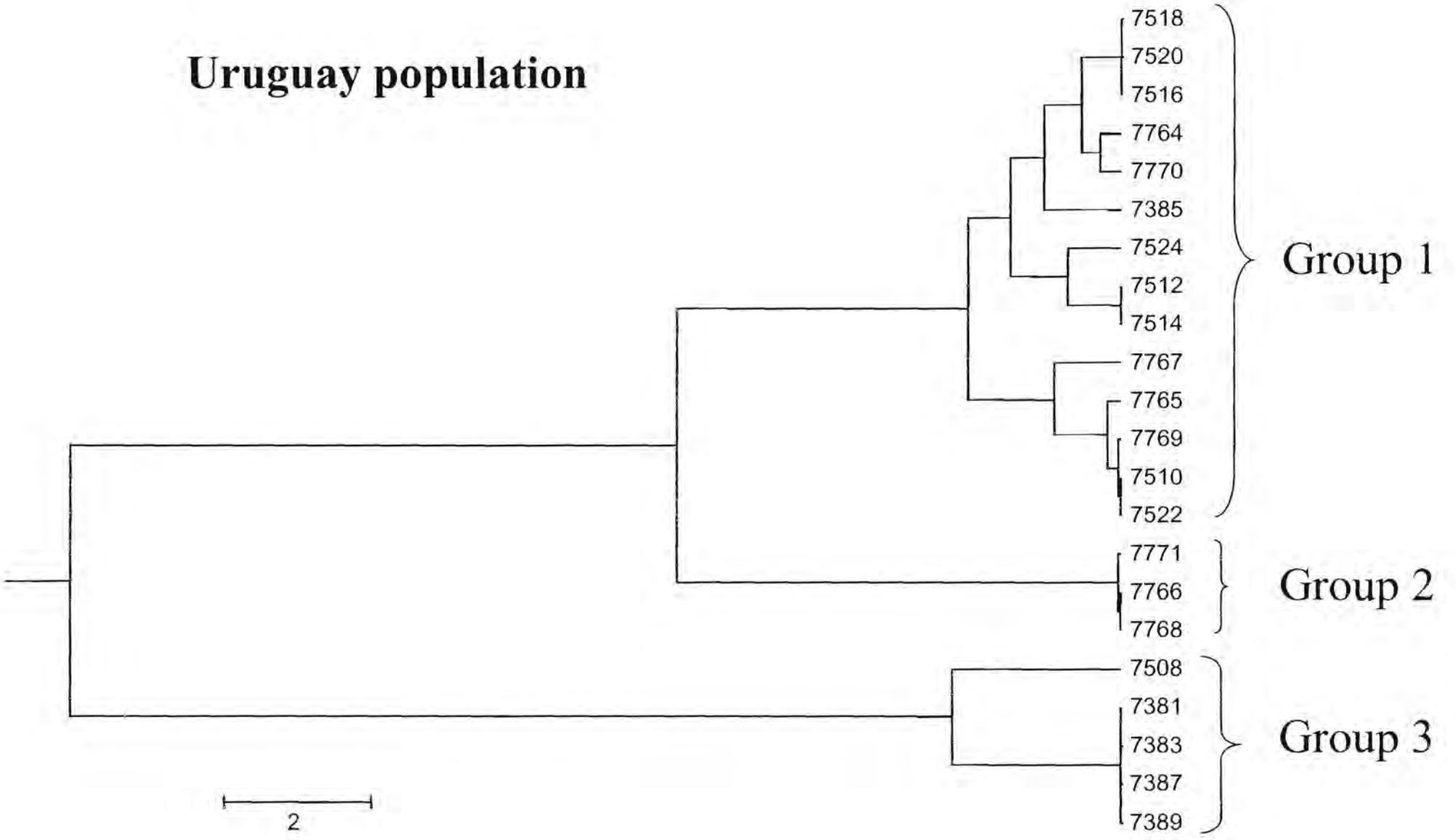

Uruguay population
7518
7520
7516
7764
7770
7385
7524
7512
7514
7767
7765
7769
7510
7522
Group 1
7771
7766
7768
Group 2
7508
7381
7383
7387
7389
Group 3
2

Figure 4: UPGMA dendrogram of the combined populations of *C. fimbriata* from Congo, Colombia and Uruguay. Genetic distances were calculated using $\delta\mu^2$ on the total nucleotide length of the alleles. Duplicate genotypes found within a population were removed in the analysis. The combined populations of *C. fimbriata* show substantial substructure, with a G_{ST} value of 0.39. The Colombian, Group 1 and the Uruguay Group 1 were genetically differentiated from the other isolates and were thus labelled 'unique'. The remaining groups from these two populations were intermixed, especially with the isolates from Congo. 'Col' refers to isolates from Colombia, 'Con' from the Republic of Congo and 'Uru' from Uruguay.

Combined populations of *C. fimbriata* from Congo, Colombia and Uruguay

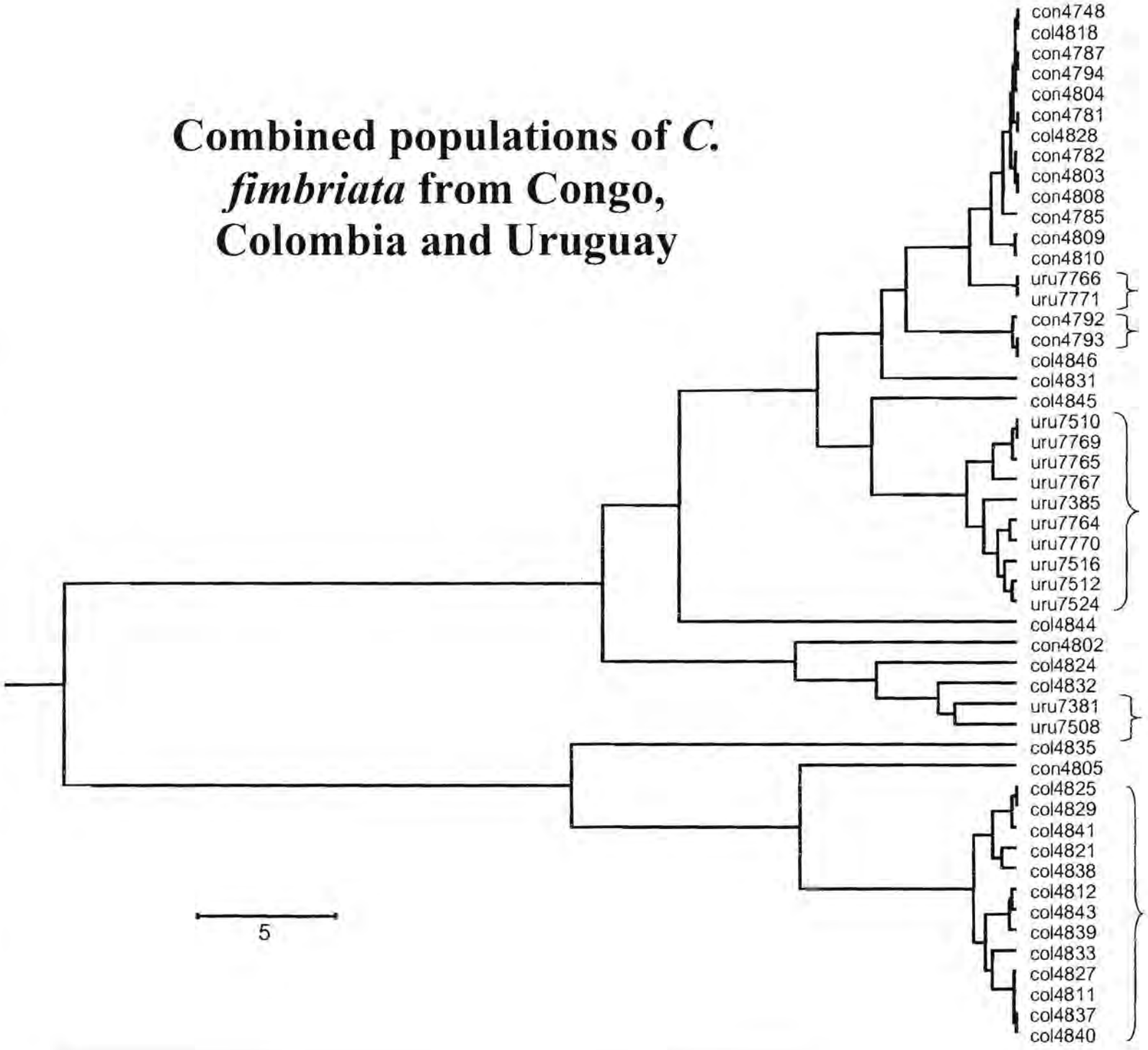


Figure 5: UPGMA dendrogram of *C. fimbriata* from Congo, Colombia and Uruguay. Congo and Uruguay have the least genetic distance compared with the Colombian population. Both the populations from Congo and Uruguay are from *Eucalyptus* spp. while the Colombian population is from *Coffea* spp.

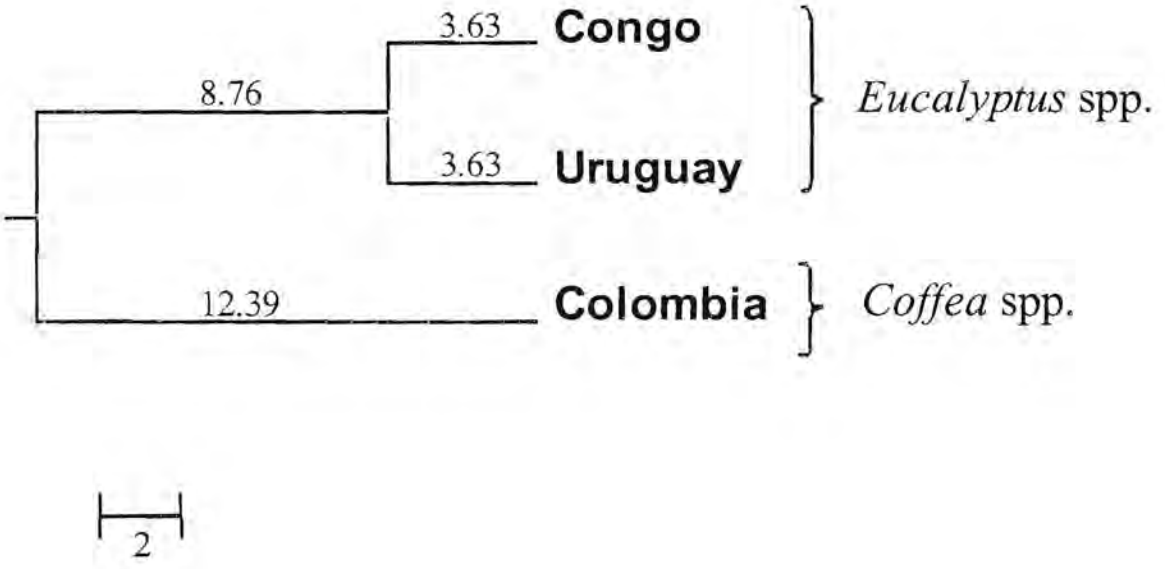
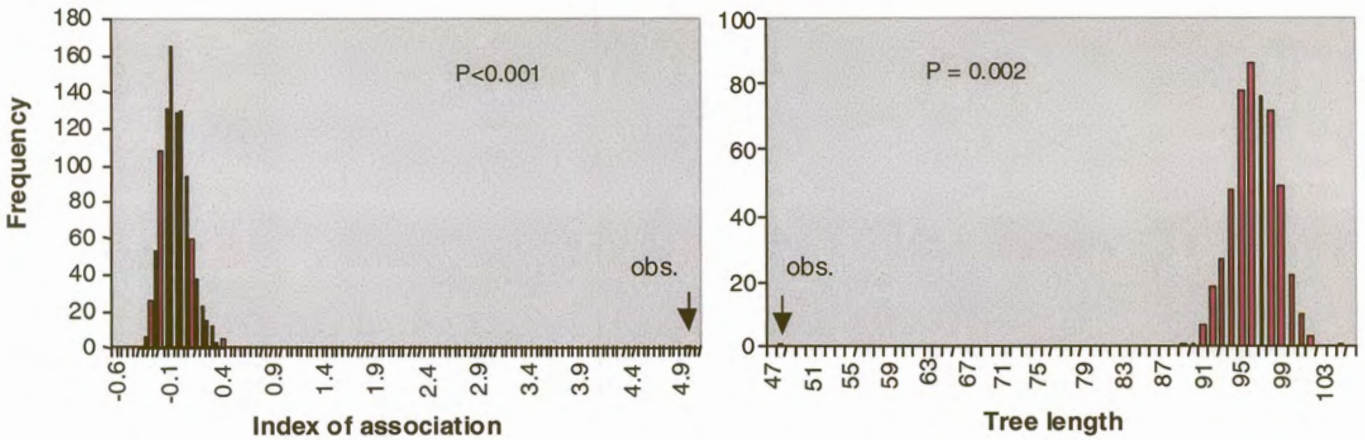


Figure 6: Histograms representing the distribution range of randomly recombining populations using a) the index of association (I_A) with 1000 randomized datasets b) parsimony tree lengths (PTLPT) based on 500 randomisation events. All three populations of *C. fimbriata* were analysed. The observed values for (I_A) in all three populations falls well beyond the distribution range for the recombining population, indicating significant correlation of alleles across loci ($P<0.001$). Similarly, the observed tree length was significantly shorter ($P = 0.002$) than that for the randomised data set based on permutation tests. Both tests indicate a clonal reproductive mode for the populations of *C. fimbriata* from Congo, Colombia and Uruguay.

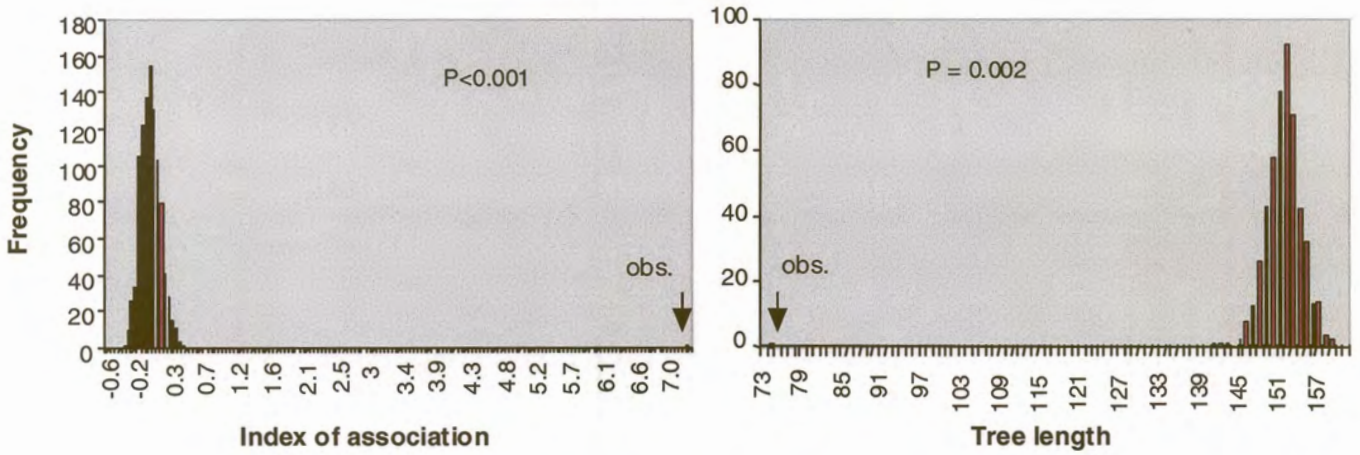
a) I_A TESTS

b) PTLPT

Congo population



Colombian population



Uruguay population

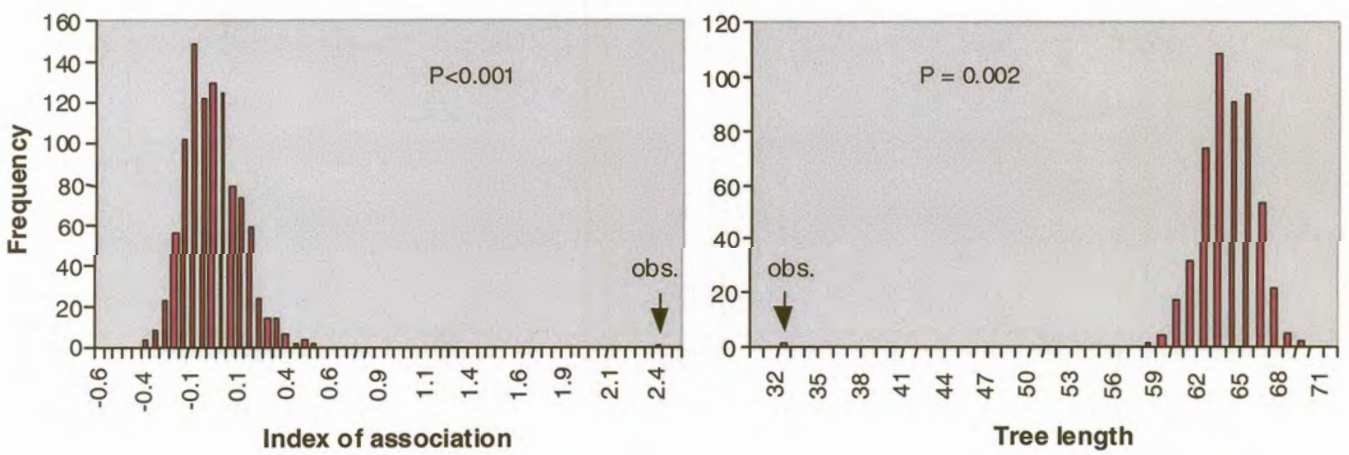
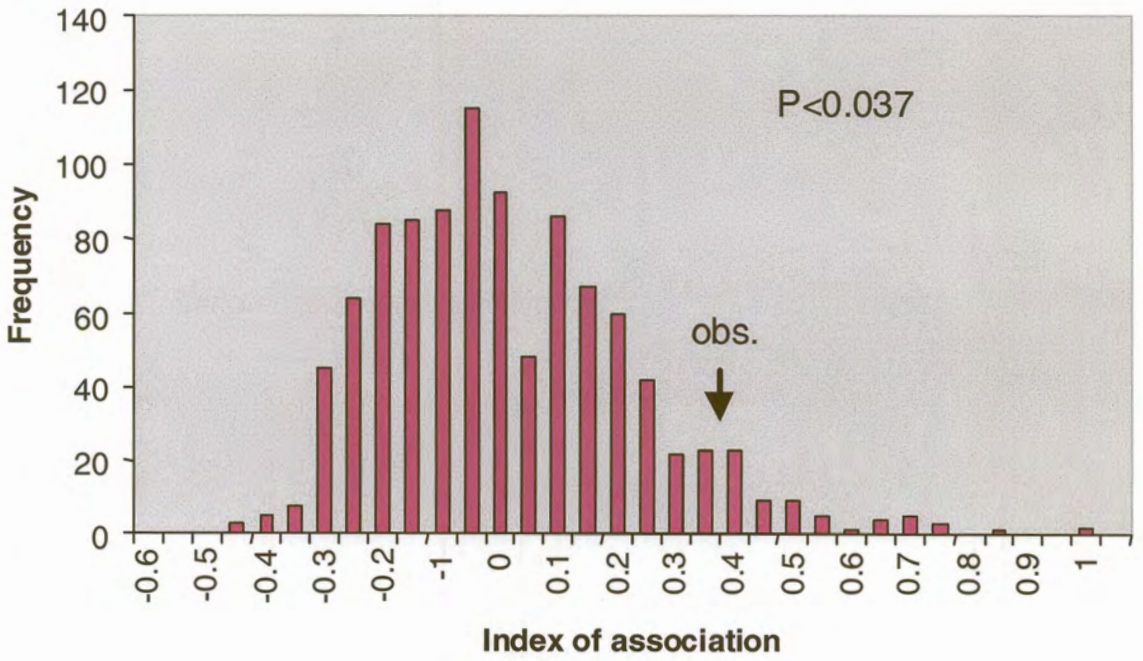


Figure 7: Histograms representing the frequency distribution range of 1000 randomly recombining populations using the index of association. Observed values were calculated for the two distinct groups differentiated in the UPGMA microsatellite distance analysis in the Colombian population. a) Colombian Group 1 and b) Colombian Group 2. In both cases, the observed value falls within the distribution range of the simulated recombining population. The null hypothesis that recombination is occurring, is supported.

Colombia Group 1



Colombia Group 2