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REVIEW

Of mushrooms and chocolate trees: aetiology and phylogeny of witches' broom and frosty pod diseases of cacao

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The troubled history of the two major diseases of the chocolate tree (*Theobroma cacao*) in South America, witches' broom and frosty pod, is reviewed, concentrating on critical aspects of the aetiology as well as the phylogeny of the causal agents. Both diseases are caused by sister species within the genus *Moniliophthora*, belonging to the *Marasmiaceae* family of mushrooms. The witches' broom pathogen, *Moniliophthora perniciosa*, evolved on the Amazonian side of the Andes and induces brooms not only in cacao and its relatives in the genera *Theobroma* and *Herrania* (Malvaceae), but also in species in the plant families Bignoniaceae, Malpighiaceae and Solanaceae, on which the mushrooms (basidiomata) are produced. *Moniliophthora roreri,* the type species of the genus, evolved as a pod pathogen on endemic *Theobroma* species on the western side of the northern Andean Cordillera. Because *Moniliophthora* was described originally as the asexual form of an unknown basidiomycete, the generic diagnosis is amended here to accommodate species with agaricoid basidiomata. In addition, the new variety *M. roreri* var. *gileri* is designated for the morphotype occurring on *Theobroma gileri*, in northwest Ecuador. Cytology studies indicate that the supposed conidia of *M. roreri* are, in fact, sexual spores (meiospores) and it is posited that the fruiting structure represents a much-modified mushroom. Finally, based on preliminary data from pathogenicity testing, it is hypothesized that the true causal agent of both diseases is an as yet unidentified infectious agent vectored into the host by the fungus.

Keywords: *Marasmiaceae*, *Moniliophthora*, taxonomic novelties, *Theobroma cacao*, tritrophic interactions

Introduction

Cacao, and its relatives in the genera *Theobroma* and *Herrania* (Malvaceae), evolved in and are native to northern South America (Schultes, 1958; Cuatrecasas, 1964). The establishment of the Amazonian basin as we know it today occurred with the final upsurge of the Northern Andean Cordillera, some three million years ago (Ribas *et al.*, 2012). This resulted in a geographic separation of plant populations, as well as of their natural enemies; in particular, the western or Pacific side of the Andes became depauperate in representatives of the genus *Theobroma*, most notably cacao (Cuatrecasas, 1964; Thorold, 1975).

Theobroma cacao, variously known as cacao, cocoa or the chocolate tree (Young, 2007; Grivetti & Shapiro, 2009), originated in the Upper Amazon region, bordering eastern Ecuador and Peru (Bartley, 2005). Here, uniquely amongst the world's crops, there is no evidence that it was ever cultivated in its 'botanical birthplace' (Schultes, 1984; Motamayor *et al.*, 2008; Clement *et al.*, 2012). Hence, when unknown fungal diseases moved, naturally or human assisted, into the previously disease-free, exotic plantations of cacao outside of the Amazon basin, their very novelty delayed their eventual diagnosis. Here, the long and troubled history of these two critically important diseases of cacao are charted in the pre-molecular era, then the available molecular data are analysed in relation to the phylogeny of the causal agents. Finally, there is a discussion on where best to place them, and those areas of research that should be prioritized to make the future less uncertain.

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A Troubled Past

Witches' broom disease

The first of these diseases to be reported and investigated scientifically was witches' broom, known locally as 'krulloten' disease, when it appeared in the extensive cacao plantations of Dutch Guiana (Surinam), towards the end of the nineteenth century (van Hall, 1914). This author documented the early history of the new disease that was sweeping through the plantations inducing gross distortion and malformation of the meristematic tissues, resulting in the diagnostic witches' brooms (Fig. 1a). Samples were sent to mycologists in the Netherlands, UK and

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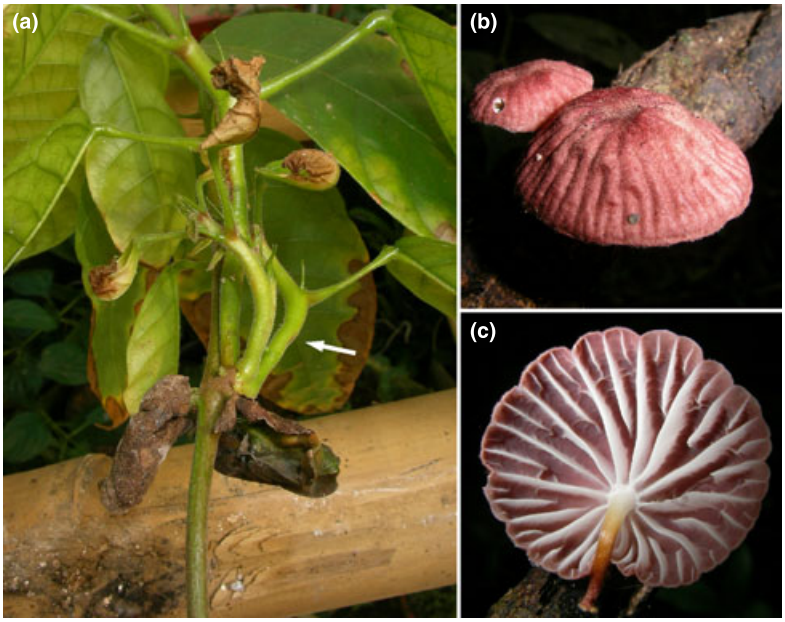

Figure 1 (a) *Moniliophthora perniciosa* infection on cacao seedling, showing a terminal broom with lateral brooms developing around the cotyledonary node (arrow) – note the swollen leaf petioles and pulvini; (b) basidiomata of *M. perniciosa* developing on an unknown fallen vine in forest understorey, Minas Gerais, Brazil; (c) mature mushroom showing details of stipe and gills.

USA. Opinions about the identity of the causal agent were diverse: *Fusarium* (Howard, 1901), *Lasiodiplodia* (Charles, 1906; based on material received from Brazil) and a new species of the ascomycete genus *Exoascus*, *E. theobromae* (Ritzema Bos, 1900, 1901). The Dutch mycologist F. A. Went visited Surinam and published the first illustrations of the symptoms and discovered similar mycelium within both witches' brooms and diseased pods (previously considered to be due to *Phytophthora* disease). Detailed drawings of the mycelium in green brooms clearly show intercellular colonization by the highly specialized (monokaryotic, endophytic) parasitic mycelium (Went, 1904; see Fig. 2a); a defining characteristic of the pathogen which was not investigated until many decades later (Evans, 1980). This was followed by a more sustained study over several years by scientists resident in Surinam whose field observations indicated that an anthracnose was consistently associated with diseased tissues, especially pods, and that '*Colletotrichum* fruiting bodies were the only fructifications formed in cultures of the witch-broom fungus' (van Hall & Drost, 1909). The new species *Colletotrichum luxificum* was described and proposed as the causal agent (van Hall & Drost, 1907, 1909), despite the fact that Koch's postulates were never proven. Moreover, evidence from histological studies revealed colonization of the living brooms

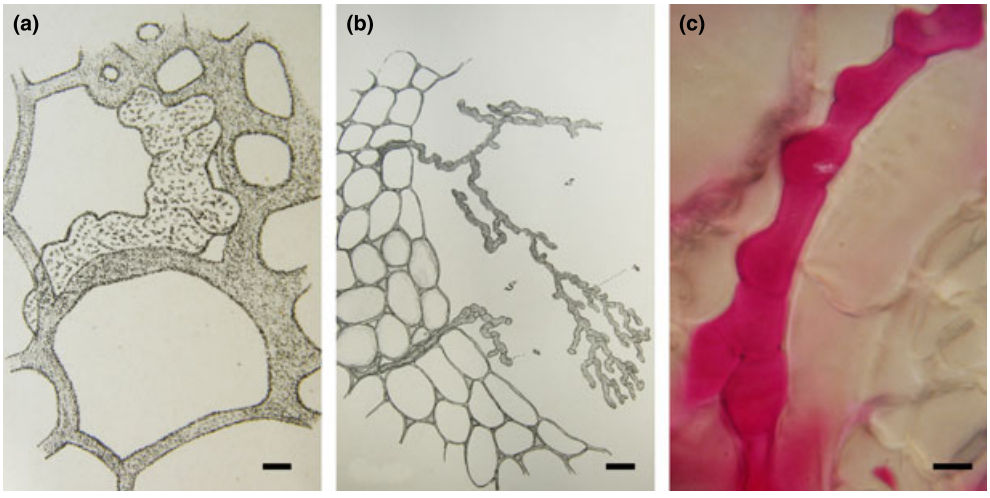

Figure 2 Swollen convoluted intercellular (endophytic) monokaryotic mycelium typical of the biotrophic phase of *Moniliophthora perniciosa* (and *M. roreri*), as illustrated by: (a) Went (1904); (b) van Hall (1910); (c) stained with lacto-fuchsin in cacao broom (bars = 10 μm; 50 μm; 10 μm).

and pods by a distinctive intercellular mycelium, not typical of essentially necrotrophic fungi such as *Colletotrichum*, as illustrated by Went (1904) and later by van Hall & Drost (1909; see Fig. 2b). van Hall (1910) also linked the disease with an indigenous forest tree, concluding that 'the wild *Theobroma speciosum* is still more liable to the disease than the ordinary cacao', and noting that 'the mycelium of the *Colletotrichum luxificum* was found easily on microscopical examination'.

Subsequently, Massee (1910) included a full description of *Colletotrichum luxificum* as the pathogen responsible for 'witches' brooms of cacao' in his book, *Diseases of Cultivated Plants and Trees*. However, it was obvious that this diagnosis was not accepted universally as investigations continued, leading Rorer (1910) to test the pathogenicity of a species of *Colletotrichum* cf. *luxificum* isolated from 'abnormal growths' on cacao in Trinidad, with negative results. Later, Rorer (1913) availed himself of the opportunity to isolate from cacao brooms and pods in Surinam and noted that the cultures from diseased tissues consistently produced clamp connections and, therefore, that the true pathogen must be an unknown basidiomycete. This supposition proved to be correct when, shortly afterwards, Stahel (1915) found that if necrotic witches' brooms, bearing pink to crimson mushrooms (basidiomata, see Fig. 1b,c), were suspended above cacao seedlings, typical disease symptoms developed. He named the fungus *Marasmius perniciosus*, a novel agaric species producing its mushrooms only on the dead host tissues, including necrotic pods, as well as brooms. However, when material from Ecuador was sent to Stahel (1924), shortly after witches' broom first appeared there, he noted differences, in particular the darker red colour of the basidiomata, and he erected a new variety, var. *ecuadoriensis*.

Nearly three decades after the original description, *M. perniciosus* was transferred to the genus *Crinipellis* as *C. perniciosa*, within the section *Eu-Crinipellis*, subsection *Iopodinae* (Singer, 1942), based on the tough, thick-walled pileal hairs or setae. Eventually, Singer (1976) recognised *Iopodinae* as a distinct section, placing *C. perniciosa* in the new subsection *Insignes* and noting, prophetically: 'The fact that the fungus is still frequently quoted as *Marasmius perniciosus*, 30 years after its transfer to *Crinipellis*, is a good illustration of the "conservatism" of some phytopathologists and their reluctance to adopt the results of mycological work'. Perhaps, he was reflecting on the statement by Baker & Holliday (1957) that because: 'The name *Marasmius perniciosus* is so well known… we do not recommend the general adoption of this transfer'. Prior to this, Dennis (1951) had accepted its inclusion in the genus *Crinipellis* in his revision of the *Agaricaceae* of Trinidad, and also listed *Marasmius scalpturatus* from Cuba, as a synonym. Evans (1977) considered that there was insufficient material for a positive identification, but noted that 'the twigs have all the appearance of small cushion brooms as seen on diseased cocoa trees'. However, Singer (1976) in his monograph on neotropical *Marasmieae* also examined a fragment of the type specimen held at Harvard Museum (details in Singer, 1978), and chose to place it in *Crinipellis siparunae*. Conversely, Pegler (1978) endorsed the earlier conclusion of Dennis (1951) that the Cuban material, deposited as *M. scalpturatus* in the Kew Herbarium on an unidentified woody plant, is *C. perniciosa*. Because no published description of *M. scalpturatus* could be found, the name was included as a *nomen nudum*. Further intrigue surrounds this record from the mid-nineteenth century because witches' broom disease has never been reported from Cuba. Indeed, the first official record from the Caribbean region was from Trinidad after its arrival in 1928 (Stell, 1928). The mystery deepened still further when the host wood was identified at the Kew laboratories as *Theobroma* (Evans, 1977; Pegler, 1978). So, is this the earliest official record of the disease and was the fungus introduced accidentally with cacao by the Spanish during the colonial era, or is it the closely related *C. siparunae*, coincidentally growing on cacao?

Pegler (1978) also recognized morphological differences in the mushrooms collected in Ecuador, based predominantly on variation in size and colour intensity, and agreed with Stahel (1924) that they should be afforded varietal status. In addition, another variety, var. *citriniceps*, was described from a single collection on a cacao broom from Ecuador with citron-yellow basidiomata. However, there is little doubt that the latter is based on an aberrant or mutant strain lacking red pigmentation, and there have been no records since. Similarly, the status of var. *ecuadoriensis* still remains uncertain.

Frosty pod disease

Historical records show that whilst witches' broom disease was making inroads into cacao plantations on the eastern side of northern South America, another novel disease was causing problems to the burgeoning and lucrative cacao industry on the Pacific coast of western Ecuador at the turn of the nineteenth century (Jorgensen, 1970). Jorgensen translated the diary of a plantation owner from 1895 that matched the critical symptom: 'most of the pods become white whilst maturing on the trees'. This describes perfectly one of the local names for a pod condition encountered by van Hall (1914), who appears to have been the first to have reported scientifically on the disease during a consultancy visit to Ecuador in 1909, which was considered to be due to unseasonably cold weather: 'A rather sudden decline of temperature brings on what is called "helada" [frost] of the pods, which causes an abnormal growth of the pods and beans' (see Fig. 3).

Mirroring almost exactly the events in Surinam for witches' broom disease, these preliminary investigations by van Hall were followed up by Rorer (1918), who submitted a report to the cacao growers of Ecuador on the nature and management of the new disease. Specimens were sent to a specialist in the USA (R.E. Smith, University of California) and the fungus was identified as a species of *Monilia*, close to *M. fructicola* (Rorer, 1918, 1926), with obvious analogies being drawn with this

Figure 3 (a) Cacao pod *c.* 3 months after infection by *Moniliophthora roreri*, entering the necrotrophic phase marked by the appearance of the white 'pseudostroma'; (b) more advanced infection with the pseudostroma covering the pod surface and the beginnings of sporogenesis.

temperate pathogen of stone fruits, not least its placement in the *Sclerotiniaceae* (*Ascomycota*). However, more than a decade passed before the species was described formally and named *Monilia roreri* by R. Cifferi, after specimens were sent to him in Italy from Ecuador (Ciferri & Parodi, 1933). This included the first use of the Italian descriptor 'Moniliasi' for the common name, which has persisted in one form or another in the cacao literature ever since.

Over 40 years later, the aetiology of the disease and the morphology of the pathogen were investigated in more depth, in particular, using advanced microscopy techniques (SEM, TEM) to elucidate both sporogenesis and hyphal ultrastructure. The former was found to be basipetalous, rather than acropetalous as in *Monilia*, in which spore chains are formed by yeast-like budding at the apex, whilst the presence of dolipore septa in the mycelium confirmed its basidiomycetous affiliation. The new hyphomycete genus *Moniliophthora* – literally, *Monilia*-destroyer – was erected to accommodate this supposed anamorph or asexual state of an unknown basidiomycete (Evans *et al.*, 1978), with *Moniliophthora roreri* as the type species. In addition, an attempt to terminate the erroneous association with the ascomycete fungus *Monilia* was thought to be necessary by changing the universally accepted popular name 'moniliasis', which, most unfortunately, is also used as an alternative name for the human fungal disease 'candidiasis' (Kirk *et al.*, 2008). Frosty pod was proposed based on the various Spanish vernacular terms e.g. 'helada', 'hielo' (ice), 'pasmo' (wilt due to frost), which accurately describe the frosted appearance of diseased pods (Fig. 3). Nevertheless, it has not succeeded at either end of the scientific spectrum; field technicians in Latin America still refer to the disease as 'la monilia' or 'moniliasis' ('monilíase', in Brazil), although most farmers use local descriptive names rather than pseudoscientific terminology, whilst in higher scientific circles, the myths and misnomers relating to its troubled past continue unabated. Classic publications on plant pathology and mycology have painted a confused picture over the four decades since the transfer was made. For example, Agrios (1997) in the penultimate edition of his standard text on plant pathology retains the pathogen in the *Sclerotiniaceae*, described as 'Monilia pod rot of cacao, caused by *Monilia roreri*'. In the latest edition (Agrios, 2005), it remains in the *Sclerotiniaceae* with the mixed message: 'Monilia pod rot of cacao, caused by the fungus *Moniliophthora roreri*, anamorph *Monilia roreri*'. Possibly, this was influenced by the equally confusing entry in the *Dictionary of the Fungi* (Kirk *et al.*, 2001), where it is described thus: 'anamorphic *Ascomycetes* (with dolipore septa) (synanamorph *Monilia roreri*)'. However, more unfortunate are the images purporting to illustrate disease symptoms which show an unconvincing early stage in pod infection and a cluster of severely infected pods (Agrios, 2005); unequivocally, the latter are pawpaw fruits (*Carica papaya*) with a whitish spore bloom (Evans, 2007). Confusion continues, and even more recent publications exemplify this uncertainty, with the misinterpretation that Evans *et al.* (1978) and Evans (1981) had discovered that the conidia were basidiospores: 'thus to reflect this observation, they established the genus *Moniliophthora*' (Rossman & Palm-Hernández, 2008).

In fact, what had been noted in these earlier publications (Evans *et al.*, 1978; Evans, 1981), was that *C. perniciosa* and *M. roreri* share remarkable similarities in the early stages of pod infection, i.e. hypertrophy of tissues, resulting in external malformation and internal compaction of beans; intercellular colonization by a swollen, convoluted mycelium representing a well-defined, long-lasting (2–3 months) parasitic phase, leading to the conclusion: 'that they represent two evolutionary branches of a single fungal species' (Evans, 1981). At this stage, *Moniliophthora* was still considered to be the anamorph of an unknown basidiomycete fungus, potentially belonging to the genus *Crinipellis*. Indeed, surveys of the agarics occurring in Ecuadorian cacao farms were undertaken in 1976–77 to try to establish the 'missing link' (Evans, 1981).

An Uncertain Future

With the molecular era came the opportunity to test the hypotheses and address the speculation relating to the origins and relationships of these two seemingly radically different causal agents of the major diseases of cacao in the Neotropics. The first proof of concept came when the ITS and small mitochondrial rDNA sequences of *C. perniciosa* and *M. roreri* were found to be closely matched, which led to the proposal to transfer *M. roreri* to the genus *Crinipellis*; the new combination *C. roreri* (Cif.) H. C. Evans was made (Evans *et al.*, 2002). Unexpectedly, the same molecular techniques revealed that samples of *M. roreri* isolated from the pods and, as an endophyte, from the stems of a rare endemic submontane forest tree (*Theobroma gileri;* Cuatrecasas, 1953) in northwest Ecuador, were distinct from cacao isolates, based not only on the nucleotide sequence data but also on spore morphology (Evans *et al.*, 2003a). The new variety *Crinipellis roreri* var. *gileri* was proposed. Unfortunately, this was not validly published because the collection where the type material is conserved was not specified (Article 37.6 [now Art. 37.7] in the *International Code for Botanical Nomenclature*, *fide* G. J. Samuels, USDA-ARS, personal communication, 2003).

The uniqueness of these collections, from endangered remnants of the biodiverse Chocó forest region (Gentry, 1982, 1995), have since been confirmed when a study of the genetic diversity of *M. roreri* using AFLP and ISSR profiling showed that the *T. gileri* isolates from northwestern Ecuador sit in a separate clade, termed the *Gileri* group (Phillips-Mora *et al.*, 2007). However, the long-held hypothesis that the disease outbreaks that decimated the cacao plantations in coastal Ecuador originated from these forest foci on the western slopes of the Ecuadorian Andes (Evans, 1981, 2002), was disproven when the '*Gileri*' isolate was found to be non-pathogenic to cacao (Evans *et al.*, 2003a; Evans, 2007). All evidence now indicates that the cacao pathogen evolved in north-central Colombia and was introduced accidentally into Ecuador, probably by human agency (Phillips-Mora *et al.*, 2007). Cacao is not endemic in this region of Colombia but undoubtedly it has been cultivated and naturalized there for centuries, if not millennia (Bartley, 2005), and during this period the crop would have been exposed to inoculum from forest hosts. Baker *et al.* (1954) reported that *T. gileri* is 'fairly common in the plains and foothills' of northwest Colombia and, moreover, that *M. roreri* was found infecting the pods. It is reasonable to suppose, therefore, that this is the forest host on which the cacao pathogen originated. Nevertheless, the identity of this host is problematic because in the description and illustration of Baker *et al.* (1954) the leaves are significantly bigger and broader (19 × 10 cm) than the type from Ecuador (7·5–13 × 2–4 cm; *fide* Cuatrecasas, 1953; Basil G. D. Bartley, Lisbon, personal communication, 2005). In addition, the pods are produced on both the trunk (cauliflorous) and the branches. However, in Ecuador *T. gileri* is rarely cauliflorous and pods are produced predominantly in the canopy (Evans *et al.*, 2003b; H. C. Evans, personal observation). Cuatrecasas (1964) emended his original description of *T. gileri* to include the collections from Colombia, but the evidence above suggests that these may, in fact, represent a distinct taxon.

These preliminary molecular studies were soon followed by more extensive phylogenetic analyses using five nuclear gene regions to better determine the relationships of the two pathogens and their affinities (Aime & Phillips-Mora, 2005). They were confirmed as sister species forming a distinct lineage, or monophyletic clade, within the family *Marasmiaceae*. Thus, because the witches' broom fungus could no longer be retained within *Crinipellis*, which is essentially a genus of saprotrophic litter fungi, the authors were presented with a dilemma: to erect a new genus or to treat the former anamorphic genus *Moniliophthora* as pleomorphic and transfer *C. perniciosa* to it. Because keeping name changes to a minimum has always been considered to be an important aim of good nomenclature, in accordance with the *International Code of Nomenclature for algae, fungi, and plants* (ICN), the latter option was taken and the new combination *Moniliophthora perniciosa* (Stahel) Aime & Phillips-Mora was proposed. The feeling amongst those specialists involved with cacao diseases – certainly, traditional systematists – was that this is an inadequate compromise, especially as the generic diagnosis has not been amended to include species producing basidiomata as the main taxonomic feature, because the type species sits as far removed, morphologically, from *M. perniciosa* as even well-seasoned mycologists familiar with the plasticity of the fungi could envisage. In particular, it was felt that uncertainty could prevail because, based on the original description, the genus would be limited to species with hyphae lacking clamp connections but with dolipore septa, and conidia in chains, formed in basipetal succession (Evans *et al.*, 1978).

The *Amsterdam Declaration* on mycological nomenclature recommended a fundamental modification of Article 59 (Hawksworth *et al.*, 2011), originally designed to regulate the separate naming of different morphs of

pleomorphic fungi, in which only one name would be recognized, with safeguards to protect existing names becoming invalid or illegitimate. This process has now been implemented when it was ratified at the recent International Botanical Congress (IBC) in Australia (Hawksworth, 2011), despite considerable opposition (Gams & Jaklitsch, 2011; Gams *et al.*, 2012). Thus, it could be argued that the present assignment of the cacao pathogens in the genus *Moniliophthora* is correct and justified based on the pleomorphism concept. However, the complexity of the hemibiotrophic life cycles of the cacao pathogens, with pleomorphic vegetative and reproductive stages, will continue to create confusion about the concept of a genus containing species with: (i) a well-defined monokaryotic parasitic phase lacking clamp connections; (ii) a saprotrophic intracellular dikaryophase with clamp connections (*M. perniciosa*) or without (*M. roreri*) (Evans, 1980, 1981); (iii) marasmioid basidiomata producing basidiospores (*M. perniciosa*); or (iv) spores borne in basipetalous chains on a subiculum or pseudostroma (*M. roreri*). To add to the complexity, cytological studies have provided compelling evidence that the purported 'conidia' of *M. roreri* are in fact meiospores; not basidiospores *sensu Marasmiaceae*, as misinterpreted by Rossman & Palm-Hernández (2008), but a multifunctional spore adapted for dissemination, genetic recombination and survival (Evans *et al.*, 2002). Supporting evidence has come from the results of DAPI staining which reveal a highly variable nuclear condition in the spores of *M. roreri* (Griffith *et al.*, 2003). Thus, a counterargument could be considered that, either the generic description of *Moniliophthora* is amended, to better reflect its morphological and evolutionary diversity, or a new genus is erected.

A New Start

The litany of aetiological and systematic woes that have dogged the history of these two cacao pathogens needs to be addressed, comprehensively and decisively. This continues to the present day, as a recent account of cacao diseases in Amazonian Ecuador by amateur mycologists bears witness: 'The main pathogen is *Monilia*, a powdery mildew fungus that is spread by insects' (Evans & Winkler, 2011). Clearly, a clarification of the status of two fungi which have changed the history of cacao cultivation in the Americas, along with the inevitable and incalculable socio-economic impacts, has been a long time coming. It is felt that a new start is warranted, hopefully to stimulate and kick-start research into the many grey areas of their life cycles that remain contentious or unexplored. Here, the description of the genus *Moniliophthora* is amended to better accommodate both species and identify those areas of their biology most in need of investigation. The ever increasing pace of globalization suggests that these fungi could play an even greater role on the world's commodity market stage should they ever reach the Palaeotropics. The catastrophic turn of events that occurred in Brazil after the arrival of *M. perniciosa* in the main cacao-producing region of Bahia in 1989 (Griffith, 2004; Evans, 2007), with the subsequent collapse of cacao production, Brazil's seismic shift from being the world's second largest producer to being a major importer, and the ecological repercussions (Rice & Greenberg, 2000; Saatchi *et al.*, 2001; Donald, 2004), serves as a reminder of the potential impact of these diseases, not only on the chocolate industry, commerce and the consumer, but also on biodiversity and the cacao-farming communities.

Systematics

There are powerful arguments for maintaining the status quo (Aime & Phillips-Mora, 2005), as discussed previously. Not least is that relating to the significantly modified Article 59 of the ICN that regulates the separate naming of pleomorphic fungi, and the one fungus one name initiative encapsulated in the *Amsterdam Declaration* and later ratified at a meeting of the IBC (Hawksworth, 2011). Evans *et al.* (2002, 2003a) reported meiotic events in both 'varieties' of the supposed anamorphic fungus *Moniliophthora roreri* and confirmed their close relationship with *Crinipellis perniciosa*, based on molecular evidence; the inference being that the true teleomorph, typified by agaricoid basidiomata, had been lost irrevocably from the life cycle. It has been hypothesized that the uplift of the northern Andean chain, as described previously (Ribas *et al.*, 2012), was the driving force behind this dramatic change in the life cycle (Evans, 2007). Compared to Amazonia, the Chocó forest refuge on the Pacific side of the Andes (Gentry, 1982) has only scattered populations of *Theobroma* (Cuatrecasas, 1964; Evans, 2002). In conservation terms, this is now considered to be a threatened genus (Santos *et al.*, 2011), and this low host density became the evolutionary spur to increase not only spore production but also spore survival. This was achieved through transformation of the systemically colonized cacao pod into a giant 'mushroom', producing billions of long-lived (thick-walled), efficiently dispersed, infective meiospores from sporophores on an external pseudostroma. The latter is interpreted as the vestiges of the pileus and the sporophores as modified basidia. Thus, following this interpretation, *Moniliophthora* is based on a species in which the agaricoid fruit body has been modified beyond recognition.

This gross modification of the basidioma is not unique within the *Agaricales* and a similar situation in the genus *Rhacophyllus* has provoked considerable debate over the past century (Redhead *et al.*, 2000). The latter authors considered it to represent an anamorphic *Coprinus*, despite the fact that the spores ('lysomeres'), produced within sclerotial-like structures ('bulbils') in an agaricoid basidioma lacking true lamellae, had been considered to be homologous to basidia, with cytological evidence of meiosis (Moreau, 1913). Conversely, Singer (1976) and Pegler (1986) recognised two teleomorphs, based on evidence that both morphs, the *Coprinus* form and the lysomere-forming *Rhacophyllus*, as well as intermediate

forms, could develop in culture (Maniotis, 1964). Redhead *et al.* (2000) used the argument that although *Rhacophyllous* is the earliest known name for coprinoid species, its use to characterize 'normal agaric basidiomes … would create confusion'. The same logic is employed here with the name *Moniliophthora*, as this was erected for an anamorphic genus and is duly treated as such in the most up-to-date mycological texts (Seifert *et al.*, 2011). Thus, if there are no changes to the generic diagnosis, then species with true agaricoid basidiomata would not be included, or, at the very least, the situation would be as equally confusing as that of *Rhacophyllus–Coprinus sensu* (Redhead *et al.*, 2000).

The generic description is emended herein to accommodate *Moniliophthora perniciosa* and *M. roreri*: two evolutionary distinct sister species.

Moniliophthora H. C. Evans, Stalpers, Samson & Benny *Can. J. Bot.* **56**: 2530 (1978) emend. H. C. Evans, J. L. Bezerra & R.W. Barreto.

Basidiomycota, Agaricomycetes, Agaricales, Marasmiaceae. *Mycelium* of two types: intercellular, swollen, convoluted, lacking clamp connections, monokaryotic; intracellular, narrow, straight, usually with clamp connections, dikaryotic. *Basidiomata* either agaricoid or pseudostromatal; on living or dead host tissues. If agaricoid, *pileus* small, convex; pileal surface an open network of thin-walled hyphae encrusted with pigment; *pileal hairs* or setae short, strigose, generally crowded at centre and with membrane pigment; *stipe* short, cylindrical, fleshy, with bulbous base. *Gills* distant, thin, white, fleshy; *basidia* clavate, 4-spored; *basidiospores* ellipsoid, hyaline, thin-walled, inamyloid; spore print white; *cheilocystidia* clavate to fusoid, hyaline, thin-walled. In non-agaricoid forms, *basidiomata* reduced to a pseudostroma with modified *basidia*, producing spores in basipetal chains; *meiospores* globose to subglobose, sub-hyaline, thick-walled, powdery.

Type species: ***Moniliophthora roreri*** (Cif.) H. C. Evans, Stalpers, Samson & Benny (1978)

= ***Crinipellis roreri*** (Cif.) H. C. Evans, in Evans, Holmes, Phillips & Wilkinson, *Mycologist* **16**: 151 (2002)

= ***Monilia roreri*** Cif., in Ciferri & Parodi, *Phytopath. Z.* **6**: 542 (1933)

Based on morphological, as well as on molecular evidence (Evans *et al.*, 2003a; Phillips-Mora *et al.*, 2007; T. L. Tarnowski, IFAS, University of Florida, USA, personal communication), isolates of frosty pod rot from *Theobroma gileri* in northwest Ecuador can be separated readily from all cacao isolates. Thus, a new variety is proposed:

Moniliophthora roreri* var. *gileri H. C. Evans **var. nov.**

Differing from *Moniliophthora roreri* in the predominantly larger, ellipsoid to globose meiospores, 8·0–16·0 (–22·0) × 5·5–11·0 μm, and in the DNA sequences.

Holotype: IMI 389647, from diseased pod of *Theobroma gileri* (Malvaceae) in primary forest, collected in Guadual-Lita, Esmeraldas Province, Ecuador, 650 m a.s.l., 14 Sept. 1999, H. C. Evans (= DIS 116, see Evans *et al.* (2003a) for additional collection details, genetic profile and illustrations).

Paratypes: IMI 389649 (= DIS 331), same host and locality, 5 Nov. 2011, H. C. Evans & R. H. Reeder; IMI 389648, same host, locality and collection details, but isolated as an endophyte from 'healthy' pod.

Additional collections: Isolates were obtained following in situ isolation from healthy stem tissues of *T. gileri* during a fungal endophyte survey in the same locality (Evans *et al.*, 2003b).

Pathology: *M. roreri* var. *gileri* appears to be host specific; and failed to infect cacao pods experimentally (Evans *et al.*, 2003a; Evans, 2007).

Other species:

Moniliophthora perniciosa (Stahel) Aime & Phillips-Mora in *Mycologia* **97**: 1021 (2005)

= ***Crinipellis perniciosa*** (Stahel) Singer in *Lilloa* **8**: 503 (1942)

= ***Marasmius perniciosus*** Stahel in *Bull. Dept. Landbouw. Suriname* **33**: 16 (1915)

***Moniliophthora* sp.** in Aime & Phillips-Mora (2005): isolated as a symptomless endophyte from a grass host (*Bouteloua eriopoda*) in New Mexico, USA; identified on DNA sequences.

It is considered highly probable that all the species assigned to the subsection *Insignes* of the genus *Crinipellis* by Singer (1976) belong to *Moniliophthora* and are cryptic endophytes of forest trees in the Neotropics: some sporulating on living trunks and branches, e.g. *C. eggersii* (H. C. Evans, personal observation, Amazonian Ecuador), *C. siparunae* (Singer, 1942, that was described from and appeared periodically on a tree of the genus *Siparuna*, imported from Brazil and housed in the Leningrad Botanical Garden; H. C. Evans, personal observation, Amazonian Brazil), whilst others, like *M. perniciosa*, sporulate only after tissue death. However, because of similar morphological features, and obvious difficulties in reliably separating them (Dennis, 1951; Pegler, 1978; Singer, 1978), it is considered prudent to wait until fresh collections of this group are made and a comparative morphological and phylogenetic study can be undertaken.

Similarly, more comprehensive collections of *M. perniciosa* from all the geographic regions, as well as from different hosts which have now been identified in the plant families Bignoniaceae, Malpighiaceae and Solanaceae ranging from western Ecuador to southeast Brazil (Bastos & Evans, 1985; Griffith *et al.*, 1994; Evans & Barreto, 1996; Resende *et al.*, 2000; Evans, 2007; T. L. Tarnowski, IFAS, University of Florida, USA, personal communication; see Fig. 4), as well as in the Malvaceae, are needed to better assess the morphological and genetic variation within this taxon. For example, in the only morphological and phylogenetic comparison between isolates of *M. perniciosa* from cacao in northeastern South

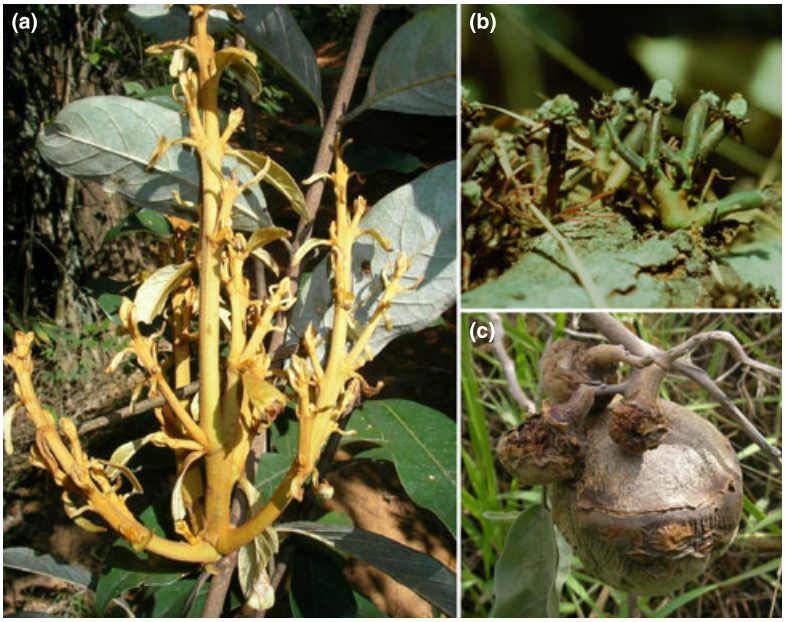

Figure 4 (a) Vegetative broom of *Moniliophthora perniciosa* on Malpighiaceae host in Minas Gerais State, Brazil; (b) systemically infected flower cushion of *Theobroma speciosum* in Amazonian Brazil, showing the parthenocarpic fruit ('chirimoyas'); (c) small (systemically infected) parthenocarpic fruit and large (spore-) infected fruit of *Solanum lycocarpum* ('lobeira') in Goiás State of Brazil.

America (Trinidad) and northwestern South America (Ecuador, Colombia) undertaken thus far, the results of the molecular analysis are inconclusive (T. L. Tarnowski, IFAS, University of Florida, USA, personal communication), especially relating to the recognition of varieties, as proposed by Stahel (1924), and validated by Pegler (1978).

Kerekes & Desjardins (2009) included three species from southeast Asia in the genus *Moniliophthora*, only one of which was included in the molecular phylogeny. Certainly, the new species *M. marginata* appears to have basidiomata typical of the *Insignes* group but the presence of rhizoids in another species (*M. canescens*) raises doubts about its relationship to *Moniliophthora*.

Aetiology

The authors believe that *Moniliophthora* is a widespread genus in the Americas; the majority of species probably live in cryptic endophytic relationships with their host plants. So, how did the two species associated with cacao 'turn-the-tables' on their hosts and become parasitic, altering the growth patterns of the invaded tissues for their own benefit and, ultimately, cause their deaths? This also proved to be a source of puzzlement for Money (2007): 'But how and why did *Crinipellis* evolve as a cacao pathogen?'.

Pilot studies in Brazil to test the cross-infectivity of *M. perniciosa* isolates, in order to answer the question 'will wild hosts of the fungus pose a threat to future cacao cultivation in Minas Gerais?' may, serendipitously, have provided the answer. Cacao was challenged with isolates of *M. perniciosa* from witches' brooms collected on species in the plant families Bignoniaceae (two isolates), Malpighiaceae (three isolates) and Solanaceae (four isolates) (Evans, 2007). A modification of the half-bean test first used to transmit *Cocoa swollen shoot virus* (Posnette, 1947) was used, chosen because of the poor or unpredictable production of basidiomata and hence insufficient amounts of basidiospores to undertake standard inoculation procedures, and a range of symptoms was observed. None of the inoculated seedlings developed witches' brooms, in contrast to the control plants treated with basidiospore inoculum from cacao brooms, but there was consistent evidence of growth abnormalities. In particular, there was stunting of the lower stem and shoot often accompanied by poor root development. In addition, isolates from two species of *Solanum*, *S. cernuum* and *S. lycocarpum*, also induced virus-like symptoms in the cacao leaves, with chlorosis, mottling and vein-banding. However, the most striking and consistent symptoms occurred with an isolate from a Malpighiaceae host with 24/40 plants showing severe stunting of both the lower (60–70% reduction) and upper (80–90% reduction) stems, accompanied by a form of 'rhizomania', characterized by highly abnormal swelling of the root system (Fig. 5b,d). A further 7/40 plants failed to develop leaves and, on harvesting the experiment 4 months post-inoculation, all plants (40/40) exhibited some form of root hypertrophy compared to the controls (Fig. 5c), with various degrees of stunting. No evidence of the biotrophic, intercellular fungal mycelium was found in sections of any of the tissues (roots, stems, leaves) examined microscopically, and the dikary-

Figure 5 (a) Cacao plant 4 months after inoculation (half-bean method) with basidiospores of *Moniliophthora perniciosa* from witches' broom on Malpighiaceae host, with marked dwarfing of stems, both upper (80–90% reduction in length) and lower (60–70% reduction), and normal-sized but abnormally thickened, rugose and distorted leaves; (b) exposed dwarf plant exhibiting 'rhizomania', lacking root hairs and with gross swelling of root tips; (c–d) details of root abnormalities compared with root system of control plant (c, left).

otic mycelium could not be isolated in culture. When leaf material from the 'dwarf' plants with normal-sized, but abnormally dark green, rugose, thickened or glabrous and twisted leaves (Fig. 5a) was ground in a pestle and mortar with buffer (pH 7), squeezed through cheesecloth and the filtrate brush-inoculated as above onto pregerminated cacao half-beans, the presence of an infectious (lethal) agent was demonstrated: the radicle became grossly swollen, the plumule failed to open (Fig. 6a), and all seedlings died within a few weeks. No fungal mycelium was evident in or isolated from the affected plants. Coincidentally, decades previously, identical symptoms were observed when pregerminated cacao beans were mass-inoculated with spores of the frosty pod pathogen, *M. roreri* (Evans, 1981; Fig. 6b).

The interpretation of these events is that when a non-host species or, in the case of *M. roreri*, a non-susceptible tissue is challenged, the intercellular mycelial invasion is halted by defence mechanisms. However, in certain circumstances, in this case, when a highly artificial inoculation method was employed, the infectious particles are released into the conducting tissues, causing uncoordinated (non-targeted) meristematic activity throughout the plant. In particular, this occurs in those plant organs never invaded by the intercellular fungal mycelium, i.e. the leaf lamina and root system. During the course of evolution, it would appear that *M. roreri* has lost the ability to invade meristems in the vegetative shoots and inflorescences and its systemic colonization is more localized and restricted to pods. Interestingly, it has also been isolated as a cryptic endophyte from healthy pods and stems of *T. gileri* (Evans *et al.*, 2003b).

Thus, there is now circumstantial evidence of a complex tritrophic relationship in action and that a third cryptic partner is the true causal agent of both diseases. This shows analogy, perhaps, with the situation reported recently by Márquez *et al.* (2007), although in the latter case there is a reciprocal three-way symbiosis. Originally, the hypothesis proposed by this group was that an endophytic fungus, *Curvularia protuberata*, enabled its grass host, *Dichanthelium lanuginosum*, to tolerate elevated temperatures in order to colonize hot springs (Redman *et al.*, 2002). However, the later study revealed that a third agent vectored by the fungus, a dsRNA virus, is essential for the system to function. In the cacao scenario, it is posited that the *Moniliophthora* fungus also acts as an endophytic vector, carrying infectious particles into the developing plant tissues via the monokaryotic, intercellular mycelium without triggering defence systems. This agent, which may be a mycoplasma, virus,

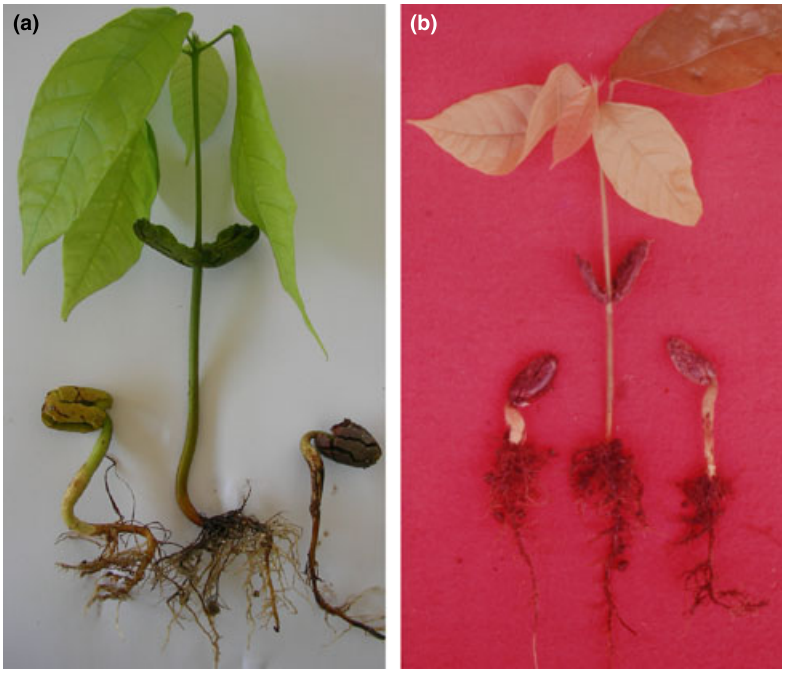

Figure 6 (a) Experiment to test infectivity of leaf sap from dwarf plant (Fig. 5a), showing results 17 days after inoculation of pregerminated cacao beans, control in centre; (b) results of inoculation of pregerminated cacao beans with spore suspension of *Moniliophthora roreri* (control in centre) in Ecuador, 1975 (see Evans, 1981).

viroid, prion or some as yet unidentified particle, then alters and controls the hormonal balance in those targeted tissues, resulting in the classic symptoms of witches' broom disease or, in the case of *M. roreri*, in the hypertrophy of pod tissues. This two-way symbiosis enables the fungus to vastly increase its sporulation capacity, whilst ensuring the propagation and transmission of the infectious agent, to the detriment of the plant host. It is further opined that a similar tritrophic interaction may be involved in the witches' brooms induced in their plant hosts by other fungal pathogens, most notably, some species of *Fusarium* and rust fungi.

Concluding Remarks

In the most recent phylogenetic examination of the genus *Moniliophthora* (T. L. Tarnowski, IFAS, University of Florida, USA, personal communication), the molecular data indicate that the previously designated Cacao (C-) and Solanum (S-) biotypes (see Griffith *et al.*, 1994, 2003) fall within the same clade (Clade 1a *Theobroma*; Clade 1b *Solanum*), as distinct from the Malpighiaceae clade (Clade 3), which corresponds with the H-biotype of Griffith *et al.* (2003), based on the malpighiaceous host *Heteropterys*, and that these represent the most recent lineages. The intermediate Clade 2 contains unidentified, seemingly disparate hosts, but it is conjectured that these may represent the oldest lineages of non-pathogenic, endophytic and heterothallic isolates from 'lianas'. Typically, the basidiomata occur on dead vines hanging in or lodged within the understorey canopy, or fallen to the forest floor with no evidence of tissue abnormalities (brooms), the so-called L-biotype (Evans, 1977, 1978) that probably belong to the Bignoniaceae, as reported subsequently (Griffith *et al.*, 1994, 2003). However, it is argued that this terminology should not be adopted because liana is used descriptively for the growth habit, and does not infer a taxonomic grouping (T. L. Tarnowski, IFAS, University of Florida, USA, personal communication).

Griffith *et al.* (1994) also determined that the biotype of *M. perniciosa* isolated from the bignoniaceous vine *Arrabidaea verrucosa* in western Ecuador lacks clamp connections and is heterothallic, as well as non-pathogenic and endophytic, and probably therefore represents the ancestor of the more specialized homothallic pathogenic biotypes from *Theobroma* and *Solanum* hosts. Subsequently, Griffith *et al.* (2003) reported *in vitro* putative hybridization between the mycelium of this heterothallic biotype and the non-clamped mycelium of *M. roreri*. The hypothesis that *M. perniciosa* evolved as an endophyte has also gained support from a recent study that identified isolates of the fungus from non-diseased branches of cacao (Lana *et al.*, 2011), mirroring the results obtained in a survey of the endophytes associated with the wild host *T. gileri* when *M. roreri* was recovered from healthy pods and stems (Evans *et al.*, 2003b). Finally, the hypothesis that this endophytic progenitor of *M. perniciosa* evolved in a series of jumps to attack hosts in various plant families, including cacao, as specialized non-outcrossing (primary homothallic) bio-

types, potentially through acquiring a pathogenic agent, would appear to be holding true (T. L. Tarnowski, IFAS, University of Florida, USA, personal communication).

Research priorities

This report prioritizes those areas of research that need to be addressed if advances in the understanding of these sophisticated pathogens are to be made and, thereby, their long-term management improved.

Proof of pathogenicity

Previously, the hypothesis that *Moniliophthora* is an endophytic genus with two well-defined nutritional forms was alluded to. These are the monokaryotic intercellular mycelium, with haustorial-feeding function, and the dikaryotic intracellular mycelium, with an extracellular-enzyme feeding mode on naturally senescing host tissues. In all probability, the relationship is symbiotic or neutral and not parasitic in most species. The new but essentially still circumstantial evidence suggests that both *M. perniciosa* and *M. roreri* acquired an infectious agent during their evolution that altered the nutritional status and caused growth changes in the host. Therefore, the priority is to further investigate these findings, and more comprehensive experiments are planned for the near future in order to characterize the causal agent. The mechanisms behind pathogenicity can then be addressed on a more informed basis.

Proof of sexuality

The meiotic events observed in *M. roreri* using standard cytology now need to be re-examined and confirmed using updated techniques to quantify the relative nuclear content at each stage of sporogenesis. Such 'cryptosexuality' has recently been demonstrated in the supposed 'urediniospores' of coffee leaf rust (*Hemileia vastatrix*) through the use of DNA image cytometry (Carvalho *et al.*, 2011). This has considerable implications for cacao breeding strategies. A sexually reproducing fungus producing billions of meiospores explains why so little resistance to *M. roreri* has been identified in cacao and why all its nearest relatives in the genera *Theobroma* and *Herrania* are susceptible (Evans, 1981, 2007). In terms of cacao production, this makes it an even more challenging and dangerous 'opponent' than *M. perniciosa*.

Genetic variation

There is obvious overlap with the above priorities but this should be a dedicated study involving an all-encompassing collection of material of both *M. perniciosa* and *M. roreri* for molecular analysis and pathogenicity screening, especially concentrating on wild hosts in the genus *Theobroma*. A start has been made (Aime & Phillips-Mora, 2005), but the genetic variation needs to be better determined within the biotypes and, in particular, their interactions with cacao. If the *M. roreri* isolates from wild species of *Theobroma* are essentially non-pathogenic to cacao, as preliminary screening suggests (Evans *et al.*, 2002, 2003a), as well as being cryptic endophytes (Evans *et al.*, 2003b), then it is important to establish the mechanisms involved and if this resistance is durable. Similarly, with *M. perniciosa* there is circumstantial evidence of considerable genetic variation within the biotypes from cacao, especially on the eastern and western sides of the Andes, but conflicting reports about the intra- and inter-species specificity of the biotypes from all the plant family hosts (Bastos & Evans, 1985; Resende *et al.*, 2000; Evans, 2007). The search for the endophytic progenitor is particularly relevant and this may possibly be represented by the heterothallic, non-pathogenic biotype reported on Bignoniaceae in western Ecuador.

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