The need to carry out re-inventory of plant pathogenic fungi

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OUTLINE

Plant pathogenic fungi have long been documented through concerted efforts of mycologists and plant pathologists; these records have served as the basis for regional and countrywide checklists which have since been put into databases listing hosts and associated fungi. They are used by governments and scientists to formulate trade quarantine policies and determine research funding, such as in plant breeding programs and disease control. With the ability to use molecular characters to study the systematics of fungi it is clear that morphologically defined species are often large complexes comprised of genetically and biologically distinct species. Use of molecular techniques to examine species complexes has revealed cryptic species in many important plant pathogenic genera, e.g. Botryosphaeria, Colletotrichum, Fusarium, and Mycosphaerella. It has occurred to such an extent that existing checklists and databases need updating. It is important that the data from these studies, including changes in taxonomy and nomenclature, be incorporated into the databases of plant pathogenic fungi to support accurate plant quarantine decisions. In addition, epitypifying fungi by re-collecting material from type habitats and isolating the organism into pure culture will provide essential materials for systematics studies to further clarify the taxonomy and phylogeny of plant pathogenic fungi. Overall, we conclude that disease lists are likely to be highly outdated and advocate the need for countrywide re-inventory of plant pathogens. As a result of these studies, tools can be developed that use morphological or molecular characters, or both, to promote accurate identification of plant pathogenic fungi.

Key words: Cochliobolus, Colletotrichum, Diaporthe, Fusarium, Phomopsis, cryptic species, disease associated fungi, quarantine.

Introduction

There have been worldwide concerted efforts by generations of mycologists and plant pathologists to document plant disease-associated fungi (Roger, 1951-1954; Holliday, 1980; Kohler et al., 1996), although the disease causal agents are probably better known in temperate than tropical regions (Hofmann et al., 2010). Surveys of plant pathogens have resulted in the publication of numerous checklists (Peregrine & Kassim, 1982; Dingley et al., 1981; McKenzie & Jackson, 1986; Hyde & Alcorn, 1993) and more recently many of these have been incorporated into databases such as: USDA, http://nt.ars-grin.gov/fungaldatabases/fungushost/fungushost.cfm; Database of Plant Disease Names in Japan, www.gene.affrc.go.jp/databases-micro_pl_diseases_en.php; New Zealand Fungi, http://nzfungi.landcareresearch.co.nz; Fungos relatados em plantas no Brasil - http://pragawall.cenargen.embrapa.br/aiqweb/michtml/micbanco01a.asp. These lists of plant pathogenic fungi have been extremely important in formulating quarantine policies and thus have an immense impact on trade and global biosecurity.

Traditionally, researchers primarily relied on morphology to identify the plant-associated fungi. Usually surveys would be carried out, disease material collected, dried and returned to the laboratory where the associated fungi were later identified (Hyde & Alcorn, 1993). Molecular data have rapidly advanced the understanding of species boundaries and relationships in several important plant pathogenic genera, revealing numerous cryptic species (Cai et al., 2009; Kvas et al., 2009; Summerell et al., 2010). Databases usually continue to cite fungal taxa recorded in dated publications, but as our knowledge of disease-associated fungi has increased, the checklists and databases have become more and more inaccurate. This has occurred to such an extent that existing checklists (e.g. Anonymous, 1960; Guba, 1961; Raabe, 1966) and databases need updating. It is therefore imperative to re-collect many of these pathogens in species complexes and re-identify them based on modern protocols and updated species concepts. For example, Phoulivong et al. (2010a) have demonstrated that the previously believed axiom that “most of the fruit rot diseases in tropics are caused by Colletotrichum gloeosporioides” was wrong. Mycologists therefore need to re-identify all these diseases and give them names that reflect their natural relationships.

In a recent paper Hyde et al. (2010) were of the opinion that plant pathologists and mycologists should re-inventory Australian plant pathogens in order to make quarantine measures more effective. The purpose of this
paper is to give examples of some selected tropical plant pathogenic genera where the utilization of updated species concept and species recognition criteria (Cai et al., 2011) has improved the discrimination for delimiting species. These examples illustrate the need for fresh inventories of tropical plant pathogens to be carried out in all countries, to ensure that countries have an accurate understanding of the fungal pathogens within their boundaries and, perhaps more importantly, are fully aware of the quarantine pathogens that need to be kept out.

**Phylogenetic Methodology**

Sequences for selected fungal genera were downloaded from GenBank and aligned using Clustal X. The alignment was optimized manually to allow maximum alignment and maximum sequence similarity. Gaps were treated as missing data. Phylogenetic analysis was carried out based on the aligned dataset using PAUP* 4.0b10 (Swofford, 2002). Ambiguously aligned regions were excluded from all analyses. Trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed, and all multiple parsimonious trees were saved. Trees were figured in TreeView (Page, 1996).

**Selected Examples**

*Cochliobolus* and its anamorphs

*Cochliobolus*, which was introduced by Drechsler (1934), is typified by *C. heterostrophus* (Drechsler) Drechsler. Previously, many species of *Cochliobolus* had been included in *Ophiobolus*, a genus with bitunicate asci and scolecospores (Drechsler, 1934; Arx & Olivier, 1952). Alcorn (1983) thought that the characters used to distinguish *Pseudocochliobolus* from *Cochliobolus* were artificial and treated the former as a synonym. However, the above treatments were all based on morphology, and the genus has not been well examined using modern protocols. The classical identification of *Cochliobolus* and its anamorphs has encountered some problems due to the plasticity of morphological traits under different growth conditions (Old & Roberston, 1969; Hosokawa et al., 2003). We predict that new molecular developments will lead to a rearrangement of taxa at the specific or subspecific level in the near future.

Currently, 56 *Cochliobolus* species names are listed in Index Fungorum and 20 are listed in the USDA database, and the Dictionary of the Fungi (Kirk et al., 2008) estimates that the genus comprises 22 species. *Cochliobolus* has anamorphs in *Bipolaris* and *Curvularia* and there are 73 estimated species of *Bipolaris* and 54 estimated species of *Curvularia* (Kirk et al., 2008); teleomorph / anamorph connections have rarely been resolved for *Cochliobolus*. An extensive re-inventory of *Cochliobolus* and its anamorphs should be carried out because 1) some species defined based on morphology often contain more than one phylogenetic species, which are increasingly being formally introduced; 2) numerous species that do not have ex-type cultures to facilitate DNA extraction and epitypifications of many taxa are urgently needed; and 3) there are still arguments regarding the synonymy of *Pseudocochliobolus* with *Cochliobolus* (Berbee et al., 1999) and placement of *Bipolaris* and *Curvularia* anamorphs which are shown to be phylogenetically distant (Groves & Skolko, 1945; Putterill, 1954; Ellis, 1966; 1971; Alcorn, 1983), and these are unlikely to be resolved until a larger number of samples from living ex-type cultures are included in analyses.

GenBank likely contains many wrongly named taxa within *Cochliobolus* (Figure 1). Twenty-four ITS sequences named as *Cochliobolus lunatus* were downloaded from GenBank and aligned with ITS sequence data derived from ex-type cultures of *Cochliobolus* species. “*Cochliobolus lunatus*” sequences are scattered throughout the phylogram containing these ex-types, indicating that numerous strains labeled *C. lunatus* in GenBank have been misidentified. The confusion among *Cochliobolus* and its anamorphs indicate that many records in countrywide checklists are likely to be wrong and a re-assessment using modern protocol is necessary.

**Colletotrichum**

The previous understanding of *Colletotrichum* species was based on morphology and to a lesser extent on cultural characters (Than et al., 2008; Hyde et al., 2009). Sutton (1980) accepted 40 species in *Colletotrichum* based on morphology in culture, and until very recently this taxonomic scheme was followed by most researchers. Hyde et al. (2009) published a list of 66 current names with notes, while Cai et al. (2009) detailed ways in which to deal with species concepts in the genus using a polyphasic approach. Prior to these publications several grass-associated *Colletotrichum* species had been epitypified and several new species formally regarded as *C. graminicola sensu lato* had been introduced (Crouch et al., 2009; Crouch & Beirn, 2009). Similarly, Damm et al. (2009) described 18 species with curved spores, of which ten were epitypes and four were new species. These publications set a standard for revising the various *Colletotrichum* species complexes and have resulted in several publications revealing and describing species within the *C. gloeosporioides* species complex (Rojas et al. 2010; Phoulivong et al., 2010b). These revisions were only possible following the epitypification of *C. gloeosporioides* (Cannon et al., 2008). *Colletotrichum acutatum* and *C. boninense* are likely to be treated in a similar way in a future issue of *Studies in Mycology* (Damm et al. pers. comm.). Hyde et al. (2009) listed all currently accepted species of *Colletotrichum*, with the information of type specimens, ex-type cultures, multilocus sequences and references to each species. Such summarizing of information will significantly increase the efficiency and accuracy for anyone who wants to identify a *Colletotrichum*.
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FIGURE 1 - Maximum parsimony phylogram generated from ITS sequence analysis of sequences named “Cochliobolus lunatus” (shown in blue) downloaded from GenBank with those derived from Cochliobolus ex-type cultures (shown in red). Data were analyzed with random sequence addition, unweighted parsimony, and treating gaps as missing data. The tree is rooted with Cochliobolus kikuchii.

pathogen. Other important pathogenic groups should also be summarized with such data.

Prihastuti et al. (2010) neotypified C. falcatum with a fresh collection from the original sugarcane field in Java, Indonesia from which the type was described. This species causes red stripe of sugarcane leaves and can be serious in some countries (e.g. India, Pakistan, Bangladesh and Taiwan, Crouch & Beirn, 2009). In a phylogenetic analysis, we downloaded all ITS sequences named C. falcatum from GenBank (accessed 1 July 2010) and aligned them with sequences from other closely related taxa, including some of the sequences from types. The C. falcatum GenBank accessions are indicated by their GenBank accession numbers in green while the reference taxa are shown in blue (Figure 2). Comparison of ITS sequence data from the neotype against sequences deposited in GenBank shows that only one ITS sequence (AB462376, generated from strain MAFF306170) is identical to the neotype (100% similarity), and only four of the 23 C. falcatum sequences cluster in the same clade in the parsimonious tree (shown in red) (Figure 2). The “C. falcatum” strains scattered throughout the tree indicate that some sequences deposited in GenBank have C. falcatum names that are almost certainly wrongly applied. The diversity of ITS sequences of C. graminicola and C. gloeosporioides deposited in GenBank is even more diverse (Crouch et al., 2009; Cai et al., 2009).

As similar changes are expected to take place in other species complexes in Colletotrichum, it is certain that records of Colletotrichum plant disease-associated fungi in all tropical countries is outdated. For example,
**FIGURE 2** - Maximum parsimony phylogram generated from sequence analysis of ITS sequences of "Colletotrichum falcatum" downloaded from GenBank and other related taxa. Data were analyzed with random sequence addition, unweighted parsimony and treating gaps as missing data. ITS sequences of "C. falcatum" are shown in green; neotypes and other identified C. falcatum strains are shown in red. Other reference taxa are shown in blue. * indicates the sequences derived from ex-type cultures. The tree is rooted with Colletotrichum cereale.

Dingley et al. (1981), following the accepted protocols and taxonomy of the time, recorded Glomerella cingulata on 66 different host plants in 32 families. In addition, Dingley et al. (1981) listed a further 25 host records, based on earlier publications, as either Glomerella sp. or Colletotrichum sp.; numerous other diseases on a broad range of hosts were recorded as Glomerella tucumanensis, Colletotrichum acutatum, C. capsici, C. circinans, C. grassipes, C. dematium, C. fructigenum, C. graminicola, C. musae, C. orbiculare and C. truncatum.Judging by the fairly wide host ranges ascribed to some of these species, and to recent knowledge on Colletotrichum taxonomy, it is extremely doubtful that many of these records can be accepted. They certainly do not provide the necessary certainty required for robust biosecurity decisions, and there is obviously an urgent need for a re-inventory of these pathogens. Although the above described situation applies to Pacific island records of these fungi, the same uncertain situation exists all over the world.

**Fusarium**

Fusarium contains economically important plant pathogens, several species that are important producers of mycotoxins, the latter being recognized as human pathogens and often isolated as endophytes (Summerell et al., 2010). For this reason the genus has been relatively well studied
and species concepts are fairly advanced. In 2001 there were an estimated 50 Fusarium species, while estimates in Kirk et al. (2008) were 111, indicating a rapid increase in the naming of species. Three basic concepts are currently used to define species of Fusarium – morphological, biological and phylogenetic (Leslie & Summerell 2006; Summerell et al., 2010); however, a single morphological species often contains several biological or phylogenetic species. The EF-1α gene is regarded as a particularly good marker for species of Fusarium (O’Donnell et al., 2009; Summerell et al., 2010). However, genes such as rpβ-2, β-tubulin, calmodulin and IGS rDNA, polygalacturonases, mitochondrial small subunit ribosomal RNA, phosphate permease, nitrate reductase, MAT 1 and MAT2 have also been used (O’Donnell et al., 2009). One example of progress in Fusarium is the understanding of the Gibberella fujikuroi complex. This complex comprises an assemblage of Fusarium species with similar and overlapping morphological traits (Leslie et al., 2001; Kvas et al., 2009). Devastating diseases of many economically important plants have been linked to species in this complex, which also produce a wide range of secondary metabolites or mycotoxins that contaminate food and feedstuffs worldwide, causing a variety of diseases in humans and animals (Kvas et al., 2009). Currently 34 morphospecies are recognized in the complex based on morphological species recognition, ten are well-characterised biological species or so-called mating populations, and phylogenetic species recognition show at least 50 distinct phylogenetic species or lineages (Kvas et al., 2009). However, the resolving power of phylogenetic species recognition far outweighs that of morphological or biological species recognition (Kvas et al., 2009).

Recognition of all known Fusarium species in the Gibberella fujikuroi complex is possible by applying only phylogenetic species recognition. However, such identifications may yield results that are not biologically meaningful (Summerell et al., 2010). Phylogenetic species recognition is therefore generally used in combination with morphological and biological species recognition in this complex. Most of the current species definitions and descriptions in this complex are based on a polyphasic or integrative taxonomic approach (Aoki et al. 2005; Kvas et al., 2009). For example, through morphological and cultural characterization, multilocus phylogenetic analyses and sexual compatibility studies, Amata et al. (2010) demonstrated that F. brevicatenulatum and F. pseudoanthophilum belonged to the same species. This integrative approach also extends to routine species identifications. Because of the rapid changes and advances in understanding the species complexes in Fusarium and the increase in numbers of cryptic species introduced (Kvas et al., 2009; Summerell et al., 2010) there is a need for re-inventory of this important group of fungi. The identification in this re-inventory should be based on multilocus phylogenetic tools and updated species concepts (Cai et al., 2011). Molecular protocol that could readily diagnose species accurately and quickly has been done for some complexes (e.g. Zhang et al., 2008).

Phomopsis

Phomopsis is an important phytopathogenic genus, introduced by Saccardo (1905). Phomopsis are anamorphic Diaporthe and occur as endophytes, pathogens or saprobes on a wide range of hosts (Mostert et al., 2001). There are over 800 species based on host associations (Uecker, 1988), but recent phylogenetic studies suggest that host association is of minor importance in the taxonomy of this genus (Rehner & Uecker, 1994; Murali et al., 2006).

Stem canker of Helianthus annuus (sunflower) was first recorded in 1980 in the former Yugoslavia, and the causative agent of this serious disease was described as Diaporthe helianthi, with a Phomopsis helianthi anamorph (Cvetkovic et al., 1981). This name often appears in the literature, and the fungus has been widely studied for its genetic variability, epidemiology and phytosanitary concerns as it causes a devastating disease on economically important hosts all over the world. We downloaded 44 ITS sequences named Diaporthe helianthi, and some of the type derived sequences of Phomopsis/Diaforthe from GenBank (accessed 31 August 2010). The phylogram generated from the analysis shows the close relationship of most of the sequences to the ex-type culture, but there are eight records that deviate significantly from the type (Figure 3). These are either due to misidentification, plasticity in the nature of morphological characters, or to obscure delimitation of morphological species boundaries.

Diaporthe helianthi shows a narrow range of host distribution compared to most other members of Phomopsis/Diaforthe. Although genetic variability among the various isolates of D. helianthi has been widely studied in Australia and Europe (Pecchia et al., 2004; Recab et al., 2004), it is unsure if all isolates in these studies have correctly applied names. The ambiguity and significant deviation of several GenBank records emphasizes the importance of the use of ex-type cultures in the identification of this important pathogen. In other words, the existence of an ex-type culture would avoid misidentification of the pathogen isolated from distant geographical locations.

The analysis of ITS sequence data for taxa within Phomopsis/Diaforthe may identify well supported groups of closely related taxa, but phylogenetic relationships for more distantly related taxa from a wide range of hosts cannot be determined with a high level of confidence. Analysis of multiple genes will be required to sort out phylogenetic relationships among species of Phomopsis/Diaforthe (Farr et al., 2002), on which to base taxonomic revision. As a result of those systematics studies, tools to accurately identify species of Phomopsis can then be developed and utilized by diagnosticians, plant pathologists, and by plant regulatory agencies.
**FIGURE 3** - Maximum parsimony phylogram generated for the ITS sequence analysis from sequences of "*Diaporthe helianthi*" available in GenBank with available sequences derived from ex-type cultures. ITS sequences of *D. helianthi* downloaded from GenBank are shown in black. The type derived sequence of *D. helianthi* is shown in red and other type derived records are in blue. Data were analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. The tree is rooted with *Corynespora cambrensis*. 
Concluding Remarks

We have discussed several important genera of plant pathogens and shown how molecular studies have revealed the existence of species complexes and made it possible to characterize and describe many new cryptic species. Similar changes are also occurring in several other important pathogenic genera such as Botryosphaeria (Denman et al., 2000; Crous et al., 2006; Phillips et al., 2008), Mycosphaerella (Crous et al., 2009; Bensch et al., 2010) and Phoma (Aveskamp et al., 2008; 2010).

The systematics and naming of plant pathogenic fungi is extremely important (Rossman & Palm-Hernández, 2008). Species concepts for many important plant pathogenic fungi have changed due to the use of molecular tools to study those organisms. Morphology has always played a key role in delimiting species and genera. Additionally, some species were described based on the host on which they occurred, because most plant pathogenic fungi were thought to be highly host specific. More recently molecular studies have shown that morphological species in plant pathogenic fungi are often species complexes that comprise genetically and biologically distant species. Previously, in studying plant pathogenic fungi, obtaining a living culture was not a significant concern for most mycologists as herbaria were used for typification (Hawksworth, 1974). In modern practice, mycologists are now using molecular characters and sequence analyses to study evolutionary relationships and classify plant pathogenic fungi. This practice relies on living cultures as high quality DNA samples are less likely to be extracted from dried herbarium samples. Because living cultures were not a strict necessity for the host-oriented and morphological species concepts, most of the currently applied names of plant pathogenic fungi do not have a living ex-type culture. This has prevented studies of the organism’s biology as well as extraction of DNA for molecular analyses.

Given the importance of living cultures for understanding the biology and systematics of plant pathogenic fungi (Abd-Elsalam et al., 2010), there is an urgent need to epitipify many fungal species and re-identify pathogens, especially those previously placed in the species complex. This may necessitate returning to the original collecting localities and original hosts to collect morphologically identical specimens and to deposit them as epitypes, supplemented with ex-epitype strains (Hyde & Zhang, 2008). Revised checklists and databases should be supported by herbarium materials, living cultures and DNA libraries to establish the link between DNA sequences and type specimens for taxonomic verification. The newly established cryptic species should, however, be further studied by plant pathologists for various characters such as host range and severity, and thus to determine if any of them bear quarantine significance. Molecular biologists will also need to develop fast and accurate protocols that can easily distinguish the quarantine-significant ones from their close relatives.

Acknowledgements

Work on the Dothideomycetes was supported by the TRF/BIOTEC Special Program for Biodiversity Research and Training grant BRT R253012 and work on Colletotrichum was supported by Mae Fah Luang University research grants 51101010029 and 52101010002. Lei Cai acknowledges funding support from CAS (KSCX2-YW-Z-1026) and NSFC (31110103906).

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in the Biosciences 12:357-358.


