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The future of coelomycete studies

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Abstract – Coelomycetes are significant fungi, being important plant pathogens responsible for a wide range of diseases worldwide and utilized in industry with some being prolific producers of novel compounds. Morphological identification of genera and species, and species complexes of cryptic taxa is difficult because of the dearth of distinctive morphological characters. With molecular techniques at our disposal we can now begin to understand the phylogeny and complexity of these poorly studied organisms. Large numbers of genera have to be recollected and restudied using molecular techniques. Herbarium material and living cultures must be deposited as a result of future studies and where possible species (and genera) must be epitypified. These efforts will help in the reclassification and phylogeny of the coelomycetes, understanding species complexes and will provide important data for future plant pathology, quarantine and industrial needs.

Asexual fungi / Cryptic species / Pathogens / Species-complex

INTRODUCTION

Coelomycetes, asexual fungi which produce conidia in conidiomata are significant as pathogens, endophytes and saprobes. These fungi have been reported as pathogens in agriculture (Ali *et al.,* 2009), forestry (Old *et al.,* 2003; Cortinas *et al.,* 2006), horticulture (Crous *et al.,* 2011) and import-export trades (Aveskamp *et al*., 2008) worldwide. Much morphological research has been carried out on major pathogenic coelomycete genera such as *Ascochyta* (Punithalingam, 1979, 1988), *Colletotrichum* (von Arx, 1957, 1970), *Pestalotiopsis* (Guba, 1961), *Phoma* (Boerema *et al.,* 1997, 1999) and *Phyllosticta* (van der Aa, 1973). These efforts have developed the basic taxonomy of these genera, but due to the dearth of morphological features a relatively poor understanding of the generic and species boundaries within the coelomycetes remains (Torres *et al.,* 2005; de Gruyter *et al.,* 2009). Precise generic and species delimitation is critical

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for understanding the ecology, conservation and for evaluating the biodiversity, and diversification of these organisms (Lumbsch & Leavitt, 2011). With the introduction of molecular based taxonomy, many mysteries have been solved such as resolution of species complexes and generic boundaries. In this paper we discuss future research that is required.

DISCOVERY OF CRYPTIC SPECIES AND RE-INVENTORY OF PATHOGENS

Molecular techniques have shown that several "species" in pathogenic genera such as *Colletotrichum, Lasiodiplodia, Phoma* and *Phyllosticta*, are actually "species complexes" (Alves *et al.,* 2008; Aveskamp *et al.,* 2008, 2010; de Gruyter *et al.,* 2009; Hyde *et al.,* 2009; Wulandari *et al.,* 2009; Cai *et al.,* 2011a, Wikee *et al.,* 2011a). *Colletotrichum gloeosporioides* (Hyde *et al.,* 2010; Phoulivong *et al.,* 2010), *Lasiodiplodia theobromae* (Alves *et al.,* 2008; Phillips *et al.,* 2008; Abdollahzadeh *et al.,* 2010) and *Phoma lingam* (Aveskamp *et al.,* 2008) are examples of species which have been shown to be species complexes. These species complexes consist of cryptic species i.e. morphologically hard to distinguishable, but resolved as distinct species based on molecular data (Alves *et al.,* 2008; Shivas & Cai, 2012), have also been described as "*populations which are phylogenetically distinct and able to reproduce themselves, by sexual means or otherwise, but which are distinguished by molecular or other features that are either not evident macroscopically or generally overlooked*" (Hawksworth, 2010). Almost all pathogenic species are complexes of cryptic species hence Crous & Groenewald (2005) stated, "*Show me a plant pathogen, and I will show you a species complex"*. Hawksworth (2010) distinguish two natures of "species complexes". 1) In most cases the populations are closely related, i.e. have a recent shared common ancestor. 2) In some cases the cryptic species do not occupy the same clade, nor do they have a recent common ancestor.

With molecular taxonomy and other biochemical methods, cryptic species have been split into two or more species or even new genera (Faris-Mokaiesh *et al.,* 1996; Fatehi *et al.,* 2003; Lee *et al.,* 2004; Verkley *et al.,* 2004; Alemu *et al.,* 2005; Cheewangkoon *et al.,* 2010; Crous *et al.,* 2011). For example, Phoulivong *et al*. (2010) showed that *C. gloeosporioides* which was previously considered to be a ubiquitous species causing anthracnose of tropical fruits, was not present from his collection of selected hosts from Thailand and Laos. Tropical fruit anthracnose in Laos and Thailand was shown to be caused by the cryptic species *C. asianum, C. fructicola, C. horii, C. kahawae* and *C. gloeosporioides*. Recently Damm *et al.* (2012) resolved the *C. boninense* complex and concluded it comprised 15 cryptic species including 12 new taxa. With the expansion of knowledge of pathogens, it is essential to update the databases including modification of nomenclature and taxonomy as it may cause confusion in many areas such as in quarantine and plant health (Ko Ko *et al.,* 2011). The correct taxonomic naming of species is also significant for plant breeding and disease management (Hyde *et al.,* 2010; Cai *et al.,* 2011; Ko Ko *et al.,* 2011). For example, the Thai chilli industry suffers from anthracnose disease, previously thought to be caused by *Colletotrichum capsici* and *C. gloeosporioides*. The chilli cultivars *Capsicum annuum* were bred with resistant against these species (Mongkolporn *et al.,* 2004). However, a careful survey of the causal agents of chilli anthracnose

by Than *et al.* (2008) showed three species *C. acutatum, C. capsici* and *C. gloeosporioides,* to be the most common anthracnose pathogens. *Colletotrichum acutatum* was newly recorded for Thailand and posed a new threat to the chilli industry. These three species were later renamed as cryptic species indicating the need to understand taxonomy before carrying out plant resistance breeding efforts. Still much more research needs to be carried out to solve the *C. acutatum* complex (Sreenivasaprasad & Talhinhas, 2005) as it is also a well known pathogen in a wide range of crops worldwide.

QUARANTINE NEEDS AND IMPORTANCE OF IMPLEMENTING TECHNIQUES FOR DETECTING PATHOGENS

Incidents have been reported recently concerning introductions of quarantined fungal taxa from one country to another on imported agriculture crops (Hyun *et al.,* 2005; Meyer *et al.,* 2012). Although the correct identification of pathogens is extremely important in quarantine, the identification is often based on characters of disease material (Hyde *et al.,* 2010) and culture based morphological approaches (Lievens & Thomma, 2005). *The reliance on the ability of the organism to be cultured, the time consuming and laborious nature, and the requirement for extensive taxonomical knowledge, all together often complicating timely disease management decisions* are limitations to a morphological approach (Lievens & Thomma, 2005). Since many pathogens are species complexes, it is also difficult to identify them precisely on the basis of morphology (Abd-Elsalam *et al.,* 2010). Correct detection of pathogens is important to producers, exporters and regulatory authorities to prevent unnecessary losses i.e. chemicals, exportation and expenses (Meyer *et al.,* 2012). Therefore, fast and reliable molecular methods are essential for the detection of quarantine pests (Aveskamp *et al.,* 2008). Molecular markers are a better solution as they are fast and may provide accurate detection (Paplomatas, 2006) as compared to ordinary techniques. Tsui *et al.* (2011) discussed some modern techniques for the pathogen identification and fungal detection. These included fluorescence in situ hybridisation (FISH), DNA array technology, Multiplex tandem PCR, and Padlock probe technology with rolling circle amplification and loop-mediated isothermal amplification (LAMP).

Pathologists are tending to use molecular based identification in plant quarantine and taxonomists have highlighted the importance of maintaining culture collections as reference herbaria with living material, instead of dried specimens in herbaria (Abd-Elsalam *et al.,* 2010). Recent molecular assays used for the identification of common pathogens are given below. For resolving complexes in *Colletotrichum*, partial actin (ACT), β-tubulin-2 (TUB1, TUB2), calmodulin (CAL), chitin synthase 1 (CHS-1), glyceraldehyde-3-phosphate dehydrogenase (GPDH), histone3 (HIS3) and internal transcribed spacer region (ITS1, 5.8S nrDNA and ITS2) were better molecular assays (Phoulivong *et al*., 2010; Damm *et al.,* 2012). Irinyi *et al.* (2009) discussed the importance of re-evaluation of *Phoma*-like (*Ascochyta* and *Phyllosticta*) pathogens which have complex generic boundaries by using protein sequences. They concluded that protein-coding genes (TEF1 and TUB) are effective within *Phoma* to infer phylogenetic relationships. Meyer *et al.* (2012) discussed the molecular assays used for the detection of *Phyllosticta citricarpa* (sexual stage *Guignardia citricarpa*)

which causes citrus black spot disease in many parts of the world except Europe and USA. Several detection methods are available to detect *P. citricarpa* and its sexual stage (Meyer *et al.,* 2006; Peres *et al.,* 2007) as it is complicated to identify this species morphologically. For the accurate identification of *Diaporthe* (*Phomopsis*), Udayanga *et al.* (2012a, b) used the combined analysis of ITS, calmodulin (CAL), partial translation elongation factor 1-alpha (TEF1) and beta tubulin (TUB) gene regions along with mating type genes. Zhang *et al.* (2012) used combined ITS, TUB and TEF1 gene regions to identify pathogenic *Pestalotiopsis*.

Molecular phylogenetic studies however, have not entirely replaced traditional culture and morphology based tests. The polyphasic approach which considers morphological characters, cultural and biochemical characters along with molecular assays is a reliable technique. Cai *et al.* (2009) and Aveskamp *et al.* (2010) discussed the use of polyphasic approaches for the quarantine significant genera *Colletotrichum* and *Phoma*. Wang *et al.* (2012) used a polyphasic approach to identify four *Phyllosticta* species, namely *P. citricarpa, P. citrichinaensis, P. capitalensis* and *P. citriasiana* that cause *Citrus* diseases in China. They used the sequences of ITS, TEF1 and partial actin gene (ACT) as molecular assays, while also using morphological, cultural and biochemical characters. Su & Cai (2012) also used polyphasic characterisation when introducing three *Phyllosticta* species. Some detection methods for important quarantine pathogens are listed in Table 1.

These methods are all rather complicated as they are time consuming and require sequencing of more than one gene. Ideally a single barcoding gene is needed that can accurately identify species, but this has often not been forthcoming. However, Wang *et al.* (2012) were able to develop a specific primer Pca8/ITS4 for *Phyllosticta citriasiana* and its corresponding PCR products were used to identify *P. citriasiana* from *P. citricarpa* and *P. capitalensis.*

Real-time polymerase chain reaction (qPCR) is another accurate, fast and powerful culture-independent molecular tool that can be used for the detection of pathogens (Schaad *et al.,* 1999; Nicholson *et al.,* 2003; Mackay, 2007). This technique uses genetic material from multiple microbial species in a single

Table 1. Examples for detection methods for important pathogens

Pathogen	*Detection method*	*References*
Colletotrichum spp.	Polyphasic approach – molecular assays, morphology, physiology, pathogenicity, cultural characteristics and secondary metabolites	Cai *et al.,* 2009; Than *et al.,* 2008
Phoma spp.	Molecular phylogenetic approach- 28S nrDNA (Large Subunit - LSU), 18S nrDNA (Small Subunit - SSU), the Internal Transcribed Spacer regions 1 & 2 and 5.8S nrDNA (ITS), and part of the β-tubulin (TUB) gene region	Aveskamp *et al.,* 2010
Phoma-like (*Ascochyta* and *Phyllosticta*)	Molecular phylogenetic approach- Protein-coding genes (tef1 and β-tubulin)	Irinyi *et al.,* 2009
Phyllosticta spp.	Polyphasic approach - phylogeny, host association, disease symptoms, colony and morphological characteristics, and biochemical characters	Su & Cai, 2012; Wang *et al.,* 2012

sample and can detect species within species complexes (Johnson *et al.,* 2005). Ioos *et al.* (2011) were able to develop real-time PCR to detect *Dothistroma pini, D. septosporum,* and *Lecanosticta acicola* which cause severe foliage diseases in conifers. Nested polymerase chain reaction-based diagnostic assay is another accurate detection technique which can be used in quarantine (Palacio-Bielsa *et al.,* 2006). Langrell (2011) used this assay to detect *Dothistroma septosporum* which is the causal agent of red band needle blight of pine.

RE-COLLECTING, TAXONOMIC PLACEMENTS AND NOMENCLATURE

Many fungi including plant pathogens may have different sexual or asexual morphological forms (Wingfield *et al.,* 2011) and these forms were often not linked (pleomorphism) (Shenoy *et al.,* 2007, 2010). Thus, understanding pleomorphism is a great challenge for taxonomists, while for plant pathologists it may be a source of confusion (Cannon & Kirk, 2000; Wingfield *et al.,* 2011). As a solution to pleomorphism, Saccardo (1904) introduced the dual system for the nomenclature of fungi, providing different names for the asexual (anamorphic) and sexual (teleomorphic) stages. Due to synanamorphism this practice has lead to a huge number of names in the fungi (Wingfield *et al.,* 2011). However, with the advent of molecular techniques it is now possible to establish taxonomic links between many sexual and asexual genera (Lee *et al.,* 2004; Cortinas *et al.,* 2006a, b; Chilvers *et al.,* 2009). Although 256 genera (26%) of coelomycetes are linked to their sexual morph and 105 genera (11%) are linked to families or orders, there remain a large number of genera (approximately 631 or 63%) that have not been linked to any sexual morph or family (Wijayawardene *et al.,* 2012). Efforts to link morphs and using of one name for a biological species is important for quarantine, plant breeding and plant health research (Ko Ko *et al.,* 2011). Quarantine organisations in some countries list asexual morphs as undesirable pests, while others list sexual morphs and this leads to confusion (Wingfield *et al.,* 2011). There is much to be done in deciding which name of the linked genera to use (Hawksworth, 2012). For example, highly polyphyletic genera such as *Phoma* need considerable study. However, Gruyter *et al.* (2012) concluded *Phoma* sensu stricto should be confined only to *Didymellaceae.*

A large number of fungi have been described, but there many are many orphaned species without higher level taxonomic affiliations (Hawksworth, 2004). These orphaned species and genera without taxonomic relationships need recollecting, sequencing and epitypifying, so as to establish their phylogenetic relationships and possibly their sexual morphs. This evaluation and revision is an essential task as many fungi are not adequately typified. For this reason some taxonomists argue and question the possibility of movement towards "one fungus, one name" (Gams *et al.,* 2012). Once the links are known a single name can be fixed to the genus and species (Hawksworth, 2012). Re-collection of genera such as *Diaporthe* (*Phomopsis*), *Pestalotiopsis* and *Phyllosticta* which were previously mostly named based on host association is also important. Recent phylogenetic studies indicate that host association is not important in the taxonomy of many species in these genera (Uecker, 1988; Rehner & Uecker, 1994; Murali *et al.,* 2006; Maharachchikumbura *et al.,* 2011; Udayanga *et al.,* 2011, 2012a; Wikee *et al.,* 2011b).

Gams *et al.* (2012) argued that in most cases the teleomorph typified name should be accepted, while the anamorph typified name should be suppressed. Further they stated if the anamorph name is accepted due to its preference such as "widely used" the teleomorph name "*still remains valid and legitimate and can still serve as a basis for names of higher-rank taxa*". Hawksworth (2012) however, explained the new situation of managing pleomorphic fungi and stated that all legitimate fungal names are treated equally. In the case of plant pathogenic fungi, asexual morphs are more prominent than sexual morphs (Cannon & Kirk, 2002) and this must be a cause of concern when deciding on appropriate names. There needs to be intelligent discussion in determining which names should be used for the sake of advancing the naming of fungi.

BIOCHEMICAL AND INDUSTRIAL APPLICATIONS

The possibility of using coelomycetes in industries such as bioremediation and the pharmaceutical industry have experimentally been shown (El Bassam *et al.,* 2002, Gangadevi & Muthumary, 2007). Species from the coelomycete genera *Bartalinia*, *Colletotrichum*, *Coniothyrium*, *Microsphaeropsis*, *Pestalotiopsis*, and *Phoma* are used in different applications. Among these, *Pestalotiopsis* is known in the pharmaceutical industry to produce taxol (Strobel *et al.,* 1996; Metz *et al.,* 2000) and many other secondary metabolites (Xu *et al.,* 2010). *Pestalotiopsis adusta* (Li *et al.,* 2008b), *P. microspora* (Strobel *et al.,* 1996) are *P. theae* (Li *et al.,* 2008a) have been shown to produce different chemicals including alkaloids and terpenoids. *Pestalotiopsis microspora* can be used as model organism for various laboratory experiments (Metz *et al.,* 2000) and some taxonomists predict the possibility of *P. microspora* being a species complex (Maharachchikumbura *et al.,* 2011). Therefore, studies on this and other coelomycete genera whose taxonomy is poorly known are needed. If we can accurately define species in a genus we can use this data to intelligently screen new and unscreened taxa for novel compound production. For example, if a seriously pathogenic species produces a useful secondary metabolite, we could screen closely related non-pathogenic species to establish if they produce the same or similar compounds (Anzai *et al.,* 2008).

BIOLOGICAL CONTROL

Biological control refers to the use of natural enemies against a pest population to reduce the pest's density and damage to a level lower than would occur in their absence in agriculture (Charlet *et al.,* 2002). It can be considered as the suitable approach to minimize pesticide usage and thus reduce environmental problems (Bale *et al.,* 2008). Coelomycetes in genera such as *Coniothyrium* sp. (Whipps *et al.,* 2008), *Colletotrichum* (Robinson & Sharon, 1999; Boyette *et al.,* 2011) and *Microsphaeropsis* sp. (Carisse & Bernier, 2002; El Bassam *et al.,* 2002) and *Phomopsis* (Ash *et al.*, 2010, Udayanga *et al.,* 2011) are utilized as biological control agents. *Prosopidicola mexicana* Crous & C.L. Lennox is an exemplary

case of a biocontrol agent used against the invasive weed *Prosopis glandulosa* (Lennox et *al.*, 2004).

At present species of pathogenic genera in species complexes (often named as forms or varieties) are used in biological control (Ash *et al.,* 2010). For example, Boyette *et al.* (2011) experimentally showed the success of using *Colletotrichum gloeosporioides* f. sp. *aeschynomene* against Hemp sesbania (*Sesbania exaltata*) in rice (*Oryza sativa*) as a bioherbicide. Therefore it is important to test the ability of other cryptic species in the *Colletotrichum gloeosporioides* complex and other species complexes in *Colletotrichum* and other genera for their ability to act as biocontrol agents. However, the efficiency of fungi may not particularly effective in the field and there needs large improvements (Butt & Copping, 2000). There is a possibility to use cryptic species as biocontrol agents and to utilize them to produce disease-resistant crop varieties.

CONCLUSION

Morphological identification of coelomycetes species was previously based on the outstanding monographs of Nag Raj (1971-1987), Sutton (1980) and Nag Raj (1993). Although these studies were based on morphological concepts they provide comprehensive data for generic placements. However, recent molecular data have shown that considerable re-evaluations are needed to understand the family placements, generic concepts and species complexes of coelomycetes (Irinyi *et al.,* 2009; Phoulivong *et al.*, 2010; Maharachchikumbura *et al.,* 2011; Udayanga *et al.,* 2011; Wikee *et al*., 2011b; Gruyter *et al.,* 2012). Additionally, non-culturable and non-sporulating coelomycetes remain as major challenges to fit in classification schemes (Tsui *et al.,* 2011) and therefore effective molecular assays are essential to identify them. Placements of coelomycetes in classes, orders and families, epitypifying species and genera and choosing a single name for one biological species which presently have names for both sexual states are challenges of this decade (Hawksworth, 2012). The journey of discovery is just beginning and this is an extremely exciting time for mycologists.

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