

Impact of molecular phylogenetics on the taxonomy and diagnostics of fungi*

P. W. Crous

Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht (Netherlands);
e-mail: crous@cbs.knaw.nl

Most phytosanitary inspections rely on a visual 'disease-free' inspection, and the question is raised whether this is sufficient, given the fact that plant pathogenic fungi may enter countries as endophytes, or latent pathogens in apparently healthy tissue. Inspectors mostly deal with lists of names of fungal species, but many pathogens are in fact species complexes, and pest lists rarely remain up to date with respect to new systematic treatments. Pathogens may also introduce new genetic diversity or mating types, which could be as devastating as the introduction of a new pathogen. It is therefore proposed that an international initiative should be funded to ensure that systematic support, accurate nomenclature, genetic material and genomic data are available to assist NPPOs in their task.

Introduction

Inadvertent introductions of fungi have had dire consequences for the environment and for cultivated crops in various continents. The economic impact of such introductions can be seen in yield loss, increased input costs for cultivation and disease control, as well as social impact. One of the best-known examples is *Phytophthora infestans* and the resulting Irish potato famine, which led to the emigration of more than 3 million people (Schumann, 1991). Introduced microorganisms can also have negative effects on the environment and ecosystems, e.g. the effect of *Cryphonectria parasitica* on chestnut trees in the USA (Anagnostakis, 1987). We are presently living in a global village, where the agricultural produce from one country will be served in households in another within days of harvest. An inextricable network of global trade in agricultural and forestry products exists and will continue in future. The occurrence of fungi is commonly used as a basis for recommending the rejection of consignments, if they have been officially specified by name as quarantine pests. This system, which due to time constraints is mostly based on visible symptoms, relies on names. A fungal name conveys a certain amount of data. At present, however, the data conveyed by names is generally insufficient to allow inspectors to make well qualified decisions. As will be argued here, well qualified quarantine actions will only be possible if more novel data can be linked to well-established names.

The following questions are raised:

- Do fungal names (commonly based on morphology only) provide a sufficient basis for phytosanitary actions, or do we need to be more precise?

- How do we obtain an integrated fungal database to address future problems?
- How can NPPOs ensure ready access to modern systematic support?
- How do we deal with old names and records?

New introductions, hybridization and new host ranges

The introduction of plant pathogens into a new environment exposes them to new biotic and abiotic influences, such as climate, vectors, hosts, and genetically different populations, which results in a potential for rapid evolution (Brasier, 1995). A newly introduced pathogen will often be subject to novel or episodic selection, which includes contact between two genetically similar, but previously geographically isolated pathogens. This creates the opportunity for modified pathogens via interspecific gene flow. Dutch elm disease is caused by *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* (including two subspecies). Although reproductively isolated, these two species can interbreed, resulting in fertile progeny with reduced virulence (Brasier, 1977; Kile & Brasier, 1990). If *O. novo-ulmi* is introduced into an area, it quickly replaces *O. ulmi* (Brasier, 1986). During this period, however, the two species are in close contact, and gene transfer can occur between them, which influences the population structure, and can have beneficial effects on the pathogen. In Oomycetes, this is also a common phenomenon. In the Netherlands, a new species of *Phytophthora* has been found on *Primula* and *Spathiphyllum*, which is actually a hybrid between *Phytophthora cactorum* (a native species) and *Phytophthora nicotianae* (an introduced species) (Man in't Veldt *et al.*, 1998). In South Africa, Campbell *et al.* (1999) induced sexual matings between the spot and net type of *Pyrenophora teres*, which causes net blotch of barley. The resulting progeny

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were found to be able to infect cultivars which are usually only susceptible to the net-type, as well as those only susceptible to the spot-type (Campbell & Crous, 2003). Hybridization thus results in a different genetic make-up, influencing not only the virulence of the pathogen but also its host range.

***Armillaria mellea* at the tip of Africa: a 300 year old time-bomb**

Armillaria root rot is a well-known disease on *Proteaceae* in different regions in the world, including Australia (Porter *et al.*, 1996), USA (California–Farr *et al.*, 1989; Hawaii–Laemmlen & Bega, 1974), Kenya (Denman *et al.*, 2000), Portugal (Madeira; Moura & Rodrigues, 2001), New Zealand (Pennycook, 1989), Tanzania (Denman *et al.*, 2000) and Zimbabwe (Masuka *et al.*, 1998). Although the disease is well known on many plant species in Africa, it has only recently been reported from indigenous *Proteaceae* in the Kirstenbosch Botanical Garden of South Africa (Denman *et al.*, 2000). In subsequent analyses of South African isolates, RFLP profiles indicated that there were two *Armillaria* species present (Coetzee *et al.*, 2003). Although it was originally expected that the *Armillaria* species in Kirstenbosch would represent African taxa, this proved not to be the case. Both species, *A. mellea* and *A. gallica*, are native to the Northern Hemisphere, and hence have clearly been introduced into the gardens. Coetzee *et al.* (2001) proved that *Armillaria mellea* s.str. was introduced into the Dutch East India Company Gardens in the centre of Cape Town approximately 300 years ago. This most probably occurred with citrus plants that were brought to the area from Europe.

Armillaria mellea s.str. is restricted to the Northern Hemisphere, and its occurrence in the Company Gardens of Cape Town is the only recorded exception (Coetzee *et al.*, 2001). It appears that the fungus has spread from the Company Gardens, where it sporulates profusely, to the nearby plants in the Kirstenbosch Botanical Garden. This has serious implications, as the whole fynbos biome of the Cape Peninsula is now threatened. A subsequent phylogenetic study done by Coetzee *et al.* (2003) indicated that the second *Armillaria* species found in the gardens was representative of *A. gallica*, suggesting that it was possibly introduced with plants obtained from Japan. These findings suggest that such introductions during the early European colonization of South Africa might be more common than previously expected. Although these pathogens were probably introduced several hundred years ago, they were always well confined in a specific garden, isolated by high buildings from the rest of the Cape fynbos. The recent finding that these pathogens now occur on the foot of Table Mountain is very serious, and could have devastating effects on the Cape fynbos as it is known today.

Pathogenic endophytes and host specificity

Mycosphaerella

The genus *Mycosphaerella* contains several thousand species (Corlett, 1991), which are usually assumed to be host-specific.

Although the author's recent molecular data indicates that some species have wider host ranges, this is generally the exception, and not the rule. A crop that has been studied rather intensively these past few years is *Eucalyptus*. *Eucalyptus* trees are commonly grown for the paper and pulp industry. Most are native to Australia and Papua New Guinea (Poynton, 1979). The genus *Eucalyptus* includes about 700 species (Potts & Pederick, 2000), and over 60 species of *Mycosphaerella* are associated with leaf spots and cankers on them. What is especially intriguing is that many of the *Mycosphaerella* species have travelled to other continents with their hosts. *M. suberosa*, for instance, was first found causing disease in South America (Crous *et al.*, 1993b), and only later in Indonesia (Crous & Wingfield, 1997), and finally in Australia (Carnegie *et al.*, 1997). *M. nubilosa*, which is a major pathogen of *Eucalyptus* in Australia, has now been informally confirmed from Africa, as well as Europe, and probably occurs much more widely than earlier supposed. *M. heimii* was first described from Madagascar (Bouriquet, 1946). Material of this species on eucalyptus was, however, later obtained from Indonesia, and subsequent collections found this pathogen to be well represented there (Crous, 1998). Similarly, many species initially described on this host from Australia, are now found elsewhere, or vice versa. Obviously, plant quarantine has not operated in these cases. The question then arises how these foliar pathogens were introduced. A recent study on *M. punctiformis* on *Quercus* (Verkley *et al.*, 2004) was the first to prove that species of *Mycosphaerella* can also occur as endophytes in apparently healthy leaves. Such species would in that case be impossible to detect by visual inspection. Further work is also required to establish whether *Mycosphaerella* spp. are seed-borne or not.

Botryosphaeria

The genus *Botryosphaeria* is commonly associated with stem cankers, leaf spots and fruit rots of many hosts. Species in this complex are notoriously difficult to identify, and until recently, most taxa were simply referred to as representatives of the *dothidealribis* complex. With new collections of these species, and by employing a multigene phylogeny, Slippers *et al.* (2004a,b) were able to distinguish *Botryosphaeria ribis*, *Botryosphaeria dothidea* and other, closely related taxa. An aspect that is extremely interesting once again concerns the host range and geographical distribution of these species. In the Southern Hemisphere, especially, it would appear that most of these records have to date been incorrect, and that they in fact represent other, recently described species. Once again, it appears that many of these species, such as *Botryosphaeria eucalyptorum* and *Botryosphaeria australis* on *Eucalyptus* in Australia and South Africa, have been introduced along with their hosts. Although morphologically similar, these pathogens differ markedly in their host range, distribution and importance as plant pathogens. Earlier work by Smith *et al.* (1996) has shown that these species commonly occur as endophytes in apparently healthy tissue. New introductions, even of the same species, could enhance the genetic diversity and fitness of

an existing population. Where to draw the line becomes an intriguing question.

Diaporthe/Phomopsis

The form-genus *Phomopsis* (anamorphs of *Diaporthe*) contains more than 800 species, most of which are recorded as being plant-pathogenic on stems, leaves, fruit or roots of various plant species (Uecker, 1988). *Phomopsis* species concepts have until recently mainly been based on host affiliation. Recent studies have shown, however, that various species of *Phomopsis* are able to infect a wide variety of hosts (Rehner & Uecker, 1994; Uddin *et al.*, 1997; Mostert *et al.*, 2001), and that host association is no longer sufficient for identification purposes. This effectively means that strains can only be identified to species level if advanced molecular techniques are employed. In a study of *Phomopsis* species causing cane and leaf spot of grapevines, Mostert *et al.* (2001) identified six distinct species. This study has recently been expanded by the author to include many more isolates, which results in the number of species known from grapevines rising to 15. Several of these taxa, however, show evidence of host switching, for instance *P. amygdali*, a known pathogen of peaches and almonds (Farr *et al.*, 1999), and *P. helianthi*, a known pathogen of sunflowers, were also found on grapevines. Several of the species could, however, not be identified to species level due to the limited molecular data presently available for this group. One of the unknown *Phomopsis* species also contained isolates from roses and cranberries. It is impossible therefore to make well qualified decisions about *Phomopsis* isolates found on plant material without molecular support. So a serious investment is now called for to obtain DNA sequence data for as many of these fungal pathogens as possible.

Guignardia/Phyllosticta

Morphological similarity of visible fungi on symptomatic material may in many cases be an insufficient basis for well qualified phytosanitary actions. A good case in point is citrus black spot, caused by *Guignardia citricarpa* (anamorph: *Phyllosticta citricarpa*). The disease was introduced into southern Africa from Australia with infected bud wood (Doidge, 1929). The disease has subsequently been found in subtropical countries that are subject to summer rainfall, and does not occur in countries or regions that are subject to winter rainfall (Baayen *et al.*, 2002). Confusion set in, however, after this fungus was recorded from numerous hosts in different parts of the world. A subsequent molecular study by Baayen *et al.* (2002) showed that two species were involved, and that one was a common endophyte with a wide host range, that could be ascribed to the species *Guignardia mangiferae*. This has resulted in several cultural and molecular tests (Meyer *et al.*, 2001) developed readily to distinguish these species on shipments of imported citrus fruits.

In all examples listed above, fungal species are possibly being introduced into new countries because their inconspicuous

endophytic growth in plant tissue makes them difficult to detect by visual inspection. In the case of *Phomopsis* and *Phyllosticta*, the situation is even more complex, as it is rarely possible to identify these organisms to species using morphology (the teleomorphs in addition being rarely available for examination), and the identification system used up till now is strongly host-based.

***Cylindrocladium scoparium*: the reality of dealing with species complexes, and complex species**

Cylindrocladium scoparium has been associated with a wide range of plant disease problems in over 30 families throughout the world (Booth & Gibson, 1973; French & Menge, 1978; Peerally, 1991; Waipara *et al.*, 1996). This species is, however, the most commonly incorrectly identified taxon in the genus (Crous, 2002). *C. scoparium* is frequently confused with other species with 1-septate, small conidia. These include *Cylindrocladium ovatum* (ovoid vesicles), species in the *Cylindrocladium floridanum* complex (sphaeropedunculate vesicles) and species in the *Cylindrocladium candelabrum* complex (obpyriform vesicles).

The presence of *C. scoparium* has only recently been confirmed from North America and Brazil (Crous *et al.*, 1993a), and most of the isolates mentioned in previous reports have been found to represent species from the complexes mentioned above (Schoch *et al.*, 1999). Because these taxa are morphologically very similar, Schoch *et al.* (1999) employed a biological species concept, and included all isolates studied in mating experiments. Isolates that underwent successful matings were subjected to further morphological and molecular comparisons. At that stage, however, data from the ITS-1, 5.8S and ITS-2 gene region proved insufficient to distinguish taxa, and thus an additional database of β -tubulin sequence data was compiled (Schoch *et al.*, 2001). In recent studies that have incorporated more isolates and gene loci (Wingfield *et al.* pers. comm.), it became clear that in some isolates speciation was not yet complete. In other words, some isolates identified based on the biological/morphological species complex contained more than one phylogenetic species. Although these isolates could be recognized as clearly different phylogenetic species, they still retained the ability to mate. This stresses the importance of combining more than one species concept when examining isolates. In addition, sufficient strains should be used and more than one informative gene region should be sequenced, as not all genes evolve at the same rate and, in some genes, evolution has proceeded more slowly, masking species differences. Accordingly, species of *Cylindrocladium* can only be identified adequately for phytosanitary purposes if enough sequence data is available in publicly accessible databases.

Conclusions

It is realistic to assume that for purposes of free trade and consumer demand, international trade in agricultural and forestry products will increase. In view of the issues discussed

above, inspectors will increasingly have to refer fungus-infected material for laboratory diagnosis. The identifications given will increasingly refer to scientific names other than those used in quarantine-pest lists in phytosanitary regulations. NPPOs accordingly need access to up-to-date specialist information on fungi. However, this information is scattered, and only a few centres remain where this is a strong nucleus of technical expertise. For the fungi, a constantly updated international online database is clearly needed. A good example of what could be done can be seen on the web page of the Centraalbureau voor Schimmelcultures (<http://www.cbs.knaw.nl>) for yeast identification. Such an approach would enable researchers and NPPO staff to do similarity searches based on morphological characteristics, DNA sequence, as well as other features. It would be valuable for NPPOs throughout the world.

A first step should be to ensure that good cultures of regulated fungi and similar pathogens are available (where appropriate), and that informative loci are sequenced for these organisms. This data should be deposited in GenBank or similar international databases, where they will add to the exponentially growing number of DNA sequence and other data. The database should be modelled to slot into the databases presently linked via the Global Biodiversity Information Facility (G-BIF), so that it is constantly updated, and can address new issues as these occur.

In conclusion, most plant pathogens are currently known by the diseases they cause, and by their general morphological characteristics. Few have been characterized in relation to living cultures, DNA and sequence. Phytosanitary diagnostics must keep abreast of new developments, and strategic decisions are needed now, at an international level, to determine how this can be funded and achieved.

Impact de la phylogénétique moléculaire sur la taxonomie et le diagnostic des champignons

La plupart des inspections phytosanitaires se base sur une inspection visuelle 'd'absence de maladie', et la question se pose de savoir si cela suffit, étant donné que certains champignons phytopathogènes semblent être importés comme endophytes ou à l'état latent dans des tissus apparemment sains. Les inspecteurs utilisent surtout des listes de noms d'espèces alors que les champignons phytopathogènes sont souvent des complexes d'espèces, et que les listes tiennent rarement comptes des nouveaux traitements systématiques. Les pathogènes peuvent aussi introduire une diversité génétique ou de nouveaux types de compatibilité, qui peuvent être aussi nuisibles que l'introduction d'un nouveau pathogène. Il est donc proposé qu'un projet international soit financé pour rendre disponible un soutien relatif à la systématique, les noms corrects des champignons, le matériel génétique et les données génomiques afin d'aider les ONPV dans leur mission.

Воздействие молекулярной филогенетики на таксономию и диагностику грибов

Большинство фитосанитарных досмотров базируются на визуальном досмотре на предмет «отсутствия болезни»,

поэтому справедливо поставлен вопрос о его достаточности, принимая во внимание тот факт, что патогенные для растений грибы могут попадать в страны как эндофиты или латентные патогены в здоровой на вид ткани. Инспекторы как правило имеют дело со списками названий видов грибов, однако на деле многие патогены представляют собой сложные комплексы видов, в то время как списки вредных организмов редко обновляются в соответствии с современными систематическими изменениями. Патогенами может также внедряться новое генетическое разнообразие или новые типы совместимости, которые способны быть столь же разрушительными, что и внедрение нового патогена. Поэтому было предложено профинансировать новую международную инициативу, позволяющую обеспечивать систематическую поддержку, правильную номенклатуру, генетические материалы и геномные данные, помогающие НОКЗР в осуществлении ими своих задач.

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