Reviews, Critiques and New Technologies

Biology and recent developments in the systematics of *Phoma*, a complex genus of major quarantine significance

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Species of the coelomycetous genus *Phoma* are ubiquitously present in the environment, and occupy numerous ecological niches. More than 220 species are currently recognised, but the actual number of taxa within this genus is probably much higher, as only a fraction of the thousands of species described in literature have been verified *in vitro*. For as long as the genus exists, identification has posed problems to taxonomists due to the asexual nature of most species, the high morphological variability *in vivo*, and the vague generic circumscription according to the Saccardoan system. In recent years the genus was revised in a series of papers by Gerhard Boerema and co-workers, using culturing techniques and morphological data. This resulted in an extensive handbook, the "*Phoma* Identification Manual" which was published in 2004. The present review discusses the taxonomic revision of *Phoma* and its teleomorphs, with a special focus on its molecular biology and papers published in the post-Boerema era.

Key words: coelomycetes, *Phoma*, systematics, taxonomy.

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Introduction

The genus *Phoma* is geographically widespread and consists of a large group of fungi that are found in numerous ecological niches. Besides several harmless saprobic species, *Phoma* has also been shown to be an important fungal plant pathogenic genus occurring on economically important crops. Several *Phoma* species are also of quarantine significance, posing serious problems to organisations that are involved in plant health quarantine regulation. Identification of isolates found on possible infected material is frequently carried out under extreme time constraints. Because morphological studies are time-consuming, expensive, and require highly skilled personnel, the chance of successfully identifying strains to species level in such laboratories is often low. Therefore, there is a need for the development of fast, reliable molecular methods for the detection of quarantine actionable *Phoma* species. Several molecular methods to detect quarantine species have been developed in the past decade, e.g. for *P. macdonaldii* (Miric *et al*., 1999), *P. cucurbitacearum* (Somai *et al*., 2002b; Koch and Utkhede, 2004), *P. foveata* (Macdonald *et al*., 2000; Cullen *et al*., 2007) and *P. tracheiphila* (Balmas *et al*., 2005; Licciardella *et al*., 2006), but the validation of these methods is heavily questioned as the species concepts of these taxa are not yet fully understood. In this literature review we will provide an outline of the biology of the genus, and circumscribe its taxonomic boundaries. We will also focus on the methodologies that can be used to obtain a better understanding of speciation within *Phoma*.

Phoma biology

The generic name *Phoma* was in the first instance solely reserved for plant stem pathogens (Saccardo, 1880), but nowadays the genus comprises pathogens, opportunists as

well as saprobes from a much wider range of substrates. The more ubiquitous species such as *P. herbarum*, *P. glomerata*, *P. pomorum* var. *pomorum* and *P. eupyrena* have been found on inorganic materials; isolates are known from asbestos, cement, oil-paint and plaster (*P. herbarum*), chemicals, paint (*P. glomerata*) and crockery (*P. pomorum* var. *pomorum*), along with many other inorganic substrates. These ubiquitous fungi probably also play an important role in the degradation of organic materials, together with other, more specialized species. In contrast to these harmless saprobes, more than 50% of the species described thus far are known to be able to occur in living tissue, either as opportunists or as primary pathogens.

Phoma infections commonly occur in humans and animals. Zaitz *et al*. (1997) and De Hoog *et al*. (2000) referred to nine species that were isolated from humans. Recently, an additional species, *P. exigua*, was added to the list of human pathogens (Balis *et al*., 2006). Several severe vertebrate diseases are also associated with *Phoma*, such as bovine mycotic mastitis (Costa *et al*., 1993) and fish-mycosis in salmon and trout (Ross *et al*., 1975; Hatai *et al*., 1986; Voronin, 1989; Faisal *et al*., 2007). Soil associated organisms such as arthropods and nematodes can also be subject to *Phoma* infections. Chen *et al*. (1996) listed 11 *Phoma* species found in association with cyst nematodes. Furthermore, *Phoma* species have been found parasitizing other fungi and oomycetes (Hutchinson *et al*., 1994; Sullivan and White Jr., 2000). Although lichens have been poorly studied, a key to 14 lichenicolous *Phoma* species was provided by Hawksworth and Cole (2004). Still, the vast majority of the species appear to only colonise plant material. Some species are only known from decaying leaves or wood, whereas others play a role as secondary invaders of weakened plant tissue. However, more than 110 species are known to be primary plant pathogens, mainly specialising on a single plant genus or family.

The economically most important pathogens include the widespread species *P. medicaginis* (Broscious and Kirby, 1988) and the two species that are involved in Black Leg in *Brassicaceae*: *P. lingam* and to a lesser extent the unnamed *Phoma* anamorph of *Leptosphaeria biglobosa* (Gugel and Petrie, 1992; Fitt *et al*., 2006). Although recent estimations of financial losses are rare, the impact of this genus on agriculture is highly significant. *Phoma lingam* is regarded to be the most important pathogen of oilseed rape in the Northern Hemisphere, with yield losses of up to 25% being recorded (Fitt *et al*., 2006). Besides the direct costs due to yield loss, indirect losses occur due to import and export restrictions to prevent introductions of possible pathogenic or quarantine relevant *Phoma* species. Rigorous measures, such as refusal or even destruction of a shipment that is suspected of infection with such quarantine organisms, are the cause of high additional costs to both the exporting and importing traders. These quarantine organisms include amongst others *P. andigena* and *P. tracheiphila* in the European and Mediterranean regions (Smith *et al*., 1992), *P. foveata* (also known as *P. exigua* var. *foveata*) in Southern America (Mendes *et al*., 2007), and *P. macdonaldii* in Australia (Miric *et al*., 1999).

Only in some cases the pathogenic nature of *Phoma* is regarded as helpful, by means of biocontrol agent of weeds and plant pathogens. The ubiquitous species *P. herbarum*, *P. exigua* and *P. macrostoma* may play a role as bioherbicide, effective against broadleaf weeds, such as dandelion (*Taraxacum* spp.), and chickweed (*Caryophyllaceae*) (Stewart-Wade and Boland, 2004, 2005; Zhou *et al*., 2004), clematis (*Clematis vitalba*) (Paynter *et al*., 2006) and salal (*Gaultheria* spp.) (Zhao *et al*., 2005, 2007). The antagonistic effect of *P. glomerata* and *P. etheridgei*, respectively against *Microsphaera penicillata* and *Phellinus tremulae*, has been studied in detail. However, their application as biocontrol agent is debatable, due to application problems and a low impact on the disease spread (Hutchinson *et al*., 1994; Sullivan and White Jr., 2000).

The life cycle

Although the life cycle is influenced by the occupied niche, it is relatively similar for all plant pathogenic *Phoma* species. Primary infection of hosts may occur through wounds that are caused by cultivation practices, weather conditions, or interaction with other organisms. In several species, entering a host plant through stomata or directly through the epidermis also

may occur (Williams, 1992; Agrios, 1997; Roustaee *et al*., 2000; West *et al*., 2001; Van de Graaf *et al*., 2002). Initially, the fungal hyphae grow intercellulary through plant tissues (Hammond and Lewis, 1987). Following this symptomless stage, the fungus becomes necrotrophic. Host cells are subject to phytotoxification or to a hypersensitive response, after which the fungus has acces to the resources of the dead plant tissue. This can be observed macroscopically as the formation of lesions. After a short period, often dark-coloured, mostly globose or flask-shaped pycnidial conidiomata can be observed within the lesions, which are embedded in the plant's epidermis. These pycnidia contain numerous conidia, which ooze in a pale white to pinkish coloured matrix. A detailed description of the spore production is provided by Boerema (1965), Brewer and Boerema (1965) and Boerema and Bollen (1975). In some cases extradermal mycelium may be formed. Conidia, and in some species mycelial fragments, disperse easily by water-splash, misting or wind, and can thus infect new host plants. Also birds and insects may act as vectors (Perrotta and Graniti, 1988). In absence of a suitable host, due to crop harvesting for example, most species persist as saprobes on decaying organic material in the soil. In most cases, this material is the residue of plants that were previously infected. In this mode, the mould may survive periods of stress, such as drought or extreme cold. The structures which are most suitable for long-term survival are the conidiomata, and the uni- or multicellular chlamydospores, which are formed only by a small number of *Phoma* species. From this material infection may occur in newly planted crops, and from the exuding conidiomata new conidia can emerge to facilitate onward dispersal.

Sometimes also a meiotic cycle can be observed, besides the asexual one described above, in which ascospores are formed in pseudothecial ascomata. If such sexual structures are found in nature, they are mostly observed in the saprobic part of the life cycle (Williams, 1992; West *et al*., 2001). However, such a sexual stage is quite uncommon within this genus, and is presently not known for more than 40 taxa, of which many produce the sexual reproductive structures only *in vivo*. Unfortunately, little is known about the induction of sex *in vitro*.

The abundant production of conidia, together with a relatively fast growth rate and the capability to invade a large number of hosts and substrates, are the main reasons why the genus is so cosmopolitan in distribution. The most frequent occurring species, *P. eupyrena*, *P. exigua*, *P. glomerata*, *P. herbarum*, and *P. macrostoma* are found worldwide, irrespective of the climatic conditions. *Phoma herbarum* is probably also indigenous to Antarctica (McRae and Seppelt, 1999; Tosi *et al*., 2002). Several plant pathogenic species have been transmitted worldwide with the cultivated host on seeds and other plant material.

The Phoma generic concept

The first descriptions of fungi that belong to the genus *Phoma* date back to 1821 (Sutton, 1980). Nevertheless, it took until 1880 before the generic name was officially introduced by Saccardo, and was later emended by Boerema and Bollen in 1975. In the Saccardoan system, the genus name *Phoma* applied to filamentous fungi which were capable of forming pycnidial conidiomata with aseptate, hyaline conidia that could inhabit plant stems. Fungi with the same morphological appearance, but found in association with leaf spots, were placed in the genus *Phyllosticta* (Van der Aa and Vanev, 2002; Boerema *et al*., 2004).

Identification of fungi occurred mainly *in vivo* until the fourth decade of the 20th century. Wollenweber and Hochapfel (1936) were the first to recognise the advantages of using growth characteristics on artificial substrates in *Phoma* taxonomy, whereas Dennis (1946) launched the use of basic morphology, physiology and biochemical tests *in vitro*. In a study on hyphomycetes, Hughes (1953) introduced conidiogenesis as an important taxonomic criterion, a feature that was later applied to all conidial fungi including species of *Phoma* (Sutton, 1964). In addition, new criteria for morphological differentiation were introduced to distinguish *Phoma* species from other coelomycetes such as *Ascochyta* (Boerema and Bollen, 1975), *Phyllosticta* (Van der Aa *et al*., 1990), *Coniothyrium* and *Paraconiothyrium* (Verkley *et al*., 2005).

In 1964, eight years after the designation of *Phoma* as a conserved genus name in the

International Code of Botanical Nomenclature (Lanjouw *et al*., 1956), the designated type species, *Phoma herbarum*, was restudied and lectotype material was selected (Boerema, 1964). Morphology, synonymy and ecology of this type species were later extensively described by Boerema (1970) and Morgan-Jones (1988a).

Currently *Phoma* species can be defined as filamentous fungi that produce pycnidial conidiomata with monophialidic, doliiform to flask-shaped conidiogenous cells. A collarette is present at the apex of those cells after the production of the first conidium. *In vitro* the hyaline conidia are mainly single-celled, although in several species a small percentage of transversely septate conidia may also be observed. This definition applies to more than 220 inter- and intraspecific taxa (Boerema *et al*., 2004), but it should be borne in mind that this definition does not necessarily apply to samples in nature, as bicelled or even pigmented conidia occur more often *in vivo*.

The genus *Phoma* is generally considered by most modern mycologists to be taxonomically problematic due to ambiguous morphological criteria, but also due to uncertain phylogenetic affinities. The genus is currently linked to three different teleomorph genera. If teleomorphs are known, they reside in *Didymella*, *Leptosphaeria*, or occasionally *Pleospora* (Table 1, Fig. 1). It should be noted that not all species in these genera form *Phoma* anamorphs; this is a feature that may have been lost over time, or developed multiple times in multiple ancestors. In literature, *Phoma* species have also been associated with other teleomorph genera within the Dothideomycetes, including *Mycosphaerella* (Punithalingam, 1990; Corlett, 1991; De Gruyter *et al*., 2002), *Belizeana* (Kohlmeyer and Volkmann-Kohlmeyer, 1987), and *Fenestella*, *Cucurbitaria*, *Preussia*, and *Westerdykella* (Von Arx, 1981). As many of these connections are based on association, they remain to be confirmed in culture. Furthermore, a range of synanamorphs of *Phoma* spp. have been recognised in *Stagonosporopsis*, *Epicoccum*, *Phialophora* and *Sclerotium* (Boerema and Bollen, 1975; Sutton, 1977; Boerema, 1993; Boerema *et al*., 1994, 1997; Arenal, 2000, 2004). Crous and Gams (2000) also described a phoma-like synanamorph of *Phaeomoniella chlamydospora* (*Chaetothyriales*), a fungus associated with Petri disease of grapevines (Mostert *et al*., 2006). This complexity in taxonomy is a futher complicating factor for the identification and differentiation of members of the genus *Phoma*.

Amongst the many scientists that attempted to order the numerous species belonging to *Phoma* are Saccardo (1884), Grimes *et al*. (1932), Wollenweber and Hochapfel (1936), Dennis (1946), Sutton (1964) Monte *et al*. (1990, 1991) and Rai and Rajak (1993). Morgan-Jones and co-workers described some important species in detail in several papers published in Mycotaxon (Morgan-Jones and White, 1983; White and Morgan Jones, 1983, 1986, 1987; Morgan-Jones and Burch 1987a,b, 1988a,b; Morgan-Jones, 1988a,b). The greatest effort made on *Phoma* systematics was by Boerema and co-workers, who published contributions towards a genus concept including numerous species descriptions and synonyms in a series of papers in Persoonia (De Gruyter and Noordeloos, 1992; Boerema 1993, 1997, 2003; Boerema *et al*., 1994, 1996, 1997, 1999; Boerema and De Gruyter 1998, 1999; De Gruyter *et al*., 1998, 2002; Van der Aa *et al*., 2000; De Gruyter, 2002; De Gruyter and Boerema, 2002). As a final product of their 40-year study on *Phoma* taxonomy, a taxonomic handbook was written based on the papers published in the previous decades (Boerema *et al*., 2004). The *Phoma* generic concept, and therefore also the identification key is based on the establishment of nine sections within the genus (Boerema, 1997), which are listed in Table 1.

Table 1. The nine sections of the genus *Phoma*, with their type species and associated teleomorph genera.

Section	Type species	Associated teleomorph genus
Heterospora	*P. heteromorphospora*	-
Macrospora	*P. zeae-maydis*	*Didymella*
Paraphoma	*P. radicina*	-
Peyronellaea	*P. glomerata*	-
Phoma	*P. herbarum*	*Didymella*
Phyllostictoides	*P. exigua* var. *exigua*	*Didymella*
Pilosa	*P. betae*	*Pleospora*
Plenodomus	*P. lingam*	*Leptosphaeria*
Sclerophomella	*P. complanata*	*Didymella*

Fig. 1 A-D. Teleomorphs of *Phoma*. Asci and ascospores of **A-B.** *Didymella zeae-maydis* (anam. *P. zeae-maydis*) **C.** *Leptosphaeria maculans* (anam. *P. lingam*) **D.** *Pleospora herbarum*, lectotype of the genus *Pleospora*. Scale bars = 10 μm.

Although the key is helpful for identification of species, it is still uncertain if this division into sections can be considered natural from an evolutionary perspective. In this classification system, most sections are to be based on morphological characters that imply a certain evolutionary relationship, or comprise species that share a teleomorph in the same genus. Unfortunately, several characters that are linked to a certain section, sometimes also seem to occur in species that are placed in other taxonomic groups. As an example, Punithalingam (2004) mentioned the slightly pilose species, *P. anserina* and *P. leonuri* that are not accommodated in either section *Pilosa* or *Paraphoma*. Based on the appearance of their conidia, these species have been placed in the sections *Phoma* and *Plenodomus*, respectively. This ambiguous approach has resulted in multiple overlaps between the separate sections. Furthermore, the sections *Phoma* and *Phyllostictoides* are also considered to be artificial. In stead of comprising species with a shared feature, both sections seem to be a repository for species that lack the presence of good sectional characters. According to Boerema (2004), section *Phoma* comprises species "that have much in common with *P. herbarum*". But as the majority of the species are accommodated within this section, it shows the necessity to conduct further research on classification within the genus.

Towards a generic concept for Phoma

Macromolecular approaches to systematics were introduced in mycology in the late 80's (for a review see Bruns *et al*., 1991). These techniques, although accurate, were providing only a limited number of relevant characters. The introduction of nucleotide sequences as phylogenetic characters greatly advanced fungal systematics (Bridge, 2002; Malgoire *et al*., 2004). Within the genus *Phoma* this gave rise to several novelties, and to new insights on the delimitation of *Phoma* and its teleomorphs, as described below.

The present classification system has been criticized for the unclear discrimination between *Phoma* and several related or even convergent genera. For example, Grondona *et al*. (1997) discussed the poor delineation between the *Phoma* section *Paraphoma* and the genera *Pyrenochaeta* and *Pleurophoma*. Furthermore, the relation between *Ascochyta* and *Phoma* species that produce septate conidia remains unresolved. The confusion between the two genera is illustrated by the large number of shared synonyms. Although both genera have teleomorphs in *Didymella*, Boerema and Bollen (1975) differentiated *Phoma* from *Ascochyta* on differences in conidiogenesis and conidial septation. The conidia of *Ascochyta* are basically always 2-celled, due to early euseptation during conidiogenesis, whereas in *Phoma* septate conidia are rare in culture - although it

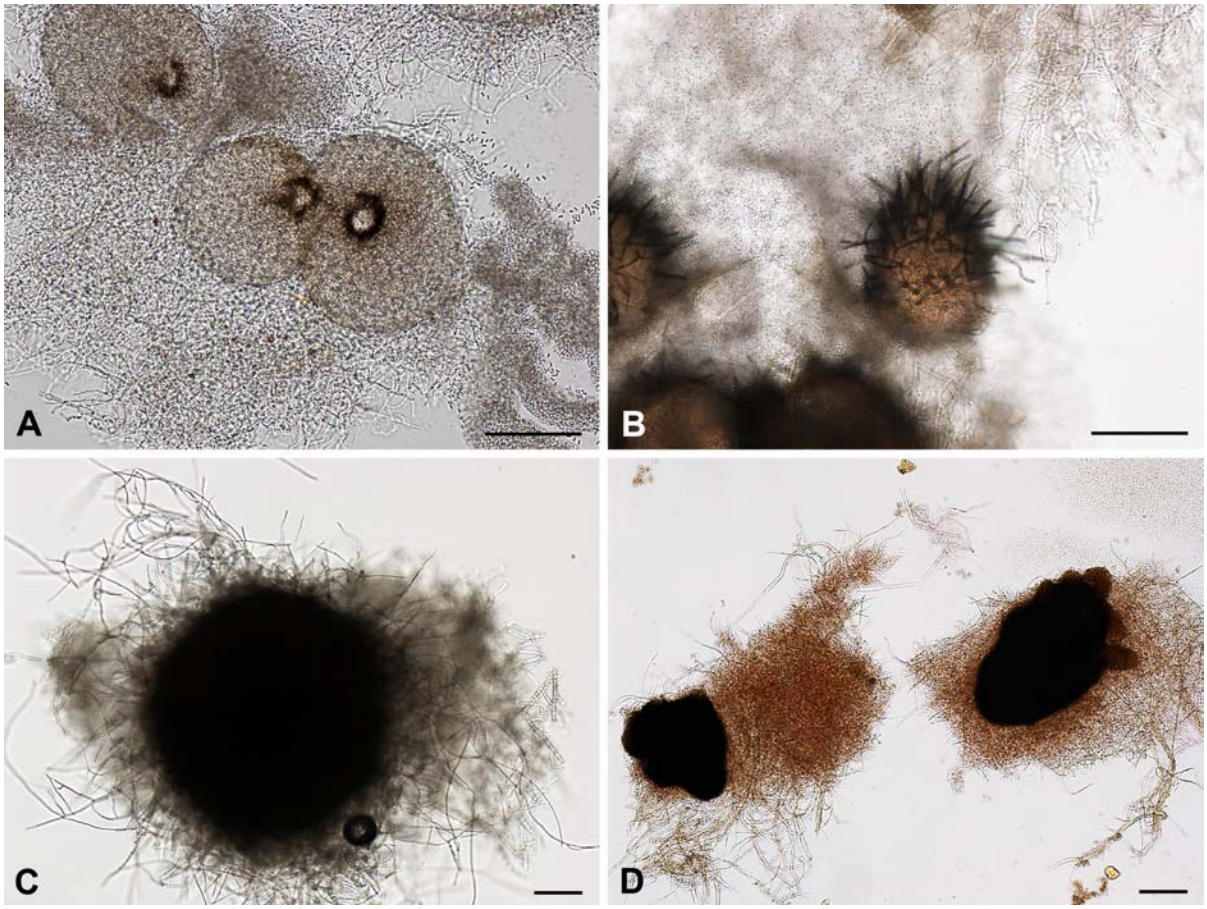

Fig. 2A-D. Pycnidial types. **A.** Regular glabrous as in *P. herbarum* **B.** Setose as in *P. carteri* **C.** Pilose as in *P. betae* **D.** Pycnosclerotia-like pycnidia as in *P. incompta*. Scale bars = 100 µm.

is not uncommon in nature - and are formed by a late euseptation. Spectrometrical analysis of crystals formed by specimens of both genera seemed to support this differentiation (Noordeloos *et al*., 1993). However, later Faris-Mokaiesh *et al*. (1995) showed that *P. pinodella* and *A. pinoides* probably are closely related, basing their conclusions on a similarity in the banding pattern of PCR and RFLP products. This relatedness between the two species was supported by sequence analyses of various gene regions (Barve *et al*., 2003; Fatehi *et al*., 2003; Peever *et al*., 2007).

A section that has been the subject of continuous discussion is *Plenodomus*. In the mid-70s, the genus *Plenodomus* was incorporated into *Phoma* as *Phoma* section *Plenodomus* (Boerema *et al*., 1981). Presently 32 taxa are accommodated in this section (Boerema *et al*., 1994, 1996, 2004; Torres, 2005b). Already in 1964, even before the official recombination of this group of fungi into the genus *Phoma*, Boerema and van Kesteren (1964) questioned the link between *Plenodomus lingam* and the genus *Phoma* due to differences in pycnidial development. Later, the authors justified the recombination based on the similarity in conidiogenesis: both taxa produced conidia from unicellular, flask-shaped phialides, a feature that is considered to be specific for the genus *Phoma* (Boerema *et al*., 1981). This decision arose again as point of discussion in 1997, when molecular data of the ITS-region revealed that *P. lingam* and its relative *P. wasabiae* were only distantly related to other phomoid fungi (Reddy *et al*., 1998). This led to the hypothesis that the former genus *Plenodomus* had been incorrectly reduced to synonymy, and should be reinstated (Reddy *et al*., 1998). This idea agrees with other, more recent studies using other genes (Pethybridge *et al*., 2004; Torres *et al*., 2005b; Schoch *et al*., 2006), although the interspecific variation in *Plenodomus*-linked *Leptosphaeria* species is relatively high (Morales *et al*., 1995; Câmara *et al*., 2002).

The synanamorphs and teleomorphs of *Phoma* have been poorly investigated thus far, except for the *P. lingam* complex. Few synanamorph relationships have thus far been confirmed by means of molecular techniques. Arenal *et al*. (2000, 2004) demonstrated *P. epicoccina* and *Epicoccum nigrum* to be synanamorphs by employing ITS sequence data, and synonymised these species after further microscopical studies.

Towards a species concept for Phoma

Numerous *Phoma* strains have been identified and characterised using mainly morphological characters *in vitro*. As in most other fungal genera, these characters include the size and shape of conidiomata, conidia and chlamydospores, but also metabolite production and growth rates and patterns on various agar media.

Characters relating to pycnidial conidiomata are mainly used for sectional differentiation (Fig. 2). Pycnidia are highly variable in shape and size, but in most species they are either globose or subglobose, or sometimes pyriform due to an elongated neck (section *Plenodomus*). In older cultures the pycnidia may aggregate. The colour varies per species from yellowish to brown-olivaceous or olivaceous-black and depends on the culturing conditions and age. Occasionally the pycnidia are setose as in section *Paraphoma* (Fig. 2B), or pilose as in section *Pilosa* (Fig. 2C). Single or multiple ostioles may be observed, although in some species (sections *Sclerophomella* and *Plenodomus*) pycnosclerotia are found (Fig. 2D). In contrast to most sections that produce thin-walled pseudoparenchymatous pycnidia, the species in *Sclerophomella* are characterised by the ability to produce thick-walled conidiomata, whereas species in section *Plenodomus* produce scleroplectenchyma in the pynidial wall. The presence of multicellular chlamydospores (Fig. 3) is often a good indication that a strain belongs to the section *Peyronellaea*, although this might not always be the case. The shape of these chlamydospores is in most cases comparable to the multicellular conidia of *Alternaria* spp., so-called alternarioid or alternarioid-botryoid (Fig. 3A-D), but in some species pseudoscleroid or epicoccoid chlamydospores can be found (Fig. 3E and 3I respectively). The formation of unicellular chlamydospores (Fig. 3F-H) is not regarded as characteristic for any particular section, but can aid identification at the species level. In the same way the characters of swollen hyphae, commonly regarded as ancestral to chlamydospores, are regarded as informative (Boerema, 1993).

Conidia are formed in pycnidia, and conidial shape and size are regarded as the most useful indicators for determining a strain up to species level, but are also used for differentiating some sections (Fig. 4). The average conidium measures ca. 2.5-10 × 1-3.5 µm. However, in the section *Macrospora* enlarged conidia occur, which are up to 25 × 9 µm (Fig. 4K-L). The section *Heterospora* comprises species that posses both normal sized conidia as well as so-called macroconidia (Fig. 4I-J). Especially in these sections there might be a high variability in conidial size, even within a single species. Studying cultures *in vitro* should be done under standardised conditions: cultures grown on different media and under different conditions may prove to be highly variable (Rai, 1998). Besides conidial measurements, conidial shape is of primary importance. Guttule number and size may provide additional valuable characters for species identification. The section *Phyllostictoides* (Fig. 4E-H) can be distinguished from the section *Phoma* (Fig. 4 A-D) by the presence of a low percentage of septate conidia in pure culture. The septation ratio is highly variable, even within species and can be influenced by the growth media. Therefore, this character is often regarded as uninformative (Onfroy *et al*., 1999). *In vivo*, the ratio of septate conidia may be up to 95%, which has led to many misidentifications in *Ascochyta* in the past (Boerema and Bollen, 1975), and a large number of synonyms for *Phoma* species in *Ascochyta* (Van der Aa *et al*., 2000).

Growth characteristics on media, such as growth rate, pigment formation and colony outline and pattern can also aid identification. The media types that are most intensively applied in *Phoma* identification include oatmeal agar, malt extract agar and, to a lesser extent, cherry decoction agar (for protocols see Boerema *et al*., 2004). Boerema *et al*. (2004) provide measurements of the diameter of

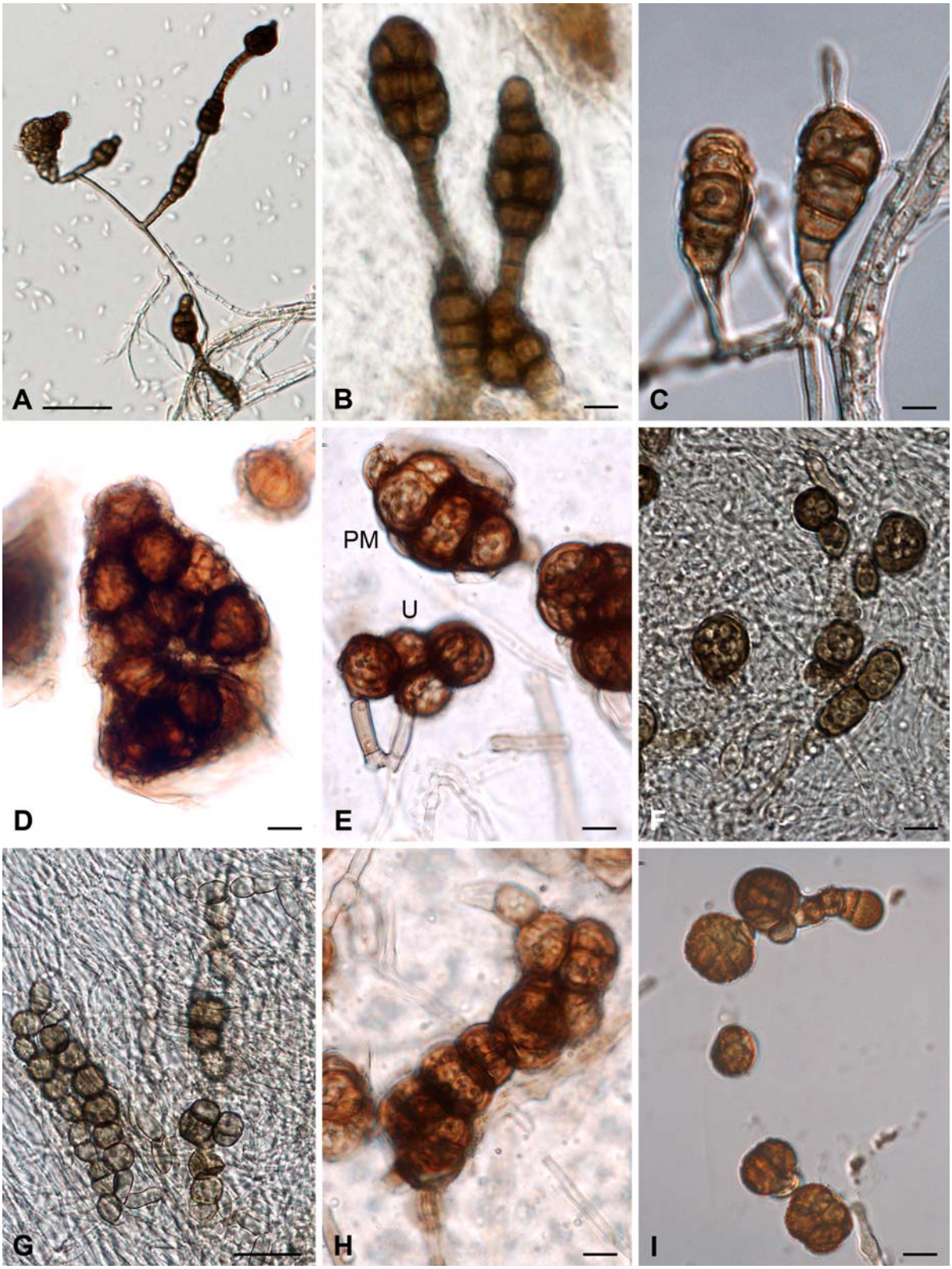

Fig. 3 A-I. Chlamydospore morphology in *Phoma*. **A.** Chain of chlamydospores of *P. glomerata*. **B.** Alternaroid chlamydospores of *P. glomerata*. **C.** Alternaroid chlamydospores of *P. jolyana*. **D.** Botryoid chlamydospore as in *P. sorghina*. **E.** Unicellular (u) and pseudosclerotoid multicellular chlamydospores (pm) in *P. chrysanthemicola*. **F.** Unicellular chlamydospores of *P. pinodella* **G.** Chain of unicellular chlamydospores in *P. clematidina*. **H.** Chain of unicellular chlamydospores in *P. chrysanthemicola*. **I.** Epicoccoid chlamydospores in *P. epicoccina*. Scale bars: A = 100 µm, G = 50 µm, B-F and H-I = 10 µm.

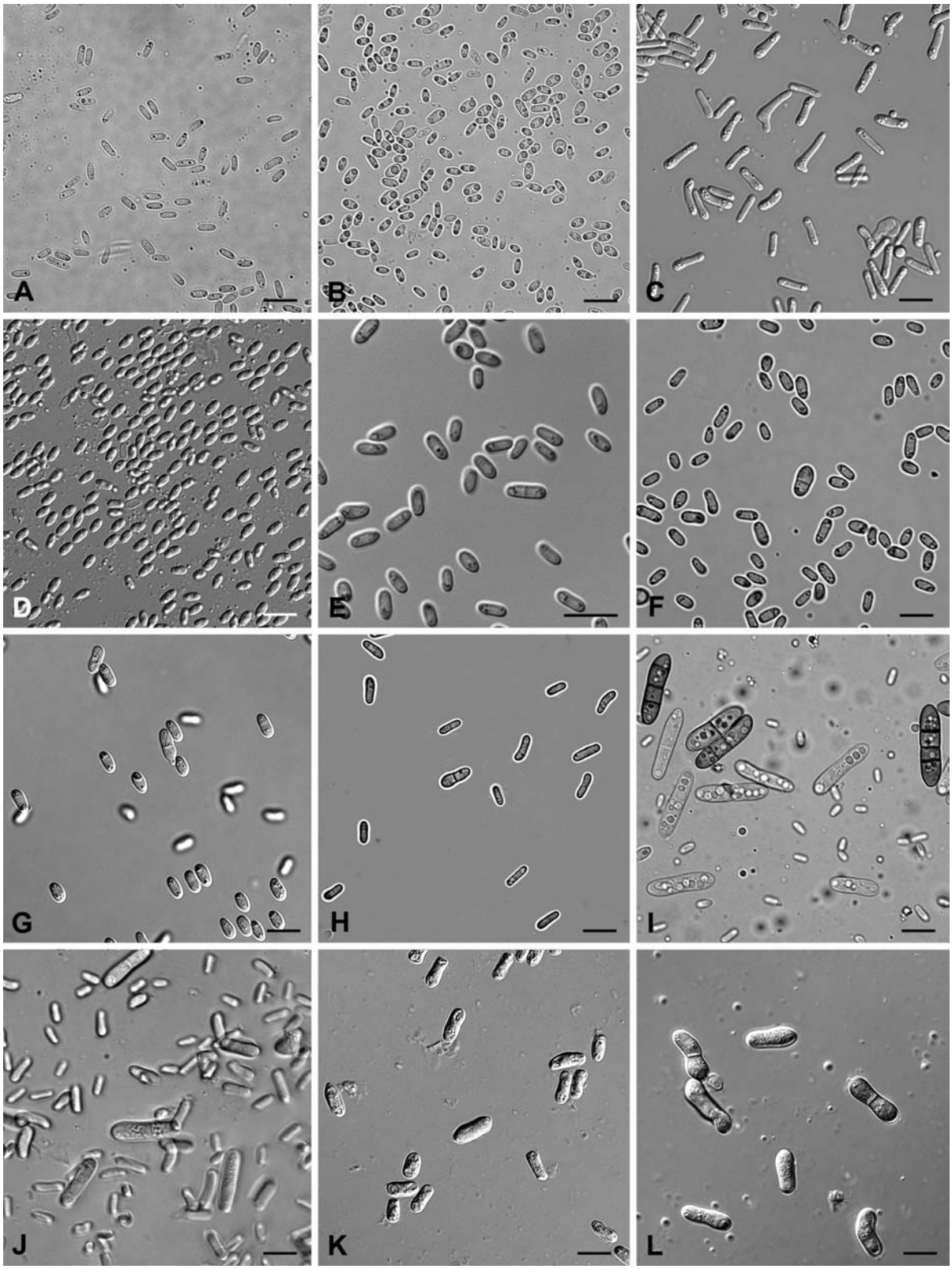

Fig. 4 A-L. Conidial morphology in *Phoma*. A-D. Conidia hyaline, aseptate small-sized as in A. *P. herbarum* B. *P. multirostrata* C. *P. astragali* and D. *P. eupyrena*. E-H. Conidia hyaline, occasionally septate, small-sized as in **E.** *P. exigua* **F.** *P. cucurbitacearum* **G.** *P. macrostoma* and **H.** *P. polemonii*. **I-J.** Conidia generally hyaline small-sized, but always also large septate and often pigmented condia occur as in **I.** *P. actaeae* and **J.** *P. schneiderae*. **K-L.** Conidia hyaline, occasionally septate, but all relatively large as in **K.** *P. rabiei* and **L.** *P. xanthina*. Scale bars = 10 µm.

Phoma colonies after 7 d of incubation at 20-22ºC in complete darkness, and complete colony descriptions after a further incubation period of 7d at the same temperature, but at a UV : dark interval of 11:13 (Boerema *et al*., 2004). Unfortunately, within a single species, wide variability may be observed, especially the presence of sector formations as mycelial or pycnidial zones. Again, standardisation is essential, as slight alterations in the media may be the cause of observed differences in growth characteristics (Rai, 1998).

The use of biochemical reactions and physiological tests to indicate the presence of certain metabolites was common practice in *Phoma* systematics (Wollenweber and Hochapfel, 1936; Dennis, 1946; Boerema and Höweler, 1967; Dorenbosch, 1970; Monte *et al*., 1990, 1991; Noordeloos *et al*., 1993). The application of alkaline reagents (KOH, NaOH) on fresh cultures is still used as it may change the colour of pH dependent metabolites and pigments (Boerema and Höweler, 1967; Dorenbosch, 1970). Furthermore, the biochemical analysis of dendritic crystals formed in older cultures can be used for the identification of a strain up to species level (Noordeloos *et al*., 1993). Although the development of such techniques in mycology is not yet fully optimised, the interest in such methods for species identification is decreasing as such specific methods can only be applied on a limited scale. Instead, molecular labs are more commonly equipped to perform more routine and popular DNA-based methods, which are thought to be more consistent.

DNA-based approaches in Phoma taxonomy

Modern DNA-based techniques can greatly contribute to identification and taxonomy of fungal species. Nevertheless, the actual number of studies using macromolecular approaches to define new species in *Phoma* and resolve species complexes within this genus is relatively low (e.g. Shoemaker and Brun, 2001; Bridge *et al*., 2003, 2004; Torres *et al*., 2005a, b).

To identify strains up to species level, a high level of expertise is required. Nevertheless, misidentifications in morphological taxonomy of *Phoma* species cannot always be avoided, as not all isolates may fully express all the species-specific characters. As a result, up to 2003 almost one fifth of the total number of *Phoma* strains of which sequences were submitted to public sequence databases such as GenBank were found to be incorrectly identified (Bridge *et al*., 2003). This trend is not unique to *Phoma*, however, as this percentage is slowly increasing over the years, and by 2006, close to 27% of the total public fungal sequences were derived from incorrectly identified strains (Nilsson *et al*., 2006). This may also be due to the existence of so-called species complexes, in which multiple morphologically indistinguishable taxa are occupying the same ecological niche, but are only distantly related from an evolutionary point of view. Further, it seems to have become common practice to sequence fungal strains without even identifying them morphologically (Hyde and Soytong, 2007). Bridge *et al*. (2004) suggested that the nucleotide sequences deposited as *P. herbarum* in GenBank probably represent at least two different taxa. Because *P. herbarum* is the type species of the genus *Phoma* (Boerema, 1964; Morgan-Jones, 1988a), it should be unambiguously clarified to which taxon the name *P. herbarum* should be applied.

Several other species complexes have been revealed using molecular techniques. In this context the *P. lingam* species complex can be mentioned. A natural variance in virulence and pathogenicity within *P. lingam* was observed in the United States (Pound, 1947) and Canada (McGee and Petrie, 1978). These findings were found to occur worldwide, as high genetic diversity amongst isolates of this species was observed by e.g. Johnson and Lewis (1990), Schäfer and Wöstemeyer (1992), Morales *et al*. (1993), Pongam *et al*. (1999), Williams and Fitt (1999), Purwantara *et al*. (2000), and Voigt *et al*. (2001). Molecular typing tools aided in distinguishing two species within *P. lingam* (teleomorph: *L. maculans*), which could hardly be separated using solely morphological characters. The teleomorph of the weakly aggressive variant was described as *L. biglobosa* with an unnamed anamorph in the genus *Phoma* (Shoemaker and Brun, 2001). *Leptosphaeria biglobosa* comprises 3 to 5 separate taxa itself, whereas the remaining isolates in *P. lingam* also seem to be a heterogeneous assemblage of cryptic taxa

(Howlett *et al*., 2001; Mendes-Pereira *et al*., 2003; Barrins *et al*., 2004; Voigt *et al*., 2005). Other species complexes that have been revealed to require further study include *P. cucurbitacearum* and *P. exigua*. Several studies on the population structure of *Didymella bryoniae*, the teleomorph of *P. cucurbitacearum*, revealed the presence of at least two separate subgroups within the American population (Somai *et al*., 2002a; Kothera *et al*., 2003). Supporting the theory of Van der Aa *et al*. (2000) on the existence of 11 varieties within *P. exigua*, Abeln *et al*. (2002), using AFLP-data, showed a widely distributed variance within *P. exigua* var. *exigua*. New, unnamed variants of this taxon appear to represent some serious pathogens, such as a recently discovered pathogen on lettuce (Koike *et al*., 2006). The discovered species complexes are probably just the tip of an iceberg, as most species have been studied less intensively. Based on their variable appearance, it can also be assumed that species such as *P. complanata*, *P. macrostoma* and *P. leveillei* are heterogeneous assemblages of multiple taxa.

Recently, a phylogenetic approach based on molecular data for the classification of fungi has aided in the discovery of two new species. This approach consisted of a DNA database, which was compared to several unidentified *Phoma* strains. The described species include *P. billsii*, that was isolated from Hawaiian soil samples, and a species from the American West Coast that provisionally - and as shown earlier in this paper, questionably - is described in the genus *Plenodomus* as *P. morganjonesii* (Torres *et al*., 2005a,b).

How many species are included in Phoma?

The unclear Saccardoan criteria for defining a new *Phoma* species and the tradition of host associated characterisation, together with a large host-range of isolates and the widespread presence of the genus, led to the description of 638 taxa in 1884 (Saccardo, 1884). The number of *Phoma* "species" that have been characterised, grew to more than 2800 in the first half of the past century (Sutton, 1980; Monte *et al*., 1991). To date, almost 3000 *Phoma* epithets have been recorded in MycoBank (Crous *et al*., 2004; Robert *et al*., 2005).

Based on the new morphological methods of identification (Boerema and Bollen, 1975), the genus *Phoma* was revised. The recently published identification manual describes 223 specific and infra-specific taxa (Boerema *et al*., 2004). Although this manual describes most of the known plant pathogenic species and common saprobic species, we can assume that a large number of *Phoma* species still have to be (re-)described. Only 2000 of the approximately 5000 existing herbarium specimens have been studied by the Boerema and co-workers during the past 45 years, and although many of those could be recombined into other species, the total number of *Phoma* taxa listed in this work is probably incomplete. Ample research has also been conducted on lichenicolous species. At least 14 lichenicolous species could not be cultured, and their morphology is only known directly from the substrate (Hawksworth and Cole, 2004). Furthermore, physiological and molecular data of those species are lacking, making a classification in the current *Phoma* taxonomic system uncertain.

A second reason why the present taxonomical system is probably lacking taxa is that a number of strains are still not recognised, as these are solely known under a synanamorph or teleomorph name. In the Identification Manual eight such species are listed, most of them known under their respective *Leptosphaeria* teleomorphs (Boerema *et al*., 2004). Furthermore, many species show a high natural variation in shape and size of microscopical structures and cultural characters. Due to phenotypical characters, pathogenicity, host range or virulence, separate species in such complexes are often hard to recognise. The aid of molecular typing tools is thus needed for the differentiation of separate taxa.

On the other hand, the Identification Manual also contains some taxa of which the validity remains debatable. For example, the three intraspecific taxa of *P. multirostrata*, the varieties *multirostrata*, *microspora* and *macrospora*, can solely be distinguished based on their conidial dimensions (Boerema, 1986). Those different varieties were not accepted by various other authors (Morgan-Jones, 1988b; Rai, 1998), because the conidial dimensions of the three varieties strongly overlap, and inter-

mediate forms are regularly encountered (Boerema, 1986; De Gruyter *et al*., 1993; Boerema *et al*., 2004). Nucleotide sequences from several house-keeping genes of the type strains of the three varieties do not show any differences (Aveskamp, unpubl. data), and the subdivision of *P. multirostrata* is therefore questionable.

In the same way as the *in vivo* phenotype may depend on the composition of the substrate, the high intra-specific variation in culture is the type and pH of the media used, as well as the growth temperature. This may have influenced the shape and size of pycnidia, conidia and chlamydospores, growth-rate, pigmentation and colony colour in many descriptive studies (Rai, 1998; Onfroy, 1999). Furthermore, the difference in condition of structures in herbarium material and freshly collected samples can be confusing in descriptive studies (Shin and Mel'nik, 2004), and could have aided in the misunderstanding of generic boundaries.

The state of our knowledge of the systematics of the genus is not much further advanced than it was in the Saccardoan system. An idea of the descriptive work that still has to be carried out is seen in the 40-year study of Boerema and co-workers. Of the 223 taxa they accepted, 110 (almost 50%) were recombined from other taxa or described as new. As only 40% of the herbarium species has been re-examined thus far, this indicates how many years of taxonomical work may lie ahead.

The future of Phoma research

Currently the concept and limits of *Phoma* are still under debate. Understanding true phylogenetic relations of species currently classified in *Phoma* requires new studies on the type materials and reference strains of its taxa and their related genera, and the generation of both phenotypic and genetic data. The strains present in the fungal collections in the world should be combined for an intensive study based on the morphology and genotypic characters of the taxa within the genus complex to obtain a better understanding of the genus, and to define a sound generic concept. At the species level much research needs to be conducted on the species complexes, which in some cases can best be explained as a recent speciation event. It must always be kept in mind that strains that look alike, and that are assigned to as the same species, might quite well be phylogenetically distinct. This is certainly the case within the subclassification of *Phoma*, for example in the sections *Phyllostictoides*, *Heterospora* and *Phoma*. But as is shown at the generic level, it might also be the other way around: morphologically distinct taxa that only recently have evolved from a shared common ancestor. Understanding how speciation in *Phoma* occurs will be one of the main challenges for the future. In the apparently closely related genus *Ascochyta*, the mating type locus has been characterised in two species: *A. lentis* and *A. rabiei* (Barve *et al*., 2003; Chérif *et al*., 2006). The mating type related genes have proven to be highly informative in reconstructing phylogeny (Barve *et al*., 2003), probably because these are much more involved in the speciation process than the house-keeping genes and the nrDNA loci, which are more commonly used in phyogenetical studies. Also the mating type sequence of *Leptosphaeria maculans*, teleomorph of *Phoma lingam*, has been determined (Cozijnsen and Howlett, 2003), and was valuable in phylogenetic reconstruction (Voigt *et al*., 2005).

To solve the problems in identification of isolates found at various ports of import and border controls, a comprehensive taxonomic system is urgently required to provide rapid identifications to aid in distinguishing plant pathogenic quarantine organisms from saprobic species and opportunists. The development of a polyphasic identification tool, based on a good collection of strains is needed to aid proper identification of isolates. One of the most promising initiatives that can aid in this is DNA-barcoding (Hebert, 2002), and the subsequent development of microcodes (Summerbell *et al*., 2005), an initiative in which a short species-specific nucleotide sequence is sought for each taxon. This approach may be of high value to invasive species identification (Armstrong and Ball, 2005). Recently, a joint programme was initiated between the CBS Fungal Biodiversity Centre and the Dutch Plant Protection Service to develop a new *Phoma* identification method and database, mainly based on this DNA-barcoding concept. The crux of this project is, however, to fully

understand the species concepts before taxon-specific nucleotide sequences can be identified.

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