

## CLASSIFICATION OF *PHOMA* SPECIES USING NEW PHYLOGENETIC MARKER

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### Abstract

The cosmopolitan *Phoma* genus contains mainly phytopathogenic, opportunistic parasite, and saprophyte fungal species. The currently applied classification is based on morphological characters. In this study, a preliminary classification for six taxa of *Phoma* were constructed, viz. *P. exigua* var. *linicola*, *P. pinodella*, *P. eupyrena*, *P. herbarum*, *P. foveata*, and *P. destructiva*, based on DNA sequence data of translation elongation factor1 subunit  $\alpha$  (EF1 $\alpha$ =tef1). The six *Phoma* species were compared to two species of the closely related *Ascochyta* genus, and was divided into two clades. The phylogenetic tree based on tef1 sequences does not support the traditional *Phoma* sections set up by morphological characters.

**Key words:** *Phoma exigua*, *Phoma pinodella*, *Phoma eupyrena*, *Phoma herbarum*, *Phoma faveata*, *Phoma destructiva*, marker

### INTRODUCTION

*Phoma* is a cosmopolitan genus of coelomycetous fungi. Many species have been reported from wide range of hosts, substrates, particularly as pathogens from plants, as well as soil-borne but predominantly saprophytic and opportunistic species have also been isolated. Almost 2000 *Phoma* species have been reported throughout the world till now (Boerema et al., 2004).

There are several ways in the traditional and modern mycology to contribute to taxonomical studies of fungi including morphology, biochemistry, nucleic acid sequences and many others.

The three most commonly discussed species concepts are Morphological Species Concept, Biological Species Concept, and Phylogenetic Species Concept. Since the beginning of mycology, studies of species concept in fungi have been mainly based on morphological elements. The most of the species and other taxa of *Phoma* have so far been determined on the basis of morphology on standardized media, and gene sequence analysis was only used as a confirmative or distinctive complement. Thus, members of the genus are primarily defined by the application of the Morphological Species Recognition (MSR).

The weakness of MSR is that species diagnosed by this often comprise more than one species when diagnosed by Biological Species Recognition (BSR) or Phylogenetic Species Recognition (PSR).

Biological species concept defining species as „...groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups” (Mayr, 1940). BSR acceptable for many fungi, where sexual reproduction occurs. But there are also fungal groups, where sexual reproduction has never been discovered. Approximately 20% of fungi are morphologically asexual and do not produce meiospores (Reynolds, 1993). Since strains of *Phoma* spp. apparently can not be crossed, the application of the BSR concept is impracticable. Though, despite reproducing asexually, many anamorphic fungi including *Phoma* spp. are known to possess a surprisingly high level of genetic variation (Khon, 1995; Talhinhas et al., 2002).

The current advances in biochemical and molecular research have provided mycologists with powerful tools that can be used for delineation of fungal taxa. The PSR species as „... the smallest aggregation of populations with a common lineage that share unique, diagnosable phenotypic characters” (Harrington and Rizzo, 1999). According to Taylor et al. (2000) seems to be well suited for fungi and likely to become very popular with mycologists, because it can be applied equally both to sexual and to asexual organisms. Taylor et al. (2000) proposed the genealogical concordance phylogenetic species recognition (GCPSR) *for species defining*, which *could be* an attractive alternative or complement to the morphological species concept, but has not been widely applied to *Phoma* spp. yet. It requires the analysis of several unlinked genes and implies that the phylogenetic position of a true species is concordant in at least some of them and not be contradicted in the others.

Up to now the characterization of *Phoma* species have been mostly applied on the basis of morphology, phenotype and physiology. Recently, a work of Boerema et al. entitled *Phoma* Identification Manual, based on morphological studies was published, which contains 223 cultural descriptions of specific and intraspecific taxa of *Phoma* Sacc. (Boerema et al., 2004).

In the middle of 90s, due to the advances in molecular and biochemical research of that time molecular markers were identified in *Phoma*. Some isozyme analyses were applied to distinct some morphologically identical *Phoma* species from each other (Kövics, 1995). Protein polymorphisms comparing to DNA polymorphisms is unfavorable, because protein electrophoresis assays the genotype indirectly, and a high proportion of the variation occurs at the DNA level may not be detectable as it does not alter the amino acid composition of the protein. Some changes in amino acid composition similarly do not change the electrophoretic mobility of the protein, and remain undetected, leading to different genotypes being assigned to the same allozyme allele.

DNA polymorphisms are based on differences in DNA sequences and have three enormous advantages over protein polymorphisms. The first is, that the sequence difference is detected directly. The second advantage is that they occur in a genome at very high frequency and finally they are not subject to selection pressure, in case they do not affect the phenotype. But morphological characterization besides molecular tools will remain a basic and powerful key in the identification of *Phoma* species.

One of the most commonly used molecular techniques for assessing phylogenetic relationships is to use the sequences of certain fungal DNA regions. Phylogenetic sequence comparisons concentrate on a comparison of the coding portions of the ribosomal genes and their RNA products, allowing discrimination at different taxonomic levels. Many phylogenetic works are based on the internally transcribed spacers (ITS), which one of the most widely used molecular markers due to their highly variability in nucleotid sequences. However, in *Phomas*, the ITS sequence analyses have not been showed significant nucleotide differences to infer relationships (Balmas et al., 2004). Because of it, there is a consequent trend toward inclusion of other gene loci in the data sets, gathered for phylogenetic analysis. Among these genes, the translation elongation factor has been found useful for investigating relationships between fungi at all levels (Roger et al., 1999).

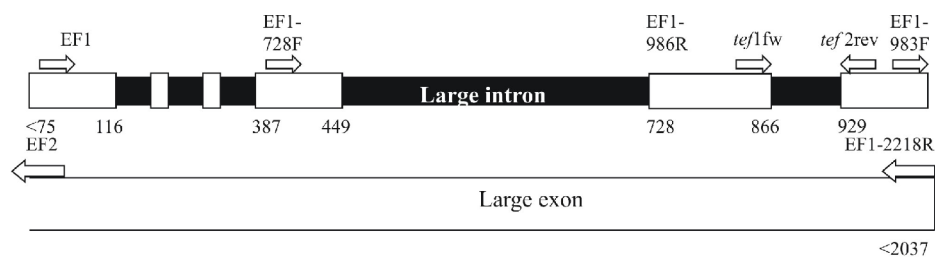
In this study we have obtained DNA sequences from translation elongation factor to resolve phylogenetic relationships among several *Phoma* species, formerly considered to closely related species.

Translation elongation factor 1 subunit alpha (EF1 $\alpha$ =*tef1*) is part of the cytosolic EF1 complex whose primary function is to promote the binding of aminoacyl-tRNA to the ribosome in a GTP-dependent process (Moldave, 1985). It is an essential component of the protein synthesis process in eukaryotes and archeabacteria. Complexed with GTP it carries the aminoacyl-tRNA to the A site of the ribosome-mRNA-peptidyl-tRNA complex; upon hydrolysis of GTP it leaves the ribosome as EF-1 $\alpha$ -GDP.

Simultaneously, elongation factor 1 $\alpha$  (EF-1 $\alpha$ ) is a highly conserved ubiquitous protein that has been suggested to have desirable properties for phylogenetic inference (Roger et al., 1999). EF-1 $\alpha$  is well suited for determining phylogenetic relationships due to its universal occurrence and presence typically as a single copy within the genome. (Baldauf and Doolittle, 1997). It has been proven to be a useful gene to resolve phylogenetic relationships at species level as well as in deeper divergences where amino acid substitutions provide phylogenetic resolution.

In this study, we employed EF-1 $\alpha$  as a potential phylogenetic markers to infer phylogenetic relationships among different *Phoma* taxa using nucleotide characters obtained by sequencing a part of the EF-1 $\alpha$  gene,

containing both introns and exons. The only disadvantage of using EF-1 $\alpha$  is being much shorter than other markers (Fig. 1). However combined gene genealogical studies have proven to be highly efficient in identification of phylogenetic species (Taylor et al., 2000), and can also be useful to delimit the *Phoma* species of the present study. The gene itself exceeds 2kb in length, and consisted of several relatively large and variable exons and introns.



**Figure 1. Schematic structure of *tef1* gene of *Phoma* spp. and location of primers used to amplify different parts of it for phylogenetic inferences**

## MATERIALS AND METHODS

The following six different *Phoma* species isolated from infected plants were obtained from the culture collection of our department: *Phoma exigua* var. *linicola*, *P. eupyrena*, *P. destructor*, *P. medicaginis*, *P. foveata*, and *P. herbarum*. All isolates were identified morphologically according to Boerama et al. (2004) based on physiological and morphological characteristics. Three more translation elongation factor partial DNA of *Ascochyta* (*Ascochyta* Georgia 12 [DQ386496], *Ascochyta pisi* strain AP2 [DQ386494]) and *Phoma* (*Phoma pinodella* CBS 318. 90 [AY831542]) species were downloaded from GenBank maintained by the NCBI.

Total Genomic DNA from each of the six isolates were extracted from freeze-dried mycelium and isolated using the E.Z.N.A.TM Fungal DNA Isolation Kit (Omega Bio-tek, Inc., USA) as following the manufacture instructions. A 250–260 bp fraction of the EF-1 $\alpha$  gene was amplified with the help of two primers (EF1-728F and EF1-986R, Druzhinina and Kubicek, 2005). The phylogenetic analysis was conducted with PAUP\*4.0b (Swofford, 2002).

## RESULTS AND DISCUSSIONS

Seven different *Phoma* and two *Ascochyta* strains were compared in this study. The morphological identification of the isolates was done following

the descriptions of Boerama et al. (2004). The obtained results indicated that the microscopical and cultural characteristics of the concerned *Phoma* strains fit the identity of *Phoma exigua* var. *linicola*, *P. eupyrena*, *P. destructor*, *P. medicaginis*, *P. foveata*, and *P. herbarum*. Sequence data of the protein coding translation elongation factor 1 subunit  $\alpha$  (EF1 $\alpha$ = *tef1*) was acquired for the seven *Phoma* strains in this preliminary experiment. One sequence from *Phoma pinodella* was obtained from the gene bank (CBS 318.90, accession number: AY831542, from GenBank maintained by the NCBI). Two *Ascochyta tef1* sequences were applied as an outgroup for the sequence analysis (accession number: DQ386494 and DQ386496).

A single product of approximately 250 to 260 bp was obtained from all the PCR amplifications with primers EF1-728F and EF1-986R for each of the 6 tested *Phoma* strains.

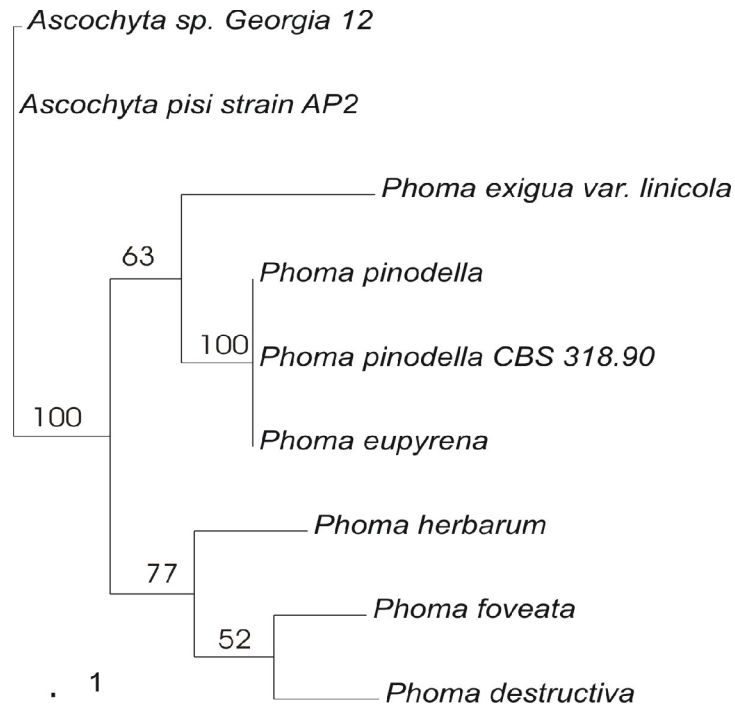
The basic sequence matrix consists of 272 sites, of with 150 (55,1%) variable characters among the six studied *Phoma* species. Including the two *Ascochyta* species the examined region (272 bp) contained 116 bp (42,6%) identical region with the *Phoma* species.

The *tef1* sequence was chosen for this analysis because it has been shown earlier to have desirable properties for phylogenetic inference for different fungal species like *Trichoderma* spp. (Druzhinina and Kubicek, 2005) and others (Roger et al., 1999). *tef1* sequences of our six *Phoma* strains and three other sequences from sequence data bank were used for the analysis. According to the phylogenetic tree based on *tef1* sequences (Fig. 2), the *Phoma* species are well separated from their closely related *Ascochyta* taxa. On the basis of the bootstrap values, the seven *Phoma* strains could be divided into two main groups with 64 and 77% probability. Both of *Phoma* main groups comprise two subgroups. Group 1 includes the *Phoma exigua* var. *linicola* subgroup and the *Phoma pinodella* subgroup. The later one contains two different *Phoma pinodella* species: one's sequence originates from the GenBank (CBS 318.90) and the other's is own isolates. The support for the *P. pinodella* subgroup to be one clade is 100% (indicated by the bootstrap analysis). The *Phoma eupyrena* also associates with this *P. pinodella* subgroup with a bootstrap value 100%, which means that the two species can not be distinguished on the basis of *tef* sequence. Group 2 includes the *Phoma herbarum* subgroup and the *Phoma foveata* associated with *Phoma destructiva* subgroup.

Within the *Phoma* genus the *P. exigua* var. *linicola*, *P. pinodella* and *P. foveata* belong to the section *Phyllostictoides*, while the *P. eupyrena*, *P. herbarum* and *P. destructor* belong to the section *Phoma* based on morphology. The phylogenetic tree based on *tef* sequences (Fig. 2) does not support the traditional *Phoma* sections based on morphological characterization as *P. exigua* var. *linicola*, *P. pinodella* and *P. eupyrena*

belong to one clade, while *P. herbarum*, *P. foveata* and *P. destructiva* belong to the other clade.

Further investigations would be necessary to clarify whether the *tef1* gene sequence as phylogenetic molecular marker is well suited for the classification of *Phoma* species.



**Figure 2. Phylogenetic relationships of 7 of *Phoma* strains and 2 of *Ascochyta* spp. inferred by the parsimony analysis of *tef1* sequences. The tree was obtained from analysis by the parsimony method using the PAUP\*4.0b program. The numbers above the lines represent the bootstrap (bootstrap=1000) values**

## REFERENCES

1. Baldauf, S.L., Doolittle, W.F., 1997, Origin and evolution of slime molds (Mycetozoa). *Proc. Natl. Acad. Sci. USA* 94: 12007–12012.
2. Balmas, V., Scherm, B., Ghignone, S., Salem, A.O.M., Cacciola, S.O., and Migheli, Q., 2005, Characterisation of *Phoma tracheiphila* by RAPD-PCR, microsatellite-primed PCR and ITS rDNA sequencing and development of species primers for *in planta* PCR detection. *European Journal of Plant Pathology* 111: 235-247.

3. Boerema, G.H., Gruyter, J., Noordeloos, M.E., and Hamers M.E.C., 2004, *Phoma* identification manual. CABI Publishing. CAB International Wallingford, Oxfordshire, UK. 470 pp.
4. Druzhinina, I. and Kubicek, C.P., 2005, Species concepts and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species cluster? *J. Zhejiang Univ. Sci.* 6B (2): 100-112.
5. Harrington, T.C. and Rizzo, D.M., 1999, Defining species in the fungi. In: *Structure and Dynamics of Fungal Populations*. J.J. Worrall (Ed.) pp. 43-70. Kluwer Academic, Dordrecht.
6. Khon, L.M., 1995, The clonal dynamic in wild and agricultural plant-pathogen populations. *Canadian Journal of Botany* 73. (Suppl. 1): S 1231-1240.
7. Kővics, G. and Gruyter, J. de, 1995, Comparable esterase isozyme analysis of some *Phoma* species occur on soybean. (A szóján előforduló néhány *Phoma* faj észteráz izoenzim mintázatainak összehasonlító vizsgálata.) *Proceedings of Debrecen Agricultural University (DATE Tudományos Közleményei)* 31: 191-207.
8. Mayr, E., 1942, *Systematics and the origin of species from a viewpoint of a zoologist*. Columbia University Press, New York.
9. Moldave, K., 1985, Eukaryotic protein synthesis. *Annu. Rev. Biochem.* 54: 1109–1149.
10. Reynold, D.R., Taylor, J.W., 1993, The fungal holomorph: An overview. In *The Fungal holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics* (D.R. Reynolds and J.W. Taylor, Eds.), pp. 15-25. CAB International, Wallingford, UK.
11. Roger, A.J., Sandblom, O., Doolittle, W.F., and Philippe, H., 1999, An evaluation of elongation factor 1 $\alpha$  as a phylogenetic marker for eukaryots. *Mol. Biol. Evol.* 16: 218–233.
12. Swofford, D.L., 2002, *PAUP: Phylogenetic Analysis Using Parsimony (and other methods)*. Version 4b10. Sinauer Associates, Sunderland, MA.
13. Talhinhas, P., Sreenivasaprasad, S., Neves-Martins, J., and Oliveira, H., 2002, Genetic and morphological characterization of *Colletotrichum acutatum* causing anthracnose of lupins. *Phytopathology* 92: 986-996.
14. Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S., and Fisher, M.C., 2000, Phylogenetic species recognition and species concepts in fungi. *Fungal Genet. Biol.* 31: 21– 32.