Thesis of doctoral (PhD) dissertation

CONVENTIONAL AND MOLECULAR COMPARISONS OF THE TAXONOMY OF *PHOMA*-LIKE SPECIES

László Miklós Irinyi

Supervisors: dr. Erzsébet Sándor and dr. György Kövics

University of Debrecen
Kálmán Kerpely Doctoral School

Debrecen, 2009

Introduction

The pathogenic fungi are responsible for most of plant diseases. Their described species number is approximately 75.000 at present, which could represent only 7% of the estimated number of all fungi species. Therefore there are lots of challenges that mycologist have to face to study and discover the biodiversity of fungi. The traditional mycological tools are widened with newer and newer powerful molecular techniques which make possible to infer phylogenetic relationships in some cases by reconstructing systems which had been already established. Our taxonomical issue contributes to the first result of this by the phylogenetic analysis of some complex and hard identifiable fungal taxa with the help of new molecular tools such as *Phoma*-like fungi which contain more than 5000 species. *Phoma* is a cosmopolitan genus of coelomycetous fungi. Many species have been reported from a wide range of hosts and substrates, particularly as pathogens from plants, as well as soil-borne but predominantly saprophytic and opportunistic species have also been isolated.

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, biological, and phylogenetic ones. 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 defines species as groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups. BSR is acceptable for many fungi, where sexual reproduction occurs. But there are also fungal groups, where sexual reproduction has never been recognized. 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.

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 defines species as the smallest aggregation of populations with a common lineage that share unique, diagnosable phenotypic characters. According to Taylor *et al.* (2000) PSR 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. They proposed the introduction of 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 can not be contradicted in the others.

Up to now the characterization of *Phoma* and *Phoma*-like species has been mostly applied on the basis of morphology, phenotype and physiology which have often resulted in uncertainty and misidentification. Recently, Boerema *et al.* (2004) published *Phoma* Identification Manual, based on morphological studies which contain 223 cultural descriptions of specific and intraspecific taxa of *Phoma* Sacc.

Till now the molecular-based phylogenetic analysis within the *Phoma* genus have only been used for defining phylogenetic relationships among isolates within one ore closely related species. Therefore our goal has been to find and apply such phylogentetic markers which are suitable for inferring phylogenetic relationships – particularly for *Phoma*-like species which are hard to identify by traditional way – at species level within the *Phoma* genus.

For phylogenetic analysis we have chosen the complete sequence of ITS region and fragments of *tef1* and *β-tubulin* genes which have already been proved to be useful as potential markers for phylogenetic studies at other different fungal taxa.

Ribosomal DNA (rDNA) has long been used as a potential marker for phylogenetic studies (reviewed in Avise, 2004). Many fungal taxonomy studies have applied ITS regions and have proved them to be suitable for resolving relationships both at genus and at species level.

Simultaneously, translation elongation factor 1 subunit alpha (EF1 α) encoding gene (*tef1*) has been proved to be a useful gene to resolve phylogenetic relationships at species level, as well as in deeper divergence of fungi.

The nucleotide sequences of tubulin encoding gene *β-tubulin* has received more and more attention for phylogenetic studies in lower and

higher taxonomic levels as well. Voigt *et al.* (2005) and Fatehi *et al.* (2003) used the *β-tubulin* gene, among others, to analyse the *Leptosphaeria maculans* (anamorph: *Phoma lingam*) species complex, to resolve molecular relatedness within the *Ascochyta pinodes* complex. The phylogenetic analysis of multiple protein-encoding genes with Genealogical Concordance Phylogenetic Species Recognition

is proposed (Taylor *et al.*, 2000) as a more robust way of determining and recognising species than analysis based on morphology or mating behaviour. Our further aim was to apply and compare different character-based phylogenetic methods (Maximum Likelihood, Maximum Parsimony and Bayesian method).

Different pathogenic *Phoma*-like species (*Phoma pinodella*, *Phoma sojicola*, *Phyllosticta sojicola*, *Phoma exigua* var. *exigua*) have been found on Fabaceae species like soybean (*Glycine max*). Since these species are symptomatically and morphologically very similar to each other, it is hard to differentiate them. Consequently, this has resulted in uncertainty and misidentification in their taxonomy. Therefore there is need to develop additional rapid molecular methods to enable accurate identification.

Objectives

I compiled and performed my research relating to the conventional and molecular comparison of the taxonomy of *Phoma*-like species on the following conception:

1. Characterization and identification of different isolates of *Phoma*-like species based on the accepted conventional morphological concept of *Phoma* genus.

2. Looking for phylogenetic markers which could be suitable for inferring phylogenetic relationships – particularly for *Phoma*-like species which are hard to identify by traditional way – at species level within the *Phoma* genus.

3. Molecular phylogenetic relatedness studies to infer phylogenetic relationships at species level within the *Phoma* genus.

Inferring phylogenetic relationships at species level within the *Phoma* genus and *Phoma*-like species.

4. Applying, assessing and comparing different character-based phylogenetic methods (Maximum Likelihood, Maximum Parsimony and Bayesian method) in the characterization of *Phoma*-like species.

5. Determination the taxonomical status of *Phoma*-like isolate deposited as *Phyllosticta sojicola* isolated from soybean.

6. Re-evaluation on molecular basis of the taxonomic status of soybean pathogen *Phoma pinodella* and closely related *Phoma sojicola* species.

Materials and Methods

Morphological analysis

Twenty-two isolates of nine different *Phoma*-like species were obtained from reference culture collections (Table 1). Seven of them were isolated from soybean, the others were collected from different hosts. All cultures were maintained on oatmeal agar at 5 °C during the study. The species were characterized morphologically according to Boerema *et al*. (2004).

The isolates were cultured on oatmeal agar and malt agar incubated at 22°C in dark, and examined 7 days later. For description of colony colours Rayner's colour chart (1970) terminology was used. Petri dishes were then exposed to cycles of 13 h NUV-light, 11 h darkness to stimulate the formation of pycnidia. 2 weeks later colony descriptions were complemented and after 3 weeks the morphology of pycnidia, conidia and other structures, such as chlamydospores, were studied from the OA cultures. The NaOH spot test was done on MA by addition of a drop of 1N NaOH on the colony margin, and changes in colour were noted. Conidia dimensions refer to 30 measurements with oil-immersion at 1250x magnifition.

Table 1 – Isolates used in the phylogenetic analyses

Isolate no.	Alternative isolate no.	Fungal species	Host plant	Location of collection	Collector	GenBank accession no.		
						Tef[a]	ITS[b]	*β-tubulin*[c]
D/035	BT-15	*Phoma pinodella*	*Glycine max*	Hungary	I. Walcz	**EU543973**	**EU573015**	**EU541416**
D/045	PD82/550	*P. pinodella*	*Hordeum vulgare*	Hungary	G.J. Kövics	**EU543971**	**EU573025**	**EU541417**
D/046	PD77/165 MYA-411	*P. pinodella*	*Pisum sativum*	Hungary	G.J. Kövics	**EU543972**	**EU573024**	**EU541419**
D/095	N.A.	*P. pinodella*	*P. sativum*	Hungary	L. Gergely	**EU543970**	**EU573027**	**EU541418**
D/159	CBS 318.90 PD 81/729	*P. pinodella*	*P. sativum*	Netherlands	M.E. Noordeloos	**EU595355**	**EU573028**	**EU595352**
D/063[d]	Ph 58 MYA-408	*P. pinodella*	*Petroselinum crispum*	Poland	J. Marcin-kowska	**EU543975**	**EU573012**	**EU541420**
D/054	MYA-406	*P. sojicola*	*G. max*	Hungary	G.J. Kövics	**EU543974**	**EU573023**	**EU541434**
D/056	CBS 567.97 PD97/2160	*P. sojicola*	*G. max*	Hungary	G.J. Kövics	**EU543976**	**EU573026**	**EU541433**
D/050	CBS 301.39	*Phyllosticta sojicola*	*G. max*	Germany	K. Böning	**EU595356**	**EU573029**	**EU595357**
D/075	N.A.	*P. exigua* var. *exigua*	*G. max*	Poland	G.J. Kövics	**EU543982**	**EU555533**	**EU541421**
D/077	N.A.	*P. exigua* var. *exigua*	*G. max*	Poland	G.J. Kövics	**EU543983**	**EU573010**	**EU541422**
D/145	N.A.	*P. exigua*	*Althaea officinalis*	Hungary	G. Nagy	-	**EU573011**	**EU541425**
D/146	N.A.	*P. exigua*	*Althaea rosae*	Hungary	G. Nagy	**EU543984**	**EU573013**	**EU541427**
D/158	ICMP 15330	*P. exigua* var. *exigua*	*Agapanthus* sp.	New Zealand	M. Braithwaite	**EU543981**	**EU573008**	**EU541428**
D/157	ICMP 13336	*P. exigua*	*Cucurbita maxima*	New Zealand	P.G. Broadhurst	**EU543980**	**EU573007**	**EU541429**
D/071	PD 86/73	*P. exigua* var. *linicola*	*Linum usita-tissimum*	Hungary	G.J. Kövics	**EU543979**	**EU573009**	**EU541423**
D/072	PD 75/907	*P. plurivora*	*Medicago sativa*	Australia	J. de Gruyter	**EU552929**	**EU573018**	**EU552932**
D/155	ICMP 6875	*P. plurivora*	*Pennisetum clandestinum*	New Zealand	P.R. Johnston	**EU552930**	**EU573019**	**EU552931**
D/034	AI-416	*P. glomerata*	*G. max*	Hungary	G.J. Kövics	**EU543969**	**EU573016**	**EU541424**
D/156	ICMP 15788	*P. glomerata*	*Yucca sp.*	New Zealand	C.F. Hill	**EU543968**	**EU573017**	**EU541426**
D/048	PD 76/1021	*P. foveata*	*Chenopodium quinoa*	Netherlands	G. H. Boerema	**EU543985**	**EU573021**	**EU541431**
D/044	PD 77/508	*P. multirostrata*	*Phylodendron* sp.	Netherlands	G. H. Boerema	**EU543986**	**EU573022**	**EU541430**
D/144	N.A.	*Ascochyta rabiei*	*Cicer arietinum*	Australia	N.A.	**EU595354**	**EU595358**	**EU595353**
D/160	CBS 581.83A	*Didymella rabiei*	*C. arietinum*	Syria	H.A. van der Aa	**EU543978**	**EU573020**	**EU541432**

AI refers to Agrobotanical Institute, Tápiószele, Hungary
BT refers to Fodder-plant Research Institute of Pannon University, Iregszemcse-Bicsérd, Hungary
CBS refers to Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands;
D refers to the Culture Collection of the Plant Protection Department of University of Debrecen, Hungary
ICMP refers to International Collection of Microorganisms from Plants
MYA refers to American Type Culture Collection, (ATCC), USA.
N.A. = not available
PD refers to Plantenziektenkundige Dienst; Dutch Plant Protection Service Collection, The Netherlands

[a] partial sequence data of the translation elongation factor coding gene (*tef1*)

[b] 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, complete sequence; 26S ribosomal RNA gene, partial sequence

[c] partial sequence data of the *β-tubulin* coding gene

[d] D/063: *P. pinodella* misidentified as *'P. exigua* var. *exigua'*

DNA extraction

For PCR based methods the studied cultures were grown in 100 ml of malt broth (MB, containing 2% malt extract) for 48 hours at room temperature in the dark on a rotary shaker (125 rpm).Mycelium was harvested by vacuum filtration. Total genomic DNA was extracted from freeze-dried mycelium and isolated using E.Z.N.A.® TM Fungal DNA Isolation Kit (Omega Bio-tek, Inc., USA) according to the protocol, followed the manufacturer's instructions. DNA concentrations were estimated in comparison to known standard in agarose gels stained with ethidium bromide (EtBr).

PCR reaction and DNA sequencing

Amplifications of 50 µl PCR reaction contained 25 µl 2X PCR Master Mix 40-40 pmol each primer, 20-40 ng of DNA and nuclease free water were run out. Primers used to amplify approx. 520bp of the ITS region containing the internal transcribed spacer regions 1 and 2, moreover the 5.8S rDNA are based on published composite sequences, SR6R and LR1 (White *et al*., 1990) with the following amplification protocol: 3 min initial denaturing at 95 °C, followed by 5 cycles of 1 min at 95 °C, 1 min annealing at 50 °C, 1 min at 72 °C and 25 cycles of 1 min at 90 °C, 1 min annealing at 50 °C, 1 min at 72 °C and 15 min final extension at 72 °C. The large intron (approx. 300bp) of the *tef1* gene was amplified by the EF1-728F and EF1-986R primer pair (Druzhinina & Kubicek, 2005) according to the previously described protocol with a temperature of 56 °C rather than 50 °C. Primers Bt2a and Bt2b (Glass & Donaldson, 1995; O'Donnell & Cigelnik, 1997) were used to amplify a fragment (approx.

300bp) of *β-tubulin* gene and PCR conditions were carried out as described above with an annealing temperature of 58 °C. PCR was performed in a Primus thermocycler (MWG Biotech). Amplification products were subjected to electrophoresis in a 0.7 % agarose gel containing EtBr and visualized by UV illumination. The PCR products were purified by using YM-100 Microcon Centrifugal Filter Devices (Millipore). Purified amplification products were sequenced by MWG Biotech Company in Germany.

Data analysis

The obtained DNA sequences were aligned first with ClustalX (Thompson *et al*., 1997) and manually checked for ambiguities and adjusted when necessary using Genedoc (Nicholas *et al*., 1997). Single gaps were treated either as missing data or as the fifth base and multistate characters as uncertain. For the Bayesian and Maximum Likelihood analyses, models of sequence evolution were evaluated for each dataset and model parameter estimates obtained with Modeltest v.3.7 (Posada & Grandall, 1998). The character based phylogenetic studies were performed by Paup*4.0b (Swofford, 2002) and MrBayes (Huelsenbeck, 2000).

Results

Morphology

On the basis of morphological characters we studied different morphological characters on five *Phoma pinodella* (D/035, CBS 318.90, D/095, PD82/550, PD77/165), two *Phoma sojicola* (D/054, CBS 567.97),

three *Phoma exigua* var. *exigua* (D/075, D/077, Ph 58), one *Phyllosticta sojicola* (D/050) and one *Phoma exigua* var. *linicola* (D/071) isolates.

The morphological characters of *Phoma pinodella* (L.K. Jones) Morgan-Jones & K.B. Burch and *Phoma sojicola* (Abramov) Kövics et al. showed high resemblance and often overlap because of the high variability of the isolates. Cultural characteristics of certain isolates were very variable even in standardized conditions. It occurred that the same (*P. pinodella* PD77/165) isolates transferred at the same time in the same media, and cultured at the same temperature even showed different morphological characters. We have experienced similar variability at other isolates too, and we observed that the morphological characters may change after certain number of transfers on media *in vitro* as well. The *P. pinodella* generally produces crystals on malt-extract agar after one week while at *P. sojicola* misses this feature. In our study we have not experienced crystal formation that feature of *P. pinodella* species at none of the isolates, which can due to the fact that they can loose this character temporarily during several transfers on the media. In our experiments, the cultural characteristics of both species (*P. pinodella* and *P. sojicola*) were very similar without any well identifiable and strong features. The colours of the two colonies were very variable even in the same isolate in the same conditions. The size and shape of pycnidia and conidia showed also such a high similarity that we could not differentiate the two species reliably.

We have encountered similar difficulties differentiating the isolates of *Phyllosticta sojicola* Massalongo and *Phoma exigua* Desm. var. *exigua* (syn.: *Ascochyta phaseolorum* Saccardo). Studying the morphological features we can assess that there are no significant differences in none of the cultural characteristics, the size and shape of pycnidia and conidia and

that the morphological variability is rather high among isolates. The only difference we have found between the two isolates was the “E metabolite” production, which is not a feature of *P. sojicola* but a frequent character of *P. exigua* var. *exigua*. However, this absence of “E-metabolite” production might be a result of a genetic mutation.

In pursuance of examination, the taxonomical status of the isolate of *Phoma exigua* var. *exigua* Ph 58 has been found ambiguous on morphological characters. According to the MSR, the isolate shows high similarity to *P. pinodella* except the only difference in the “E-metabolite” production only. The Ph 58 isolate showed a positive NaOH spot test, which is a distinctive feature of *Phoma exigua* var. *exigua* but nor a characteristic of *P. pinodella*. All phylogenetic analyses classified the isolate Ph 58 in the clade of *P. pinodella* that is why we suppose that the isolate Ph 58 has been misidentified as *P. exigua* var. *exigua* and actually it belongs to *P. pinodella* species.

Since these species and isolates are very similar to each other both symptomatically and morphologically, and rather variable depending on strains it is hard to delimit them. Consequently, this has resulted in uncertainty and misidentification in their taxonomy. Therefore there is need to develop additional rapid molecular methods to enable accurate identification.

Molecular features

Sequence analysis of the tef1 gene

The topology of phylogenetic trees (Figs 1, 2, 3) obtained by the analyses of *tef1* sequences by three character based methods (Maximum Parsimony,

Maximum Likelihood, Bayesian method) was very similar. One part of the *Phoma* isolates (*P. pinodella*, *P. exigua*, *P. plurivora, P. destructiva, P. glomerata*) were well separated from the other *Phoma* isolates as well as the other parts constitute clades.

Parsimony analysis of *tef1* revealed 173 parsimony informative sites, 16 polymorphic sites, and 121 sites that were constant among all isolates.

Species represented with more than one isolate (*P. pinodella*, *P. exigua* var. *exigua*, *P. glomerata*, *P. plurivora*) were placed in the same clade. Interestingly, *P foveata* and *P. multirostrata* were also placed in one, highly supported group (100% PP) which raises the issue of misidentification on the behalf of depositor.

Besides *Phoma* isolates, the *Ascochyta* isolates (*Ascochyta lentis* / teleomorph: *Didymella lentis*) were also clustered in one group which has proved that the *tef1* sequences are well suited for delineating phylogenetic relationships within the *Phoma* as well as *Ascochyta* genera.

The isolates of *P. sojicola* (MYA-406, EU543974 and PD 97/2160, EU543976) grouped together with a *P. pinodella* isolates since their *tef1* sequences were completely identical.

The isolate deposited as *Phyllosticta sojicola* (CBS 301.39, EU595356) associated with the *Phoma exigua* var. *exigua* subgroups, which has supported the statement that the two species are identical (Kövics *et al.*, 1999).

The distances between each taxon have proved to be long and different enough so that we can consider the results or the phylogenetic tree well established.

The high bootstrap and Bayesian posterior probability values supported the solidity of the well separated clades and true coherence of the trees.

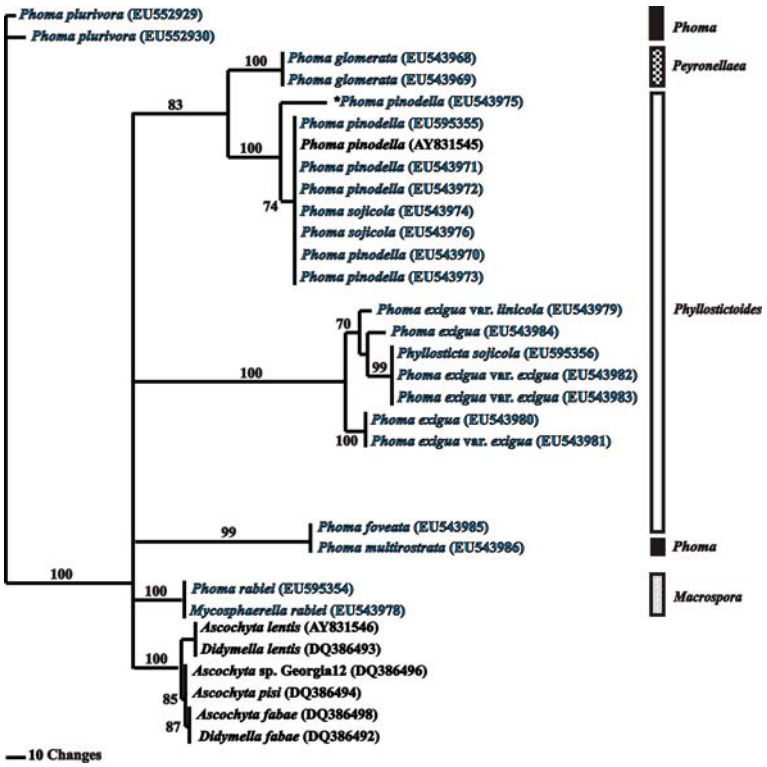

Fig 1. Phylogenetic relationships of *Phoma* strains inferred by Parsimony analysis of *tef1* sequences. The numbers above the lines represent the bootstrap (bootstrap=1000) values. The columns on the right side represent the *Phoma* sections based on morphological characterization (Boerema *et al.*, 2004). **P. pinodella* (Ph 58) misidentified as*'P. exigua* var. *exigua'*

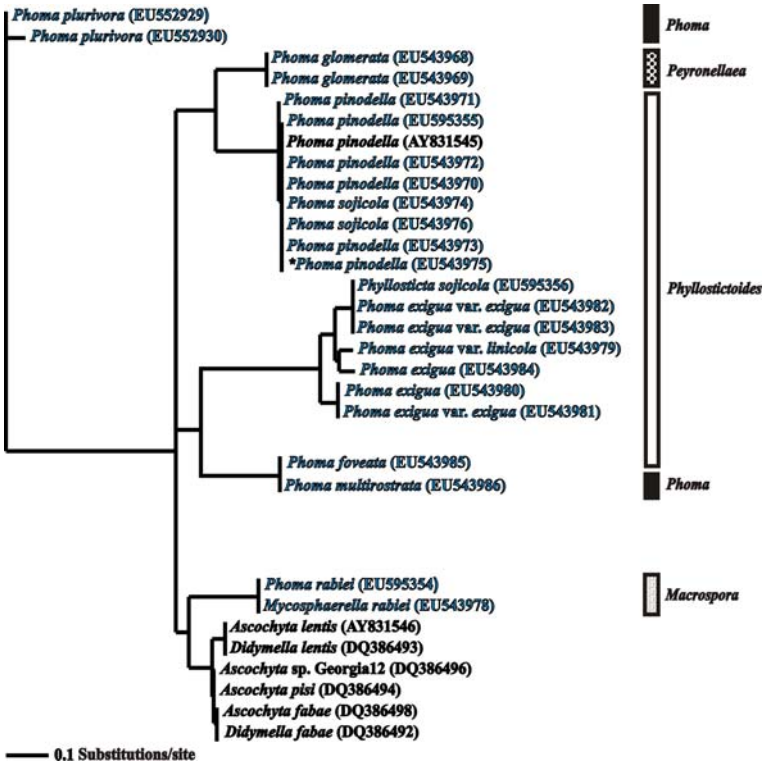

Fig 2. Phylogenetic relationships of *Phoma* strains inferred by Maximum Likelihood analysis of *tef1* sequences. The columns on the right side represent the *Phoma* section based on morphological characterization (Boerema *et al.*, 2004). **P. pinodella* (Ph 58) misidentified as *'P. exigua* var. *exigua'*

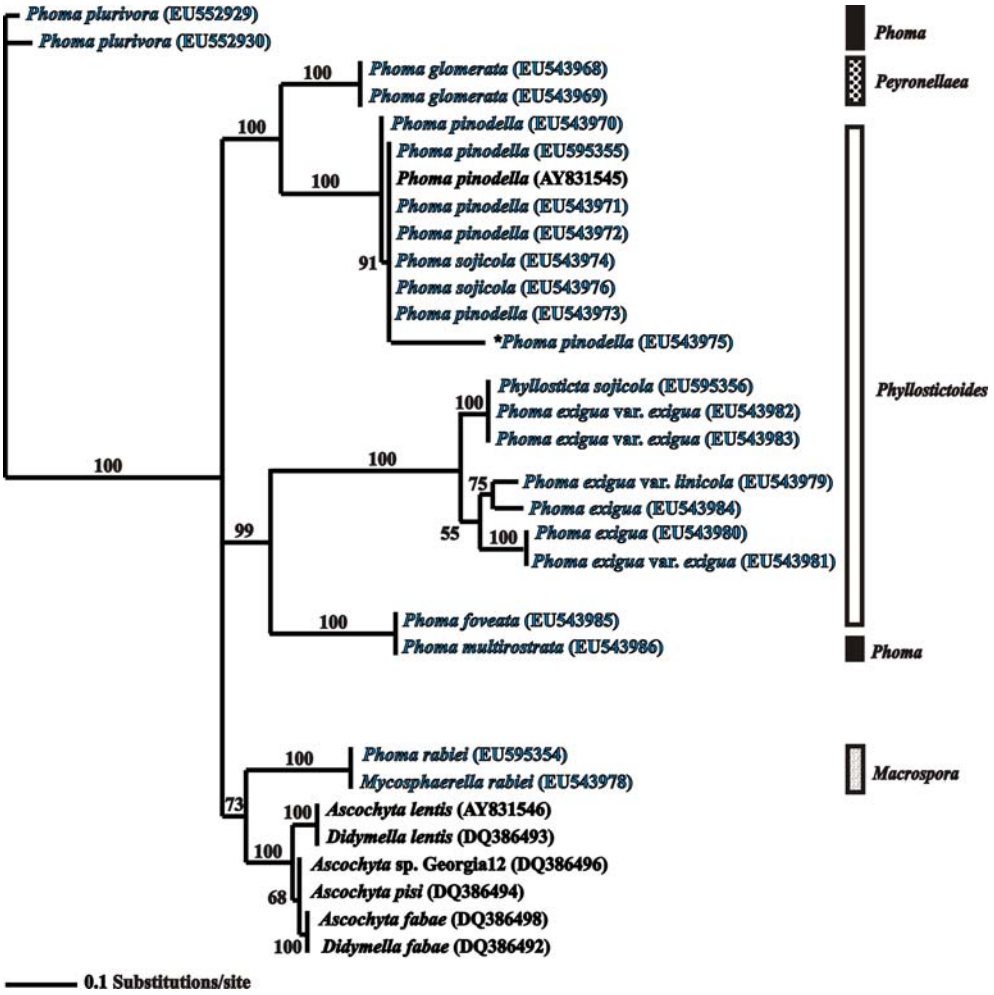

Fig 3. Phylogenetic relationships of *Phoma* strains inferred by Bayesian analysis of *tef1* sequences. The numbers above the lines represent the Bayesian posterior probability values. The columns on the right side represent the *Phoma* sections based on morphological characterization (Boerema *et al.*, 2004). **P. pinodella* (Ph 58) misidentified as *'P. exigua* var. *exigua'*

Sequence analysis of the ITS region

The obtained phylogenetic trees (Figs 4, 5, 6) were well resolved with similar clades recovered by tef1 analysis. Parsimony analysis of the ITS revealed 32 parsimony informative sites, 5 polymorphic sites, and 47 sites are constant among all isolates.

The differences between the isolates of *Phoma* and *Ascochyta* species were not significant so it is questionable, that the selected ITS region is suitable for phylogenetic studies at species level within the *Phoma* genus. The bootstrap values of *Phoma* and *Ascochyta* clades were low 854-69) therefore it is not possible to draw reliable conclusions for the phylogenetic

relationships of the species of the two genera. However the Bayesian posterior probability values (93-99) were higher than that of the bootstrap.

Species represented with more than one isolate (*P. pinodella*, *P. exigua* var. *exigua*, *P. glomerata*, *P. plurivora*) were placed in the same clade which supported the solidity of the well separated clades and coherence on the trees. Interestingly, *P. foveata* and *P. multirostrata* were also placed in one, highly supported group (100% PP and 100% BS) which raises the issue of misidentification on behalf of the depositor.

The high bootstrap and Bayesian posterior probabilities values of the two biggest clades (*P. pinodella* and *P. exigua*) of the trees confirm that the two taxa were well separated both from each other as well as other taxons.

The isolates of *P. sojicola* (MYA-406, EU543974 and PD 97/2160, EU543976) groupped together with a *P. pinodella* isoletes since their ITS sequences were completely identical.

The isolate deposited as *Phyllosticta sojicola* (CBS 301.39, EU595356) associated with the *Phoma exigua* var. *exigua* subgroups, which support the statement that the two species are identical (Kövics *et al.*, 1999).

The high bootstrap and Bayesian posterior probability values supported the solidity of the well separated clades and the true coherence of the trees.

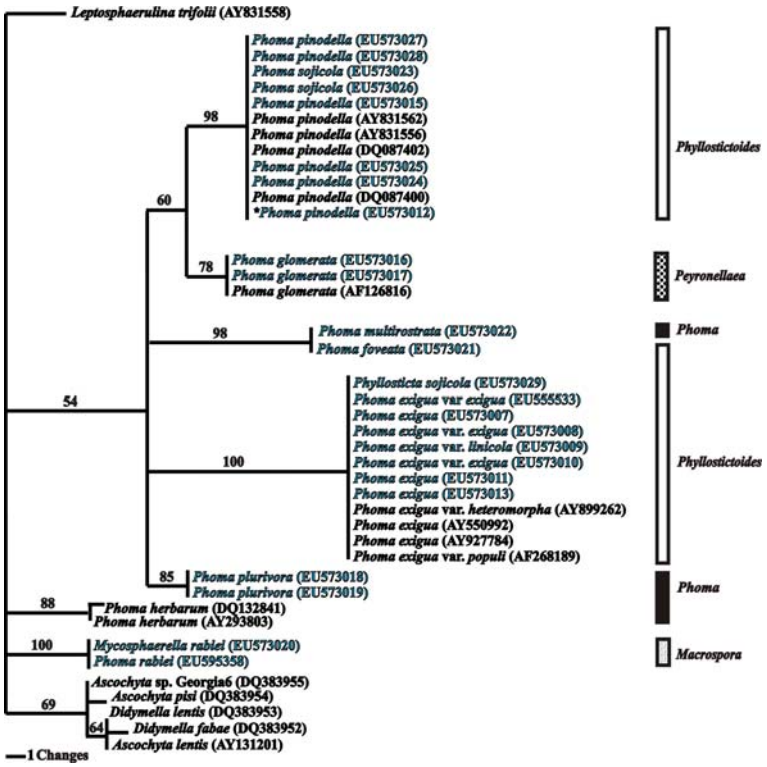

Fig 4. Phylogenetic relationships of *Phoma* strains inferred by Parsimony analysis of ITS sequences. The numbers above the lines represent the bootstrap (bootstrap=1000) values. The columns on the right side represent the *Phoma* sections based on morphological characterization (Boerema *et al.*, 2004). **P. pinodella* (Ph 58) misidentified as*'P. exigua* var. *exigua'*

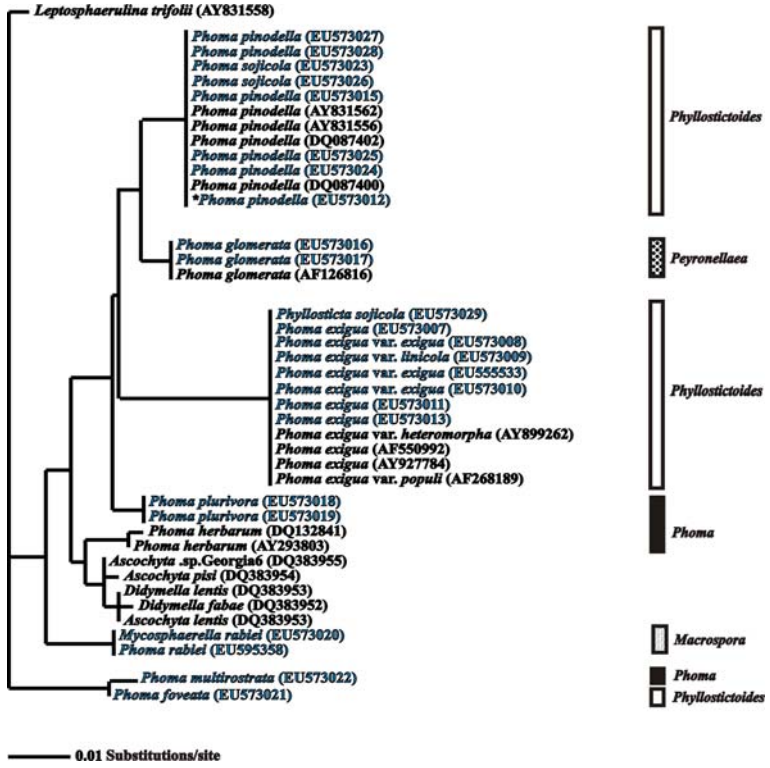

Fig 5. Phylogenetic relationships of *Phoma* strains inferred by Maximum Likelihood analysis of ITS sequences. The columns on the right side represent the *Phoma* section based on morphological characterization (Boerema *et al.*, 2004). **P. pinodella* (Ph 58) misidentified as *'P. exigua* var. *exigua'*

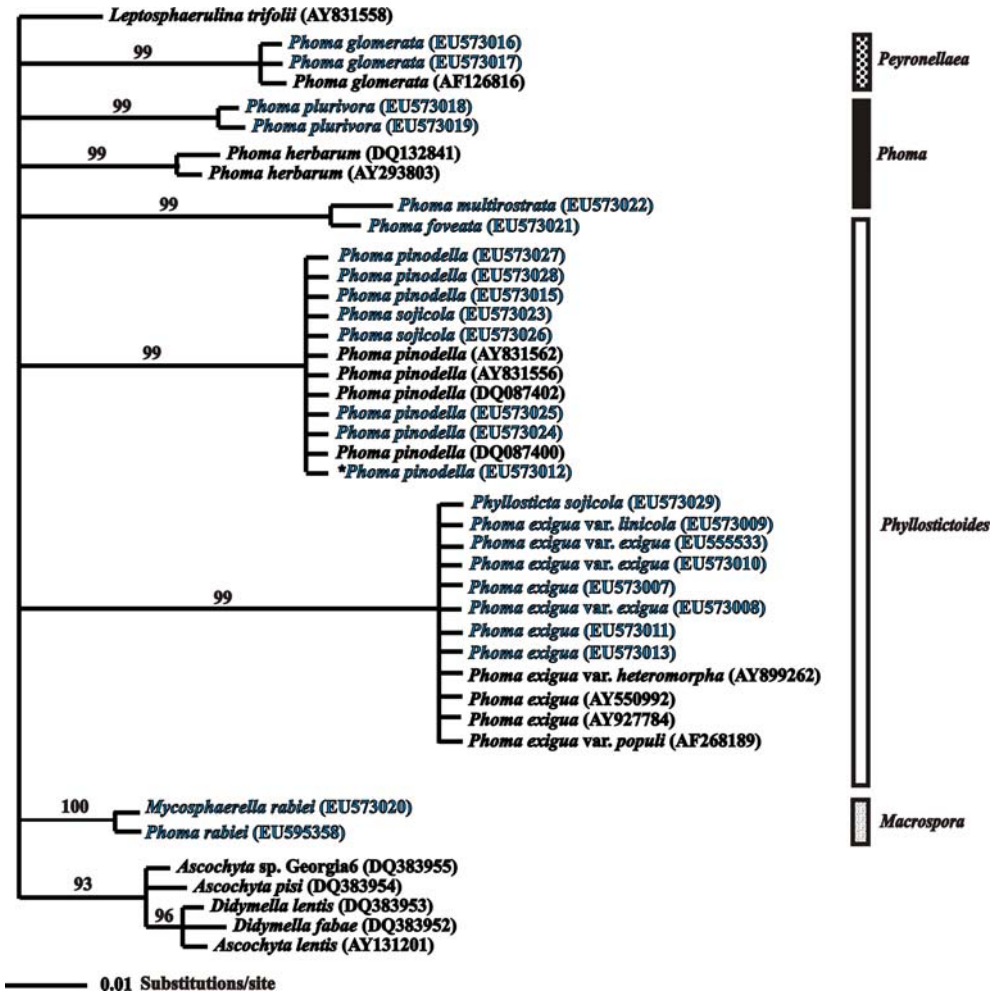

Fig 6. Phylogenetic relationships of *Phoma* strains inferred by Bayesian analysis of ITS sequences. The numbers above the lines represent the Bayesian posterior probability values. The columns on the right side represent the *Phoma* sections based on morphological characterization (Boerema *et al.*, 2004). **P. pinodella* (Ph 58) misidentified as *'P. exigua* var. *exigua'*

Sequence analysis of the β-tubulin gene

The obtained phylogenetic trees by the analysis of *β-tubulin* sequences (Figs 7, 8, 9) bore a resemblance to the *tef1* and ITS trees. The trees were well resolved by Bayesian inference and Parsimony analysis with similar highly supported clades. Parsimony analysis of *β-tubulin* revealed 49 parsimony informative sites, 20 polymorphic sites, and 229 sites that were constant among all isolates.

The *Phoma pinodella* clade contained the *Phoma sojicola* sequences (100% PP and BS support), and *Phyllosticta sojicola* groupped together

with *Phoma exigua* var. *exigua* (100% PP and BS support). Interestingly, the two *P. exigua* isolates that originated from New Zealand groupped together both in the *β-tubulin* and *tef1* analyses. Similarly to the tef1 analysis, species represented with more than one isolate, specifically *P. pinodella* and *P. exigua*, were placed in the same highly supported clade.

The high bootstrap and Bayesian posterior probability values supported the solidity of the well separated clades and the true coherence of the trees.

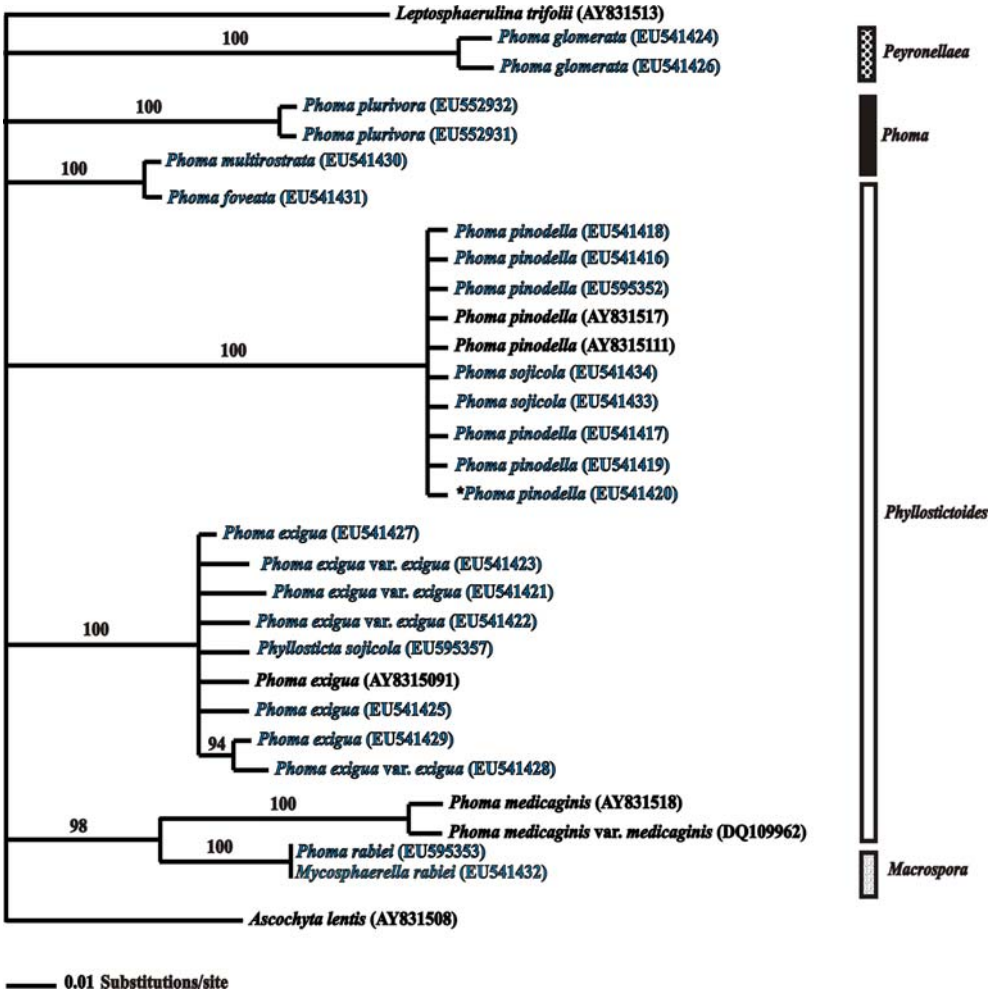

Fig 7. Phylogenetic relationships of *Phoma* strains inferred by Bayesian analysis of *β-tubulin* sequences. The numbers above the lines represent the Bayesian posterior probability values. The columns on the right side represent the *Phoma* sections based on morphological characterization (Boerema *et al.*, 2004). **P. pinodella* (Ph 58) misidentified as *'P. exigua* var. *exigua'*

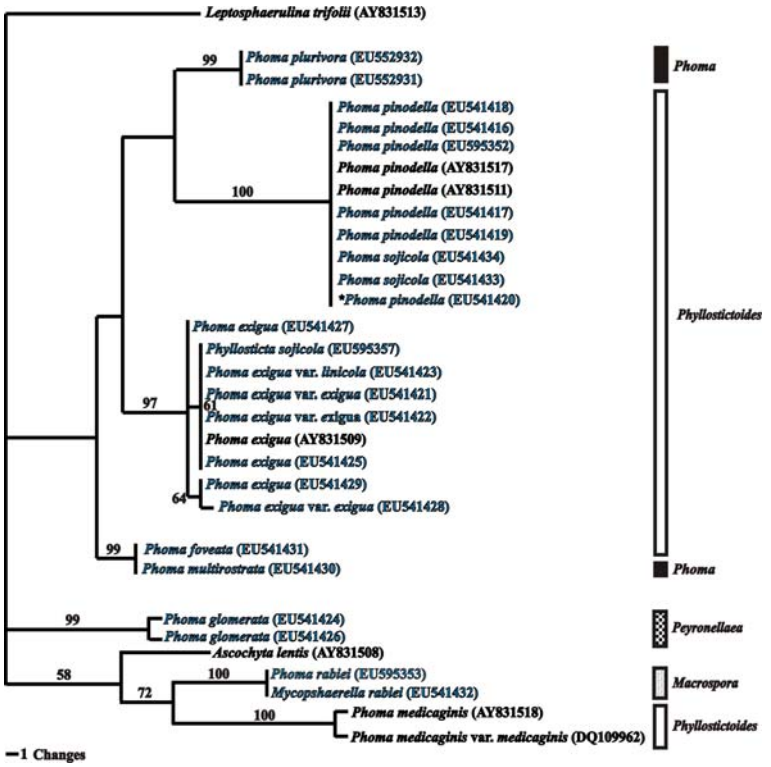

Fig 8. Phylogenetic relationships of *Phoma* strains inferred by Parsimony analysis of *β-tubulin* sequences. The numbers above the lines represent the bootstrap (bootstrap=1000) values. The columns on the right side represent the *Phoma* sections based on morphological characterization (Boerema *et al.*, 2004). **P. pinodella* (Ph 58) misidentified as *'P. exigua* var. *exigua'*

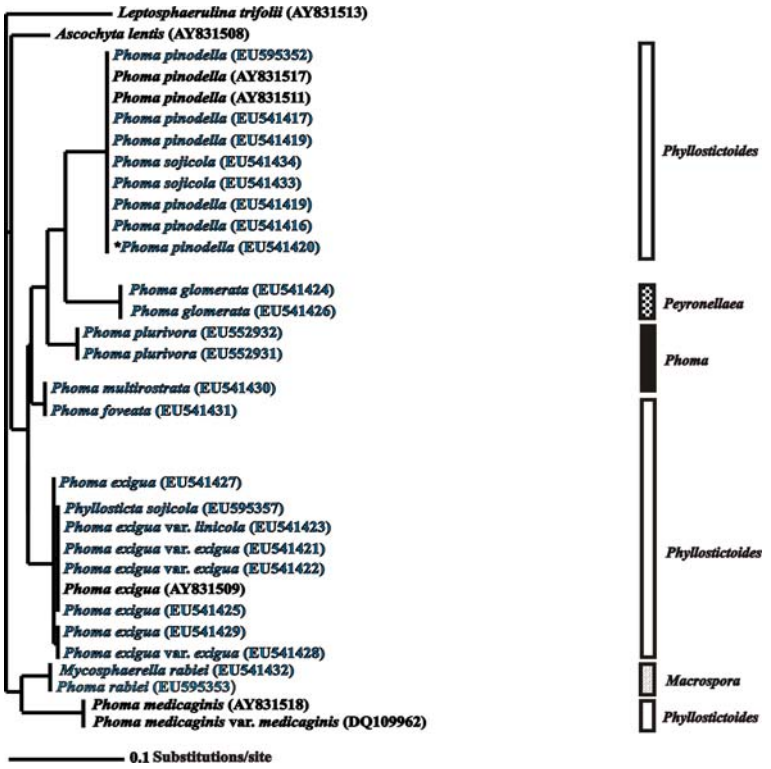

Fig 9. Phylogenetic relationships of *Phoma* strains inferred by Maximum Likelihood analysis of *β-tubulin* sequences. The columns on the right side represent the *Phoma* section based on morphological characterization (Boerema *et al.*, 2004). **P. pinodella* (Ph 58) misidentified as *'P. exigua* var. *exigua'*

Conclusions and recommendations

The taxonomy of Phoma species has not been made completely clear up to now. It includes numerous several uncertainties. It is getting more and more obvious that even the most complex morphological studies can not give reliable result to the actual taxonomical relatedness of species.

The speciation of the genus *Phoma* was based on host-alone and later on, the trend was to study different species of *Phoma* in pure culture. On the basis of morphological studies on different culture media, several species were found to be identical.

In our research we have studied twenty-two isolates of nine different *Phoma*-like species on the basis of morphological and molecular characters. Our morphological studies were carried out on the basis of the accepted conception of the taxonomy of *Phoma* species (Boerema *et al.*, 2004). While the morphological identification of certain isolates was obvious in other cases it has been charged with uncertainty since the morphological characters overlap (on the basis of culture size, color and shape as well as the size of conidia and pycnidia certain taxa can not be differentiated without any doubt. We observed that the morphological characters can change after certain number of transfers on media *in vitro* as well.

The *Phoma sojicola* (Abramov) Kövics et al. shows high similarity to *Phoma pinodella* (L.K. Jones) Morgan-Jones & K.B. Burch, however Kövics *et al.* (1999) have found representative characteristics in culture morphology, pycnidia production and crystal formation. The *P. pinodella* produces crystals on malt-extract agar after one week while the *P. sojicola* misses this feature. In our study we could not managed to detect crystal

production in neither of the species. According to Boerema *et al.* (2004) the crystal production is a contingent feature depending on cultural conditions and/or other factors (longevity of storage, temperature, number of transfers). In our experiments, the cultural characteristics of both species (*P. pinodella* and *P. sojicola*) were very similar without any well identifiable and strong features. The colours of the two colonies were very variable even in the same isolate in the same conditions. The size and shape of pycnidia and conidia showed also such a high similarity that we could not differentiate the two species reliably.

We have encountered similar difficulties differentiating the isolates of *Phyllosticta sojicola* Massalongo and *Phoma exigua* Desm. var. *exigua* (syn.: *Ascochyta phaseolorum* Saccardo). Kövics *et al*. (1999) have suggested that the two species were supposedly identical. Studying the morphological features we can assess that there are no significant differences in none of the cultural characteristics, the size and shape of pycnidia and conidia and that the morphological variability is rather high among isolates. The only difference we have found between the two isolates was the "E-metabolite" production, which is not a feature of *P. sojicola* but a frequent character of *P. exigua* var. *exigua*. However it sometimes might be completely missing in some strains. But this absence of "E-metabolite" production can be a result of a genetic mutation.

In pursuance of examination, the taxonomical status of the isolate of *Phoma exigua* var. *exigua* Ph 58 has been found ambiguous on morphological characters. According to the MSR, the isolate shows high similarity to *P. pinodella* except the only difference in the "E-metabolite" production. The Ph 58 isolate showed a positive NaOH spot test which is a distinctive feature of *Phoma exigua* var. *exigua* but does not a

characteristic of *P. pinodella*. All phylogenetic analyses classified the isolate Ph 58 in the clade of *P. pinodella* that is why we suppose that the isolate Ph 58 has been misidentified as *P. exigua* var. *exigua* and actually belongs to *P. pinodella* species.

Up to now, molecular based phylogenetic analyses within the *Phoma* genus have only been used for defining phylogenetic relationships among isolates within one or closely related species (Fatehi *et al.*, 2003; Mendes-Pereira *et al.*, 2003; Balmas *et al.*, 2005; Voigt *et al.*, 2005). For the first time, we have tried to find such molecular markers which can be suitable for delineating phlyogenetic relationships at species level within the *Phoma* genus.

For our studies, we have selected the complete sequence of the ITS region and fragments of the *tef1* (translation elongation factor coding) and *β-tubulin* genes which have been used as potential marker in many fungal taxonomy studies. The phylogenetic analysis of multiple protein-encoding genes with the GCPSR is proposed as a more robust way of determining and recognizing species than analysis based on morphology or sexual recognition (Taylor *et al.*, 2000).

The translation elongation factor 1 subunit alpha (EF1α) encoding gene *(tef1)* has been proved to be a useful gene to resolve phylogenetic relationships at species level, as well as in deeper divergence in fungi. However, this gene has not been used for the genetic analysis of the *Phoma* genus yet. Ribosomal DNA (rDNA) has long been used as a potential marker for phylogenetic studies (reviewed in Avise, 2004; White *et al.*, 1990). Many fungal taxonomy studies have applied ITS regions for resolving relationships at genus and species level. Mendes-Pereira *et al.* (2003) used ITS sequences for studying the molecular phylogeny of

Leptosphaeria maculans – L. biglobosa species complex. Balmas *et al*. (2005) inferred phylogenetic relationships among isolates of *Phoma tracheiphila* on the basis of ITS sequences as well as Fatehi *et al*. (2003) in *Ascochyta pinodes* complex. Several studies proved that *β-tubulin* at the nucleotide level can be suitable for phylogenetic studies at low taxonomic levels within Ascomycetes (Baldauf *et al.*, 2000; Yli-Mattila *et al.*, 2004). Voigt *et al*. (2005) used *β-tubulin* gene among others to analyze *Leptosphaeria maculans* (anamorph: *Phoma lingam*) species complex, as well as Fatehi *et al*. (2003) to refer molecular relatedness within *Ascochyta pinodes* complex.

Species represented with more than one isolates (*P. pinodella*, *P. exigua* var. *exigua*, *P. glomerata*, *P. plurivora*) were placed in the same clade, which proved the suitability of the examined makers for phylogenetic studies not only within species (Mendes-Pereira *et al.*, 2003; Balmas *et al.*, 2005), but also species complex (Fatehi *et al.*, 2003) as well as species level in *Phoma* genus. The different *Phoma* species were well separated from the closely related species of *Ascochyta* genus. Since the separation of the *Phoma* and *Ascochyta* species is often problematic in the isolates of pseudo-*Ascochyta* species (Fatehi *et al.*, 2003) this new molecular feature can provide a useful tool for mycologists to identify clearly an unknown species.

Phylogenetic analysis of the two protein-encoding genes, *tef1* and *β-tubulin* together with the complete ITS sequences yielded consensus results. *P. sojicola* isolates always formed one clade with all the examined *P. pinodella* isolates, while representative isolate of *Phyllosticta sojicola* groupped together with *P. exigua* var. *exigua*. The highest resolution could

be revealed with the analysis of the *tef1* gene, while sequences of *β-tubulin* showed the lowest polymorphism among the examined *Phoma* species.

On the basis of phylogenetic trees obtained by different character based methods (Maximum Likelihood, Maximum Parsimony, Bayesian method) the studied *Phoma*-like isolates were well separated from each other and the closely related *Ascochyta* genus. The topologies of the trees showed high similarity, which has proved the confidence of different phylogenetic analyses respectively within the *Phoma* genus. Comparing the advantages and disadvantages of the three studied phylogenetic methods we can conclude the following statements: during the analysis one of the most important aspects is the speed, which was the strength of the Maximum Parsimony but it does not offer the opportunity of the selection of the evolution models which the other two methods do. The most accurate and confident tree was given by the Maximum Likelihood (ML) analysis since it takes every possible mutation step into account, which could resulted the topology of the given tree that is why this method approaches the best the real evolutionary changes at the expense of the required time and calculation capacity for the analysis. It took much more time with our data than the other two methods as well as we could not do bootstrap analysis because the calculation was outstandingly time-consuming.

On the basis of our experiences, among the different character based methods the Bayesian one has seemed to be the best choice because of its easy controllability, speed and high confidence. However we have to emphasize that the other two methods are also suitable for correct coherence analysis within the *Phoma* genus at species level.

New results

1. – Twenty-two isolates of nine different *Phoma*-like species by the comparison of morphological and molecular features. Seven of them were isolated from soybean, the others were collected from different hosts.
2. – The different *Phoma* species were well separated from the closely related species of *Ascochyta* genus. Since the separation of the *Phoma* and *Ascochyta* species is often problematic in the isolates of pseudo-*Ascochyta* species (Fatehi *et al.*, 2003) this new molecular feature can provide a useful tool for mycologists to identify unknown species.
3. – In pursuance of our study we have found such molecular markers (*tef1*, ITS, *β-tubulin*), which have proved suitable for phylogenetic studies at species level in *Phoma* genus. Among these, it is the *tef1*, which has not been used until now for phylogenetic studies in *Phoma* genus, proved to be the most confident marker.
4. – The soybean pathogenic *Phoma sojicola* (Abramov) Kövics et al. (syn.: *Ascochyta sojicola*), which was described as a new combination in 1999 by Kövics *et al.* But in our present studies the morphological differences were small and delimitation was made basically on the absence of crystal production on MA, in contrast to *P. pinodella*. However this feature appears to be an unstable character, *viz*. in our experiments none of the isolates produced crystals. All phylogenetic relationship analyses classified *Phoma sojicola* in the same clades of *P. pinodella*. Based on the presented GCPRS and morphological results we suggest the re-classification of *Phoma sojicola* as synonymous with *P. pinodella* (Irinyi *et al.*, 2009).

5. – The isolate originally deposited as *Phyllosticta sojicola* – the isolate, which caused a serious disease in Germany in the 1930s – was groupped with the *Phoma exigua* var. *exigua* clade on the basis of all studied phylogenetic markers. As besides the variability of morphological characters the *Phyllosticta sojicola* can be identical to *Phoma exigua* var. *exigua* on the basis of molecular results, we propose the usage of *Phyllosticta sojicola* species name as a synonymous of *Phoma exigua* var. *exigua*.
6. – We extended the molecular gene bank database (NCBI) with the *tef1*, ITS and *β-tubulin* sequences of twenty-two isolates of nine *Phoma*-like species.
7. – We contributed to clarify the plant pathology of symptomatically identical diseases that damage on Fabaceae species. Damage can be caused, beside others, by

   Phoma exigua Desm. var. *exigua (*syn.: *Ascochyta phaseolorum* Saccardo*)* (new synonym *Phyllosticta sojicola* Massal.);

   Phoma pinodella (L.K. Jones) Morgan-Jones & K.B. Burch (new synonyms: *Phoma sojicola* (Abramov) Kövics et al., *Ascochyta sojicola* Abramov (as, „*A. sojaecola*").

Summary

In our research we have studied twenty-two isolates of nine different *Phoma*-like species on the basis of morphological and molecular characters. The isolates were characterized and identified morphologically in details according to an accepted concept based on standardized *in vitro* physiological and morphological characters of *Phoma*-like species.

Different pathogenic *Phoma*-like species (*Phoma pinodella*, *Phoma sojicola*, *Phyllosticta sojicola*, *Phoma exigua* var. *exigua*) have been found on Fabaceae species, including soybean (*Glycine max*). Since these species are very similar to each other both symptomatically and morphologically, and show strain-dependent variability it is hard to delimit them. Consequently, this has resulted in uncertainity and misidentification in their taxonomy. Therefore there is a need to develop additional rapid molecular methods to enable accurate identification.

Phoma sojicola (Abramov) Kövics et al. highly resembles morphologically to *Phoma pinodella* (L.K. Jones) Morgan-Jones & K.B. Burch. According to Kövics *et al*. (1999) there are profound morphological differences in cultural characteristics, pycnidia production and crystal formation between the two aforementioned species. However, in our studies, crystal formation was not observed in either of the species which can be attributed to that this feature may depend on cultural conditions and/or other factors (longevity of storage, temperature, number of transfer).

On the basis of morphological characters, the *Phyllosticta sojicola* showed great similarity to *Phoma exigua* var. *exigua*. The sporadical incidence of *P. sojicola* on soybeans and the examination of living cultures makes its identification difficult.

We have selected phylogenetic markers (*tef1*, ITS, *β-tubulin*) that were suitable for phylogenetic studies and identification at species level in the *Phoma* genus. Phylogenetic studies of the two protein-encoding genes *tef1* and *β-tubulin*, together with the ITS sequences yielded consensual results. The highest resolution could be revealed by the analysis of *tef1* gene, whereas sequences of ITS and *β-tubulin* showed the lowest polymorphism among the examined *Phoma* species.

According to the phylogenetic trees based on molecular markers, the *Phoma* species were well separated from the closely related *Ascochyta* taxa: *Ascochyta rabiei* /teleomorph: *Didymella rabiei/*, *Ascochyta lentis* /teleomorph: *Didymella lentis/*. As the identification of *Phoma* and *Ascochyta* genera based on morphological characteristics is often confusable, these new phylogenetic markers can be useful tools for mycologists to identify unknown species.

The species represented by more than one isolate were classified in the same subgroup (*P. pinodella*, *P. exigua*, *P. glomerata*, *P. plurivora*), which prove that the molecular sequences are well suited for delineating phylogenetic relationships within the *Phoma* genus.

For phylogenetic analysis, three different character-based methods were used (Maximum Likelihood, Maximum Parsimony, Bayesian method) to study the advantages and disadvantages of each marker. In our experience, the most suitable method seemed to be the Bayesian analysis due to its complexity, reliability and fastness.

Soybean pathogenic *P. sojicola* (syn.: *Ascochyta sojicola*) was described as a new combination (comb. nov.) in 1999 by Kövics *et al*. In our presented performed studies the morphological differences were small and delimitation was made basically on the absence of crystal production on MA, in contrast to *P. pinodella*. However, this feature appears to be an unstable character, viz. in our experiments none of the isolates produced crystals. All phylogenetic relationship analyses classified *Phoma sojicola* in the same clades of *P. pinodella*. Based on the presented genealogical concordance phylogenetic species recognition and morphological results, we suggest the re-classicfication of *Phoma sojicola* as synonymous with *P. pinodella* (Irinyi *et al.*, 2009).

The morphological characters of the *Phyllosticta sojicola* isolate (CBS 301.39, deposited by Böning in 1939) discussed in this study, together with its molecular features, proved that *Phyllosticta sojicola* is synonymous with *Phoma exigua* var. *exigua*. This supports our hypothesis based on examined type material, that *P. sojicola* on leaves of *Glycine max* (Massalongo, 1900) represents the plurivorous *P. exigua* var. *exigua*.

References

Baldauf, S. L., Roger, A. J., Wenk-Siefert, I., Doolittle, W. F. (2000). A kingdom-level phylogeny of eukaryotes based on combined protein data. Science 290: 972-977.

Balmas, V., Scherm, B., Ghignone, S., Salem, A.O.M., Cacciola, S.O., 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.

Boerema, G.H., de Gruyter, J., de, Noordeloos, M.E., Hamers, M.E.C. (2004). *Phoma* identification manual. Differentiation of species and infra-specific taxa in culture. CABI Publishing, CAB International Wallingford, Oxfordshire, UK.

Druzhinina, I., 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.

Fatehi, J., Bridge, P.D., Punithalingam, E. (2003). Molecular relatedness within the "*Ascochyta pinodes*"-complex. Mycopathologia 156: 317-327.

Glass, N.L., Donaldson, G.C. (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Applied and Environmental Microbiology 1323-1330.

Huelsenbeck J.P., Ronquist F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754-755.

Irinyi L., Kövics, G.J., Sándor, E. (2009). Taxonomic re-evaluation of *Phoma*-like soybean pathogenic fungi. Mycological Research 113: 249-260.

Kövics, G.J., de Gruyter, J., van der Aa, H.A. (1999). *Phoma sojicola* comb. nov. and other hyaline-spored coelomycetes pathogenic on soybean. Mycological Research 103: 1065-1070.

Mendes-Pereira, E., Balesdent, M.-H., Brun, H., Rouxel, T. (2003). Molecular phylogeny of the *Leptosphaeria maculans-L. biglobosa* species complex. Mycological Research 107: 1287-1304.

Nicholas, K.B., Nicholas, H.B.Jr., Deerfield, D.W. II. (1997). GeneDoc: Analysis and Visualization of Genetic Variation, Embnew. News 4: 14.

Posada, D., Grandall K.A. (1998). Modeltest: Testing the model of DNA substitution. Bioinformatics 14: 817-818.

Rayner, R.W. (1970). A mycological color chart. Commonwealth Mycological Institute, Kew, Surrey, and British Mycological Society.

Swofford, D.L. (2002). PAUP: Phylogenetic Analysis Using Parsimony (and other methods). Version 4b10. Sinauer Associates, Sunderland, Massachusetts.

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S., Fisher, M.C. (2000). Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology 31: 21-32.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24: 4876-4882.

Voigt, K., Cozijnsen, A. J., Kroymann, J., Pöggeler, S., Howlett, B.J. (2005). Phylogenetic relationships between members of the crucifer pathogenic *Leptosphaeria maculans* species complex as shown by mating type (MAT1-2), actin and *β-tubulin* sequences. Molecular Phylogenetics and Evolution 37: 541-557.

White, T.J., Bruns, T., Lee, S., Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. pp. 315-322. In: PCR protocols. A guide to methods and applications. Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.) Academic Press, Inc., New York.

Yli-Mattila, T., Mach, R.L., Alekhina, I.A., Bulat, S.A., Koskinen, S., Kullnig-Gradinger, C.M., Kubicek, C.P., Klemsdal, S.S. (2004). Phylogenetic relationships of *Fusarium langsethiae* to *Fusarium poae* and *Fusarium sporotrichioides* as inferred by IGS, ITS, *β-tubulin* sequences and UP-PCR hybridization analysis. International Journal of Food Microbiology 95: 267-285.

László Irinyi's publication list

Referred Articles in English:

Irinyi L. – Kövics, G.J. – Sándor, E. (2009). Taxonomic re-evaluation of *Phoma*-like soybean pathogenic fungi. Mycological Research 113: 249-260.

Impact faktor: 1.861

Irinyi, L. – Sándor, E. (2008). Bayesian inference in the phylogeny of *Phoma* taxons. Cereal Research Communications 36: 1061-1064.

Impact faktor: 1.190

Book chapter in English

Irinyi, **L.** – Gade, A.K. – Kövics, G.J. – Rai, M.K. – Sándor, E. (2009). Morphology and Molecular Biology of *Phoma*. pp. 171-204. In: Current advances in molecular mycology. Gherbawy, Y., Mach, R.L., Rai, M.K. (Eds.) Nova Science Publishers, Inc., New York, USA.

Referred Articles in Hungarian:

Irinyi, L. – Kövics, G.J. – Sándor, E. (2008). *Phoma* fajok filogenetikai vizsgálata maximum likelihood analízissel. Agrártudományi Közlemények 2008/30: 37-46.

Irinyi, L. – Kövics, G.J. – El-Naggar, M. – Sándor, E. (2007). *Phoma* fajok filogenetikai vizsgálata. Agrártudományi Közlemények, Különszám 2007/26: 100-107.

Conference issues:

Irinyi, L., Kövics, G.J., Sándor, E. (2009). *Phoma*-szerű gombák filogenetikai vizsgálata Bayesian analízissel. 70-76. pp. in: XIX. Keszthelyi Növényvédelmi Fórum, Keszthely, 2009. február 4-6. Pannon Egyetem, Keszthely.

Irinyi, L. – Kövics, G.J. – Sándor, E. (2008). Phylogenetic estimation of *Phoma*-like fungus by Bayesian approaches. Acta Microbiologica et Immunologica Hungarica 55: 199. ISSN: 1217-8950.

Irinyi, L. – Kövics, G.J. – Sándor, E. (2008). Szóján előforduló *Phoma*-szerű gombák filogenetikai vizsgálata Bayesian módszerrel. 78-97. pp. in: Kövics Gy.J. - Dávid I. /szerk./ (2008): 13. Tiszántúli

Növényvédelmi Fórum. Előadások – Proceedings. Debrecen, 2008. október 15-16. Debreceni Egyetem, Debrecen.

Irinyi, L. – Kövics, G.J. – Sándor, E. (2008). Bayesian módszer alkalmazása a *Phoma* taxonok filogenetikai vizsgálatában. 4-12. pp. in: XVIII. Keszthelyi Növényvédelmi Fórum, Keszthely, 2008. január 30.-február 1. Pannon Egyetem, Keszthely.

Irinyi, L. – Kövics, G.J. – Sándor, E. (2007). A phylogenetic study on different *Phoma* species. Acta Microbiologica et Immunologica Hungarica 54: 51.

Irinyi, L. – Kövics, G.J. – Sándor, E. (2007). Szóján előforduló *Phoma*-szerű gombák filogenetikai vizsgálata. 107-127. pp. in: Kövics Gy.J. - Dávid I. /szerk./ (2007): 12. Tiszántúli Növényvédelmi Fórum. Előadások – Proceedings. Debrecen, 2007. október 17-18. Debreceni Egyetem, Debrecen.

Irinyi, L. – Kövics, G.J. – Rai, M.K. – Sándor, E. (2006). Studies of evolutionary relationships of *Phoma* species based on phylogenetic markers. pp. 99-113. In: 4th International Plant Protection Symposium at Debrecen University, Recent Developments of IPM. Proceedings. Kövics, G.J. – Dávid, I. (Eds.). Debrecen University Centre for Agricultural Science, Faculty of Agriculture. 18-19 October, 2006, Debrecen.

Irinyi, L. – Kövics, G.J. – Sándor, E. (2006). New phylogenetic marker for the classification of *Phoma* species. The 4th International Symposium „Natural resources and sustainable development". (Ed.: M.T. Teodor). University of Oradea, Oradea 10-11 October, 2006. 253-260.

Irinyi, L. – Kövics, G.J. – Sándor, E. (2006). A study of the utility of translation elongation factor 1 as a phylogenetic marker for *Phoma* genus. Acta Microbiologica et Immunologica Hungarica 53: 279-280.

Irinyi, L. – Kövics, G.J. – Sándor, E. (2006). Classification of *Phoma* species using new phylogenetic marker. Analele Universittăţii Din Oradea, Fascicula Agricultură – Horticultură, Editura Universittăţii Din Oradea, Volume XII, Anul 12: 91-97.

Deshmukh, P. – Rai, M.K. – Kövics, G.J. – **Irinyi, L.** – Sándor, E. (2006). *Phoma* – Can these fungi be used as biocontrol agents and sources of secondary metabolites? (A review). pp. 224-232. In: 4th International Plant Protection Symposium at Debrecen University, Recent Developments of IPM. Proceedings. Kövics, G.J. – Dávid, I. (Eds.). Debrecen University Centre for Agricultural Science, Faculty of Agriculture. 18-19 October, 2006, Debrecen.