

Molecular biology characterization of *Cryphonectria parasitica* (MURR.) BARR isolates from different Carpathian-Basin plots

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SUMMARY

The *Cryphonectria parasitica* (MURR.) BARR fungus is one of the most serious chestnut disease in Europe and North America. It originated from Eastern Asia and it was introduced into North America at the beginning of 20th century where it devastated the American chestnut (*Castanea dentata*, Fagaceae, Fagales) causing serious damage in orchards and in forests (Anagnostakis, 1987). Carpathian-Basin - can be found in the Mecsek, the Zala hills, the Somogy hills in Hungary, and the Foothills of the Alps in significant populations but also near the precinct of the Danube Bend and certain parts of the North Hungarian Mountains. Beyond our borders, it is produced in several regions of Slovakia and Ukraine, slopes of the Carpathian Mountains, the southern slopes and valleys of Kőhát and Gutin mountain around in Northern Transylvania, Nagybánya as well as the on the borders of Croatia, Slovenia and Austria. In Hungary, the first time when the symptoms of the disease was reported by Körtvély in 1969, but for nowadays the chestnut blight is an important and widespread plant disease in Hungary. The fungus kills the infected tree branches and the rapid death of the entire tree take place which is causing high environmental and economic concerns. In this study, we have tried to characterize the *Cryphonectria parasitica* isolates collecting from different Hungarian plots. Several studies using different molecular markers have been published to infer population genetic parameters and genetic diversity among the isolates originated from different regions (Hoegger et al., 2000; Marra et al., 2004; Breuillin et al., 2006; Milgroom et al., 2008). In this study we employed a part of the translation elongation factor 1 subunit alpha (EF-1 α =tef1) containing both introns and exons and ITS region containing the internal transcribed spacer regions 1 and 2 and the 5.8S rDNA, as a potential genetic markers to characterize among *Cryphonectria parasitica* isolates.

Keywords: *Cryphonectria parasitica*, ITS region, *tef1*, genetic diversity, Carpathian-Basin, chestnut

INTRODUCTION

Chestnut - also native to the Carpathian-Basin - can be found in the Mecsek, the Zala hills, the Somogy hills in Hungary, and the Foothills of the Alps in significant populations but also near the precinct of the Danube Bend and certain parts of the North Hungarian Mountains. Beyond our borders, it is produced in several regions of Slovakia and Ukraine, slopes of the Carpathian Mountains, the southern slopes and valleys of Kőhát and Gutin mountain around in Northern Transylvania, Nagybánya as well as the on the borders of Croatia, Slovenia and Austria. The heterothallic ascomycete fungus, *Cryphonectria parasitica* (Murr.) Barr (Syn. *Endothia parasitica* [Murr.] And.) and the casual agent of chestnut blight disease, is one of the most important fungal pathogens of chestnut in Europe and North America. It originated from Eastern Asia and it was introduced into North America at the beginning of 20th century where it devastated the American chestnut (*Castanea dentata*, Fagaceae, Fagales) causing serious damage in orchards and in forests (Anagnostakis, 1987). In the middle of the last century the pathogen was transferred into Europe (Braghi, 1946) and infected the European chestnut populations (*Castanea sativa* Mill). The pathogen kills the infected tree branches and the rapid death of the entire tree take place which is causing high environmental and economic concerns (Figure 1).

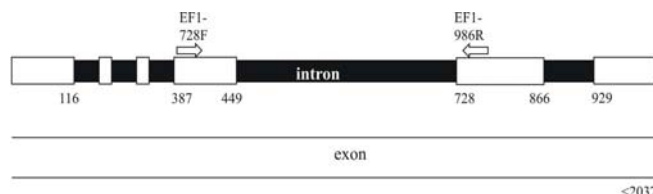
Figure 1: The damage of the *Cryphonectria parasitica* (MURR.) BARR in chestnut trees



In this study we employed a part of the translation elongation factor 1 subunit alpha (EF-1 α =tef1) containing both introns and exons and ITS region containing the internal transcribed spacer regions 1 and 2 and the 5.8S rDNA, as a potential genetic markers to infer phylogenetic relationships among *Cryphonectria parasitica* isolates. Translation elongation factor 1 (Figure 2) subunit alpha (EF1 α =tef1) is part of the cytosolic EF1

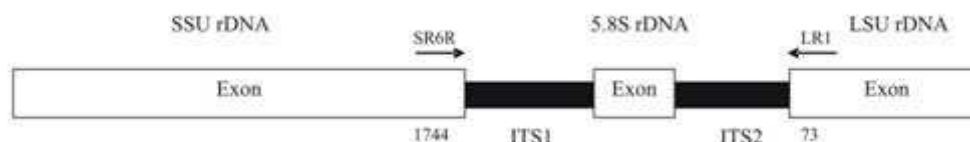
complex, whose primary function is to promote the binding of aminoacyl-tRNA to the ribosome in a GTP-dependent process (Moldave, 1985). Simultaneously, elongation factor 1 α (EF-1 α) is a highly conserved ubiquitous protein that has been suggested to have desirable properties for phylogenetic inference and population genetic studies (Roger *et al.*, 1999).

Figure 2: Schematic structure of *tefl* gene and location of primers for phylogenetic analyses



Ribosomal DNA (rDNA) has long been used as a potential marker for phylogenetic studies (reviewed in Avise, 2004). rRNA genes are organized in clusters of tandemly repeated units, (Figure 3.) each of which consists of coding regions (18S, 5.8S, and 28S; Gerbi, 1985) and 2 internal transcribed spacers (ITS) and intergenic spacer (formerly called the „ Non-Transcribed Spacer, NTS) region. While the coding regions are evolutionarily conserved and have been utilized for phylogenetic inferences for major phyla (reviewed in Hills and Dixon 1990), the 2 ITS regions are appropriate for detecting differences between co-specific individuals and are hence potentially useful markers to study the relationships of populations and closely related species in fungal, plant, and animal taxa due to their relatively rapid evolutionary rates (Baldwin, 1992; Schlötterer *et al.*, 1994; Mai and Coleman, 1997; Weekers *et al.*, 2001).

Figure 3: Schematic structure of ITS region in *Phoma* spp. and location of primers for phylogenetic analyses



MATERIALS AND METHODS

Isolates

In this study we analyzed 40 *Cryphonectria parasitica* strains isolated from the Carpathian-basin (Table 1 and Figure 4). Bark samples were collected from chestnut trees located in geographically determined (GPS) areas. In each site, bark samples were collected from the margin of cankers on at least 10 evenly distributed, blighted stumps. Only one bark sample was collected from each stump to avoid sampling clones. Individual samples were surface disinfected in 70% ethanol, rinsed in sterile water and then placed on PDA (40g potato dextrose agar, Scharlau, 1 l tap water). Plates were incubated at 24 °C in the dark for 7 days and all cultures were checked for typical *Cryphonectria parasitica* characteristics. In the analyses, other *Cryphonectria parasitica* isolates were included from GenBank maintained by NCBI (GenBank; <http://www.ncbi.nlm.nih.gov/>).

Culture morphology and growth

Isolates were grown on PDA (40 g L⁻¹) in 90mm diam. Petri dishes are incubated at 25 °C in the dark for one week. The cultures were then exposed to diffuse daylight at room temperature on the laboratory bench, and culture morphology was recorded once a week for a period of 4 weeks.

DNA extraction

For molecular works 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 from each culture was transferred to 100 ml Erlenmeyer flasks containing 50 ml MB. The cultures were grown at room temperature for 48 hours in the dark on a rotary shaker (125 rpm). Mycelia were harvested by vacuum filtration. Total genomic DNA was extracted from freeze-dried mycelium and isolated using NucleoSpin Plant II (Macherey-Nagel, 740770) according to the protocol, followed the manufacturer's instructions. DNA concentrations were measured by NanoDrop (Thermo Scientific)

Amplification of the large intron *tefl* gene

Amplifications of 50 ml PCR reaction mixture contained 25 ml 2xPCR Master Mix (Fermentas, #K0171, Burlington, Canada), 40–40 pmol each primer, 20–40 ng DNA and nuclease free water were run out.

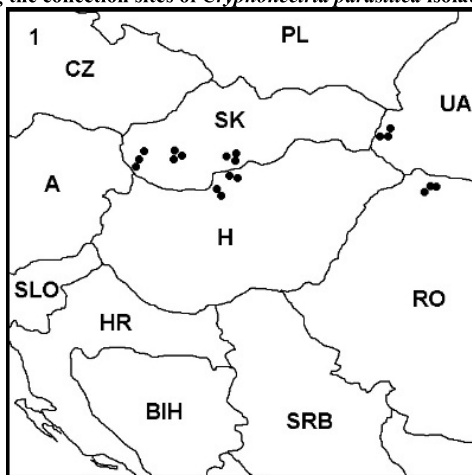
Primers used to amplify the large intron of the *tefl* gene was amplified by the EF1-728F (5'- CAT CGA GAA GTT CGA GAA GG -3') and EF1-986R (5'- TAC TTG AAG GAA CCC TTA CC -3') primer pair (Druzhinina

and Kubicek, 2005) according to the following program: 3 min initial denaturing at 95°C, followed by 5 cycles of 1 min at 95°C, 1 min annealing at 59°C, 1 min at 72°C and 25 cycles of 1 min at 90°C, 1 min annealing at 59°C, 1min at 72°C and 15 min final extension at 72°C. PCR was performed in a Primus (MWG Biotech, Martinsried, Germany) thermocycler. Amplification products were subjected to electrophoresis in a 0.7 % agarose gel containing EtBr for 1 hour and visualized by UV illumination. The PCR products were purified by using YM-100 Microcon Centrifugal Filter Devices (Millipore, Billerica, USA). Purified amplification products were sequenced by MWG Biotech in Germany.

Table 1

Origine of *Cryphonectria parasitica* isolate

Name of the samples	Country	Location	Date of the collection
B1	Hungary	Nagymaros	13 may 2011
C1	Hungary	Nagymaros	13 may 2011
E3	Hungary	Nagymaros	13 may 2011
F4	Hungary	Nagymaros	13 may 2011
N2	Hungary	Nagymaros	13 may 2011
ERSEK2	Hungary	Érsekvadkert	13 may 2011
ERSEK3	Hungary	Érsekvadkert	13 may 2011
BOB1-1	Ukraine	Bobovyshe	23 april 2011
BOB1-3	Ukraine	Bobovyshe	23 april 2011
BOB2-2T	Ukraine	Bobovyshe	23 april 2011
BOB2-3	Ukraine	Bobovyshe	23 april 2011
BOB2-4T	Ukraine	Bobovyshe	23 april 2011
BOB3-1	Ukraine	Bobovyshe	23 april 2011
BOB3-3	Ukraine	Bobovyshe	23 april 2011
RO4	Ukraine	Rostovjatica	23 april 2011
RO6	Ukraine	Rostovjatica	23 april 2011
S1	Ukraine	Serednje	23 april 2011
S3	Ukraine	Serednje	23 april 2011
VEVII	Romania	Baia Mare	7 september 2011
VEVI2	Romania	Baia Mare	7 september 2011
FEL1T	Romania	Baia Mare	7 september 2011
FEL4	Romania	Baia Mare	7 september 2011
TG2	Romania	Baia Mare	7 september 2011
TG4	Romania	Baia Mare	7 september 2011
KOBA2	Romania	Baia Mare	7 september 2011
KOBA4T	Romania	Baia Mare	7 september 2011
KEK2	Slovakia	Modrý Kameň	8 december 2011
KEK4	Slovakia	Modrý Kameň	8 december 2011
KEK6	Slovakia	Modrý Kameň	8 december 2011
KEK8	Slovakia	Modrý Kameň	8 december 2011
PAR4	Slovakia	Párovské Háje	18 january 2012
PAR5	Slovakia	Párovské Háje	18 january 2012
PAR9	Slovakia	Párovské Háje	18 january 2012
MOD1	Slovakia	Modra	18 january 2012
MOD4	Slovakia	Modra	18 january 2012
BRAT1	Slovakia	Bratislava	18 january 2012
BRAT3	Slovakia	Bratislava	18 january 2012
SVE1	Slovakia	Svätý Jur	18 january 2012
SVE3	Slovakia	Svätý Jur	18 january 2012

Figure 4: Map showing the collection sites of *Cryphonectria parasitica* isolates involved in this study

Amplification of nuclear ribosomal internal transcribed spacer (ITS) and translation elongation factor 1-alpha (*tef1*) and sequencing

Amplifications of 50 µl PCR reaction contained 25 µl 2X PCR Master Mix (ImmoMix, Bioline, 25020), 40-40 pmol each primer, 20-40 ng of genomic DNA and nuclease free water were run out.

Primers (IDT) 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 and Kubicek 2005) according to the previously described protocol with a temperature of 56 °C rather than 50°C.

PCR was performed in a Primus (MWG Biotech) thermocycler. 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 Nucleospin Gel and PCR Clean-up (Macherey-Nagel, 740609). 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) automatically 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. In phylogenetic analyses *tef1* and ITS fragments of other *Cryphonectria parasitica* from GenBank maintained by NCBI were also included. Parsimony analyses (Kluge and Farris 1969; Farris 1970; Fitch 1971) were performed using PAUP 4.0 (Swofford 2002) and consisted of heuristic searches with 1 K random addition sequences and tree bisection–reconnection (TBR) branch swapping. All characters were equally weighted and alignment gaps were treated as missing data. The stability of clades was assessed with 1 K BS replications. Phylogenetic trees were drawn by TREEVIEW (Page 1996).

RESULTS

The genomic DNA concentrations after the extraction were about 100 ng/µl. PCR amplifications resulted in single fragments of ca 350bp of *tef1* fragment and in single fragments approx. 560bp of the rDNA gene containing the internal transcribed spacer regions 1 and 2 and the 5.8S regions was amplified. There was no size variation observed among amplified *tef1* and ITS fragments.

ITS fragments to 547 bp to aid alignment with sequences downloaded from GenBank. Parsimony analysis of the ITS fragment revealed 299 constant sites, 172 parsimony-informative sites, and 76 parsimony -uninformative sites among all isolates.

The phylogenetic tree based on ITS sequence (Figure 5) is drawn by parsimony analysis. Topological robustness in parsimony analysis was estimated using 1000 bootstrap replicates. The numbers above the lines on the Fig. 4 represent the bootstrap values which support the robustness of the clades. The *C. parasitica* isolates are grouped in several clades which indicates the high variability in ITS sequences. However the clades do not represent the studied geographical regions. The isolates originated from the same regions are not grouped together, they are found mixed in different groups. The ITS sequences downloaded from the GenBank are also found in different clades on the phylogenetic trees. The robustness of the tree was supported by relative high bootstrap values.

Figure 5: Phylogenetic relationships of *Cryphonectria parasitica* strains inferred by Parsimony analysis of ITS sequences. The numbers above the lines represent the bootstrap (bootstrap=1000) values. Our isolates are indicated by different colors, the isolates downloaded from GenBank indicated by black color

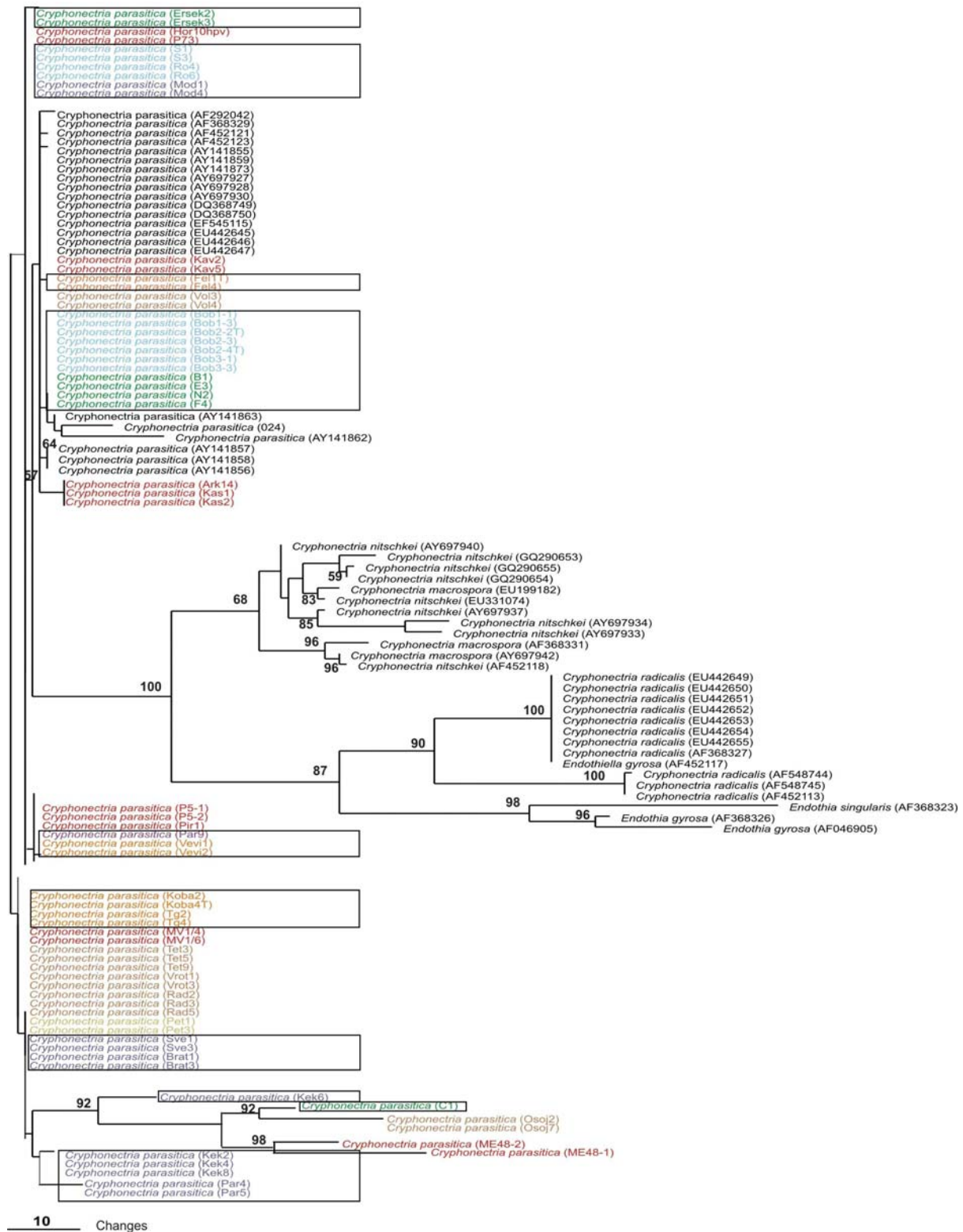
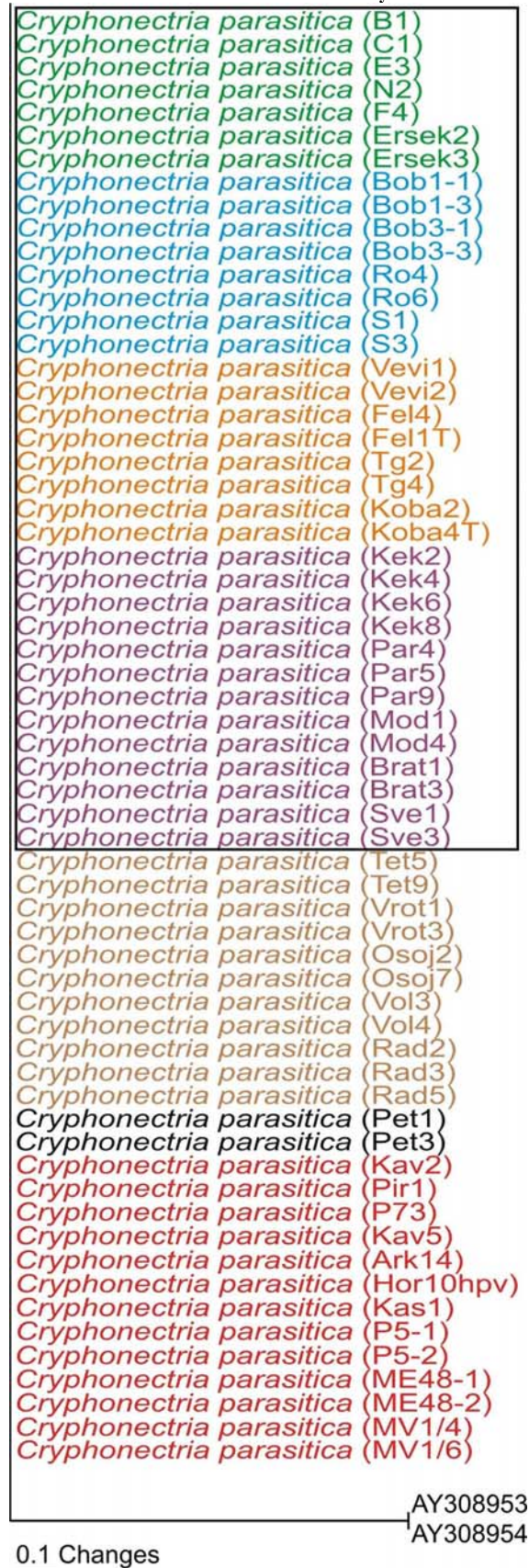


Figure 6: Phylogenetic relationships of *Cryphonectria parasitica* strains inferred by Parsimony analysis of *tef1* sequences. The numbers above the lines represent the bootstrap (bootstrap=1000) values. Our isolates are indicated by different colors, the isolates downloaded from GenBank indicated by black color



Tefl sequences were edited to 320bp for the alignment. At the time of the analyses, only two *tefl* sequences were available in the database. In the case of *tefl* fragment, no differences were found during the alignment. The *tefl* sequences of all isolates were perfectly identical.

The phylogenetic tree based on partial sequences of EF-1 α genes sequences (Figure 6.) is drawn by parsimony analysis. Parsimony analysis of the *tefl* fragment did not revealed any estimable values. Only little differences were found between the *tefl* sequences of our isolates and the ones downloaded from GenBank but this could be attributed to the mis-sequencing of the *tefl* sequences found in the GenBank. Our results show that there is no variability in *tefl* sequences among *C. parasitica* isolates collected from Europe.

CONCLUSIONS

In this study we tried to use ITS sequences and Translation elongation factor 1 subunit alpha (EF1 α) encoding gene (*tefl*) to resolve phylogenetic relationships at intra specific level among *Cryphonectria parasitica* isolates collected from different geographical regions of Central-Europe. The ITS sequences proved variable but this kind of variability does not represent the geographical regions where they were collected. Our results shows that the ITS sequences are suitable for phylogenetic studies among *C. parasitica* isolates but they do not represent geographical distributions. Further work need to be done to see whether there are other characteristics which is correlates with the variability of IT sequences. Translation elongation factor 1 subunit alpha (EF1 α) encoding gene (*tefl*) has been proved to be a useful gene to resolve phylogenetic relationships at species level, as well as in deeper divergences of fungi (Roger *et al.*, 1999; Druzhinina and Kubicek 2005). In our study, the *tefl* sequences have not showed any differences, they were identical in all isolates supporting that these sequences remained conservative within this species. The result shows that the *tefl* sequences are not well suited for delineating phylogenetic relationships within *C. parasitica* isolates collected from different regions of Europe.

In the future, it would be interesting to study the phylogenetic relationships among *C. parasitica* isolates but collected from other continent like Asia or North-America. The difference among the *Cryphonectria parasitica* isolates originated from the Carpathian-Basin was not insignificant because only six sites were considered as informative for the parsimony analysis. The bootstrap values were not enough high either to support real differences among isolates. Significant, well based differences were not found between Hungarian isolates. Little differences were found between Hungarian, Greek and isolates from database which constitute separate groups. It show also that the *tefl* sequences could be suitable for studying *C. parasitica* isolates originated from geographical places far away from each other. As the differences among the different studied isolates were insignificant, we mean that the evolutionary distance by *tefl* sequences within *Cryphonectria parasitica* isolates is too small to get well based consequences for the population diversity of this species.

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