MOLECULAR STUDIES OF HUNGARIAN *CRYPHONECTRIA PARASITICA* ISOLATES

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Abstract

A significant diversity between C. parasitica isolates may be found in Europe. In this study we have obtained DNA sequences from translation elongation factor coding gene to compare Hungarian C. parasitica isolates.

Translation elongation factor 1 subunit alpha (EF1α=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 1α (EF-1α) 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). EF-1α 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).

Keywords: chestnut, *Cryphonectria parasitica,* molecular studies

INTRODUCTION

The chestnut blight, heterothallic fungus, *Cryphonectria parasitica (Murr.) Barr* (Syn. *Endothia parasitica* [Murr.] And.), the casual agent of chestnut blight, is one of the most important fungal pathogens of chestnut in Europe and North America. *Cryphonectria parasitica*, is classified as a quarantine organism, list A2 by the EPPO and is also quarantine significance for NAPPO and IASPC. Chestnut blight is an important and widespread disease of chestnut in Hungary. The disease kills the infected tree branches and the rapid death of the entire tree take place which is causing high environmental and economic concerns.

A significant diversity between *C. parasitica* isolates may be found in Europe. In this study we have obtained DNA sequences from translation elongation factor coding gene to compare Hungarian *C. parasitica* isolates.

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. Here we have used primer pair which facilitates the PCR amplification of the large intron of *tef1* gene (Druzhinina and Kubicek, 2005, Fig. 1).

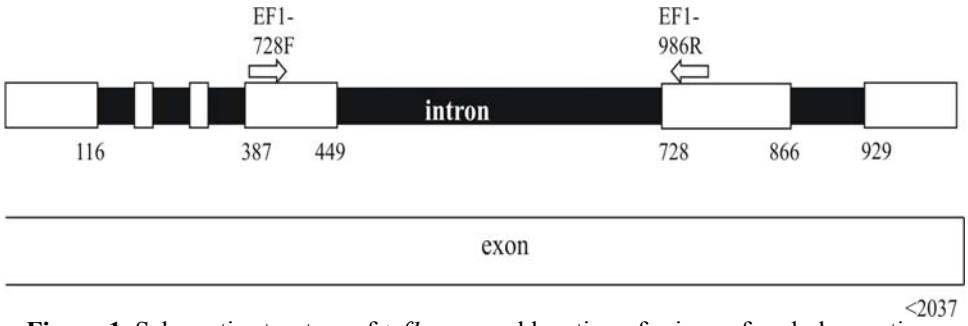

Figure 1. Schematic structure of *tef1* gene and location of primers for phylogenetic analyses

Isolates

In this study we analyzed the *tef1* region of eight *Cryphonectria parasitica* strains isolated from the Northern region of Hungary and further two Greek isolates (Table. 1). In the analyses, other two *tef1* fragments of *Cryphonectria parasitica* (only two were found until the analyses) isolates were included from GenBank maintained by NCBI (GenBank; http://www.ncbi.nlm.nih.gov/) (Table 2).

Table 1

Isolate of *Cryphonectria parasitica* from Hungary

Isolate number	Location of isolation
B1	Nagymaros (Kóspallagi utca)
C1	Nagymaros (Fehérhegy 1)
J2	Nagymaros (Őz utca)
R2	Nagymaros (Első völgy)
E2	Nagymaros (Őz utca)
N2	Nagymaros (Első völgy)
MV/16	Nagymaros (Első völgy)
MV1/4	Nagymaros (Első völgy)
P5-2	Greece
ME48-2	Greece

Table 2

Cryphonectria parasitica isolates involved in the phylogenetic analyses of *tef1* fragments

Species name	Isolation number	GenBank accession number
Cryphoncetria parasitica	CMW10427	AY308953
Cryphoncetria parasitica	CMW10431	AY308954

DNS extraction

For PCR-based methods the studied cultures were grown in 100 ml malt broth (MB, containing 2 % malt extract) for 48 h at room temperature in the dark on a rotary shaker (125 rev min_1). The mycelium was harvested by vacuum filtration. Total genomic DNA was extracted from freeze-dried mycelium and isolated using NucleoSpin Plant II Kit (Macherey-Nagel) according to the manufacturer's instructions. DNA concentrations were estimated in comparison to known standards in agarose gels stained with ethidium bromide (EtBr).

Amplification of the large intron *tef1* 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 *tef1* 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.

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* fragments, other *Cryphonectria parasitica* from GenBank maintained by NCBI were also included. Parsimony analyses (Kluge & 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 DNA concentrations after the extraction were about 100 ng/µl. PCR amplifications resulted in single fragments of ca 350bp of *tef* fragment. There was no size variation observed among amplified *tef1* fragments. *Tef1* sequences were edited to 320bp to aid alignment with sequences downloaded from GenBank.

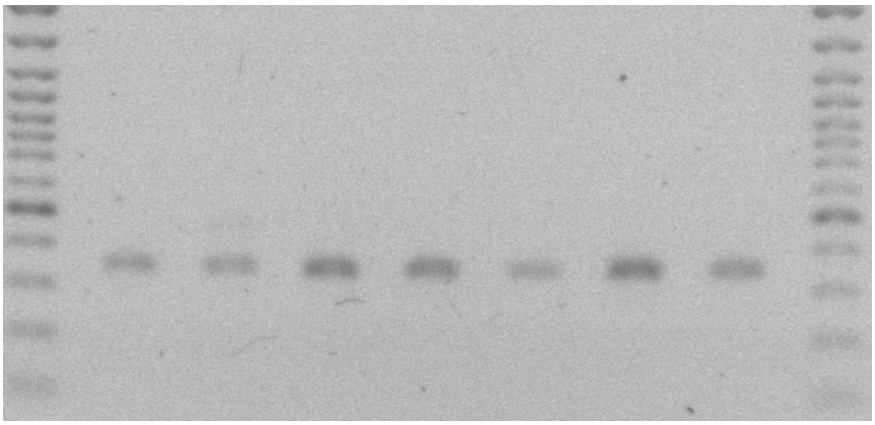

Figure2 Agarose gel stained with ethidium bromide showing amplified *tef1* fragments from Hungarian *Chryphonectria parasitica* isolates (1-7)

Hereinafter one part of the alignment of *tef1* sequences from the Hungarian *Cryphonectria parasitica* isolates (Fig. 3). The sequences were aligned with ClustalX then manually checked for ambiguities and adjusted, when necessary, using Genedoc.

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                  *       20        *       40        *       60        *       80
B1          : ATATTAATCTCCTCCCCGATCCCCAAATTCAACTTCCCATACCATCAGCAGCGGCTGCGCTGTGTTCTGTCTCAGTCGTCTGC : 83
C1          : ATATTAATCTCCTCCCC-ATCCCCAAATTCAACTTCCCATACCATCAGCAGCGGCTGCGCTGTGTTCTGTCTCAGTCGTCTGC : 82
J2          : ATATTAATCTCCTCCCC-ATCCCCAAATTCAACTTCCCATACCATCAGCAGCGGCTGCGCTGTGTTCTGTCTCAGTCGTCTGC : 82
R2          : ATATTAATCTCCTCCCC-ATCCCCAAATTCAACTTCCCATACCATCAGCAGCGGCTGCGCTGTGTTCTGTCTCAGTCGTCTGC : 82
E2          : ATATTAATCTCCTCCCC-ATCCCCAAATTCAACTTCCCATACCATCAGCAGCGGCTGCGCTGTGTTCTGTCTCAGTCGTCTGC : 82
N2          : ATATTAATCTCCTCCCCGATCCCCAAATTCAACTTCCCATACCATCAGCAGCGGCTGCGCTGTGTTCTGTCTCAGTCGTCTGC : 83
MV          : ATATTAATCTCCTCCCC-ATCCCCAAATTCAACTTCCCATACCATCAGCAGCGGCTGCGCTGTGTTCTGTCTCAGTCGTCTGC : 82
MV1/6       : ATATTCCTCTCCTCCCC-ATCCCCAAATTCAACTTCCCATACCATCAGCAGCGGCTGCGCTGTGTTCTGTCTCAGTCGTCTGC : 82
MV1/4       : ATATTCCTCTCCTCCCCGATCCCCAAAATCAACTTCCCATACCATCAGCAGCGGCTGCGCTGTGTTCTGTCTCAGTCGTCTGC : 83
AY308953    : ATATTAATCTCCTCCCC-ATCCCCAAATGCAACTTCCCATACCATCAGCAGCGGCTGCGCTGTGTTCTGTCTCAGTCGTCTGC : 82
AY308954    : ATATTAATCTCCTCCCC-ATCCCCAAATGCAACTTCCCATACCATCAGCAGCGGCTGCGCTGTGTTCTGTCTCAGTCGTCTGC : 82
P5-2        : ATATTAATCTCCTCCCC-ATCCCCAAATTCAACTTCCCATACCATCAGCAGCGGCTGCGCTGTGTTCTGTCTCAGTCGTCTGC : 82
ME48-2      : ATATTAATCTCCTCCCC-ATCCCCAAATTCAACTTCCCATACCATCAGCAGCGGCTGCGCTGTGTTCTGTCTCAGTCGTCTGC : 82
              ATATTaaTCTCCTCCCC ATCCCCAAAttCAACTTCCCATACCATCAGCAGCGGCTGCGCTGTGTTCTGTCTCAGTCGTCTGC
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Figure 3. One part of the alignment of *tef1* fragment from Hungarian *Chryphonectria parasitica* isolates

Parsimony analysis of the *tef1* fragment revealed 310 constant sites, informative sites, 2 non informative sites, and only 6 parsimony informative sites among all isolates. The phylogenetic tree based on *tef1* sequences (Fig. 4) 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.

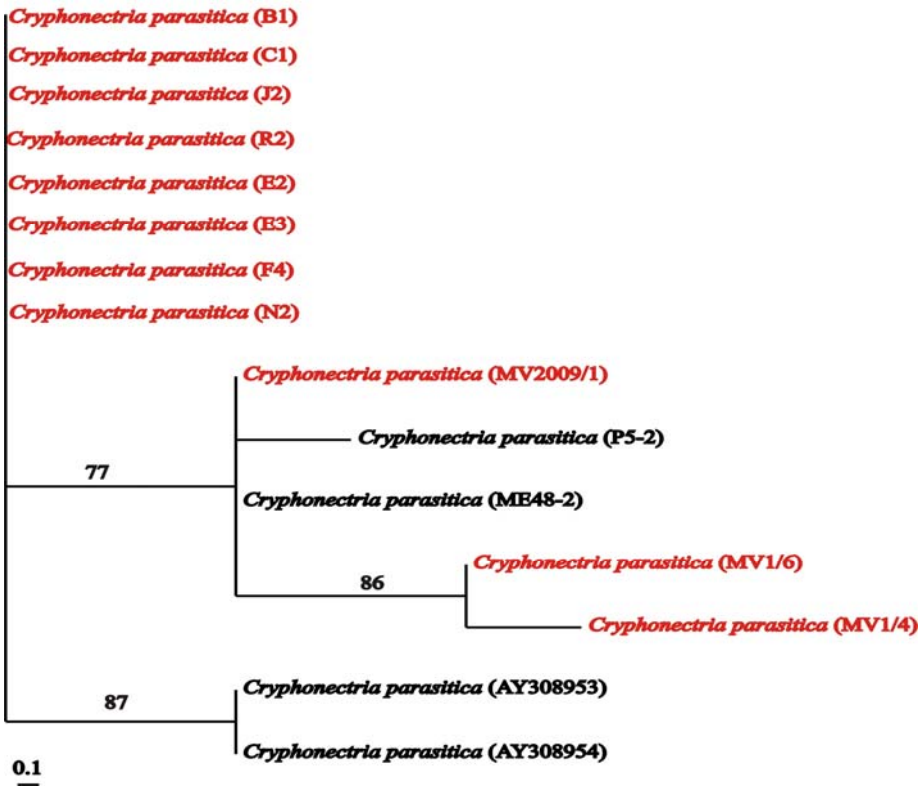

Figure 4. Phylogenetic relationships of *Cryphonectria parasitica* strains inferred by Parsimony analysis of *tef1* sequences. The numbers above the lines represent the bootstrap (bootstrap=1000) values. Our isolates are indicated with red colour.

Conclusions

- The difference among the *Cryphonectria parasitica* isolates species 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 *tef1* 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 *tef1* sequences within *Cryphonectria parasitica* isolates is too small to get well based consequences for the population diversity of this species.
- In the future, for studying the variety and diversity of *C. parasitica* at population level in Hungary, it is recommended to choose a more diverse variable molecular marker like microsatellites.

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