

**Thesis of University Doctoral (PhD) dissertation**

**COMPARATIVE MOLECULAR BIOLOGY  
ANALYSIS OF *CRYPHONECTRIA PARASITICA*  
(MURRILL) BARR SUBPOPULATIONS IN THE  
CARPATHIAN BASIN**

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## 1. BACKGROUND AND OBJECTIVES OF THE DISSERTATION

One of the most harmful pathogens in chestnut production is the fungus *Cryphonectria parasitica* (Murr.) Barr / syn: *Endothia parasitica* (Murr.) Anderson/ that is the causative agent of the chestnut blight disease. This pathogen, just as the two other significant fungi *Phytophthora cambivora* (Petri/Büsm) and *Phytophthora cinnamomi* (Rands.) have caused huge damages in chestnut populations worldwide during the past century. In particular serious chestnut blight damages were caused by the fungus in North-America and in Europe. Currently chestnut blight is spreading intensively and it is considered as the most significant pathogen of European chestnut populations (ANAGNOSTAKIS, 1987).

The disease was detected first on the old chestnut trees of the Bronx Zoo (New York city, USA) and described by a forester Herman Merkel in 1904, who defined the fungus as a lethal pathogen (MERKEL, 1906).

Although the pathogen is an obligate wound parasite, it has been observed that it is able to persist, or even sporulate on dead woody parts as a saprophyte. High amount of both sexual and asexual bodies can be produced on fallen branches or chestnut trees cut and prepared for transport (SHEAR and STEVEN, 1913).

Ascospores or conidia growing into wounds of natural or mechanical origin or cracks can be produced under favourable environmental conditions and thus cause the primer infection. Several research works have been executed in order to reveal why young plants of resistant Asian species are susceptible in their first 2-3 years to *Cryphonectria parasitica* infection, and why wooden parts of older trees covered by young bark are significantly more sensitive (UCHIDA, 1977).

Wind plays the primal and the most important role in spreading ascospores. In some cases the distribution of mycelium pieces through vectors, just as the transport of infected wooden material can become important in the introduction of the pathogen. Often no visible symptoms can be observed on such propagation materials that could be suspicious, so it is very hard for the grower to realize the presence of the disease in due time (RADÓCZ, 1995).

The disease increases the death of the infected bark and tears off. Following the pathogen appears on the bark symptoms can be observed soon on the entire wood due to re-infection. Usually several necroses develop on an infected tree, mainly on main branches, on the trunk and in forks, but the infection of neck parts is also common, especially in case of young plants and young grafts. At the end of the disease progress usually the entire tree dies (RADÓCZ, 1994).

One of the most important milestones in the control of *Cryphonectria parasitica* was the discovery of hypovirulence. It is one of the most relevant research topics, even if the symptom has been described more than 50 years ago. In case of chestnut it has been observed for the first time in Italy, in the surroundings of Genoa in a heavily infected chestnut population where healed cankers were detected on the surface of the callused bark. Till then, reformation of callus was observed as part of healing process only in case of Asian chestnut species (BIRAGHI, 1950).

Hypovirulent strains were characterized by weak sporulation ability. Regarding their virulence such strains showed significant deviances from virulent strains known before. Previous research works have revealed that causing agent of the hypovirulence can be found in the cytoplasm of fungal cells, and it can be easily transferred through hyphae anastomoses formed between two fungal individuals that are in the same vegetative compatibility group (GRENTE and SAURET, 1969).

The research of MOFFITT and LISTER in 1975 contributed to the more exact understanding of the phenomena of hypovirulence. In their study they have successfully detected the presence of double-stranded RNA (dsRNA) in the cytoplasm in two hypovirulent strains of French origin. For the first time dsRNA was isolated by MORRIS and DODDS in 1979 in using a simplified method for *Cryphonectria parasitica* isolates infected with mycovirus.

Hypovirulent strains are able to transfer dsRNA through hyphae anastomoses, and thus virulent strains lose their virulence and the slow healing of necroses can start. As this control method are proved to be the most effective in the field, several experiments have been carried out, within that researcher attempted to weaken the virulent strains using grafting methods. In these experiments it has been observed that grafting was not successful in all cases and the virus causing hypovirulence was not always transferred to 'wild' strains. The transfer of the hypovirus was successful only when vegetative compatibility presented between the two fungal strains (ANAGNOSTAKIS and WAGGONER, 1981).

In case of the combination of two fungal strains that are not compatible with each other hyphae anastomoses die even before the transfer of the double-stranded RNA particle. It was a very important detection that in some cases the transfer of double-stranded RNA (dsRNA) is possible even between incompatible individuals (members of different VCG-s), however with very low likelihood (PENNISI et al., 1992; GRANATA et al., 1992).

The number of theoretically potential VC groups may lay over 150 in case of *Cryphonectria parasitica*. In 1983 already 77 VC types were identified in the United States (ANAGNOSTAKIS, 1983; ANAGNOSTAKIS, 1998; HOGAN and GRIFFIN, 2002).

A comprehensive international research was started in Europe in order to uniformly systematize the identified vegetative compatibility types. As a result of this 31 EU-tester strains were identified from the strains of the pathogen isolated in Switzerland and Italy. According to the conclusions regarding six loci and two times two alleles there are altogether 64 such potential VCGs (CORTESI et al., 1998).

During the monitoring of Hungarian chestnut populations it has been stated that pathogen strains belonging to the same VC groups were present at particular regions. The reason of this can be that the fungus is present in Hungary relatively just for a short time. However, according to the latest research there are regions where strains of different VCG-s were identified within one chestnut population. This suggests the genetic separation or the hybridisation of the pathogen with another genotypes (RADÓCZ, 1997).

Besides the execution of vegetative compatibility tests, 3 phylogenetic markers were used for the genetic characterization of chestnut blight fungal populations. Consequently the gene is used mainly for the detection of phylogenetic relationships between species and it can be used only restricted for the characterisation of relationships within any species (CHO et al., 1995; MOREIRA et al., 1999).

The application of the not-coding ITS gene fragment of high variability is commonly current in order to describe relations between taxons. Modelling of relationships between class, family, genus and species can be executed with high reliability; therefore these gene fragments were used by several researchers in their work. Low extent of selection affects the ITS gene fragment, therefore the probability of mutations is high, variability is high and the gene fragment is evolutionary variable. This character enables the use of not-coding fragments in most of the analyses of taxonomy relationships of lower level, for example for the isolation of close relative species or populations (HILLIS and DIXON, 1991; MONCALVO et al., 1995; NICHOLSON, 1995; PINE et al., 1999).

The application of microsatellites for phylogenetical purposes in case of fungi has become common in the past years. Molecular biological markers named Simple Sequence Repeat (SSR) or Short Tandem Repeat (STR) can be effectively used for the preparation of genetic maps of high resolution. The common and large-scale applicability of microsatellites in phylogenetical and population-genetical analysis is enabled by the fact that they can be amplified very easy, they are not subjected to selection pressure and they can be found in high

quantity in the genome. It is important to notice that microsatellites are overall current in both prokaryote and eukaryote genomes, thus they can be commonly used as universal markers (HEARNE et al., 1992; PARKER et al., 1998 ZANE et al., 2002).

### **Detailed objectives of the research work**

1. Refreshment of the *Cryphonectria parasitica* sample collection available at the University of Debrecen, Centre for Agricultural Sciences, Faculty of Agricultural and Food Sciences and Environmental Management, Institute of Plant Protection, just as the collection of new samples from new test regions and the maintenance of the existing collection.
2. Molecular biological analysis of the collected samples using the molecular markers ITS and *tef1* and the comparison of the results with data available from different international databases.
3. To determine relationships and correlations associated with the genetic and geographical location of different samples.
4. The analysis of microsatellite markers, just as the definition of their applicability in the comparative analysis of different fungal subpopulations.
5. The execution of VCG tests in case of our own field collection samples, just as the comparison of the results with other research results.
6. The comparison of data of the available scientific literature and the results of our own tests, just as to reveal the reasons for any possible deviances, changes.

## **2. RESEARCH METHODS**

### **2.1 Details of sample collection**

As the first step of the present research testing regions have been selected. At the selection of the involved chestnut production regions we laid special attention on choosing regions that represent the different *Cryphonectria parasitica* subpopulations of the Carpathian Basin. As according to the classification of EPPO *Cryphonectria parasitica* is a pathogen subjected to quarantine, that can be detected in the territory of EPPO states, thus in Hungary as well and its import is subjected to a permission. Therefore we had to apply for an official permission for the samples collected from the neighbouring countries and laboratories and equipments for their analysis had to be subjected to special inspection.

During the collection of samples test regions were measured exactly with a hand GPS device and locations of GPS coordinates of trees from which bark samples were collected were also recorded. In the present research work 95 *Cryphonectria parasitica* isolates were analysed. 31 isolates were collected from Hungary, 17 from Slovakia, 10 from Ukraine, 11 from Romania, 1 from Macedonia, 2 from Bulgaria, 9 from Greece and 5 from Portugal. Samples from Portugal were provided by Helena Bragança, while those from Greece by Charikleia Perlerou.

### **2.2 Laboratory preparation of samples**

Laboratory analyses were started with the preparation of the medium. Samples were grown on PDA (Potato Dextrose Agar) medium. During the preparation bark samples were disinfected in 70% ethanol for 2-3 minutes and then rinsed with distilled water from the samples. Small pieces of bark samples were set onto the medium in the previously labelled Petri-dishes and then samples were put into a thermostat where they were grown for a week at a constant temperature of 27 C. Infected bark samples were controlled each day and when *Cryphonectria parasitica* appeared on the medium they were passaged again and grown in the thermostat. After 10 days the fungus covered the Petri-dishes and samples could be passaged onto liquid medium.

In the VCG analysis 50 mg l<sup>-1</sup> bromocresol green indicator was added to the PDA medium. The use of the indicator is advantageous because in case of the vegetative incompatible cultures the edge zone becomes far stronger visible, as the bromocresol green

indicator shows colour changing reaction in case of the acidification of the medium. However, in case of dead hyphae the medium does not show any change in its colour (POWELL, 1995).

For the preparation of the liquid MEX (malt extract) medium we followed the prescriptions of the producer: 20 g MEX medium was added to 500 ml distilled water. The medium was sterilized in an autoclave. The liquid MEX (Scharlau) medium was added into Erlenmeyer-flasks. 50 ml medium was added into each Erlenmeyer-flask. The fungus was rinsed from the solid PDA medium with 5 ml sterilized water and then this liquid was pipetted into the flasks. Then the Erlenmeyer-flasks were put into a shaker machine and were shaken at room temperature for 74 hours at a speed of 125 rpm.

### **2.3 The execution of VCG analyses**

Before the molecular biology analyses of the involved samples their classification in VC groups was executed. For the test analyses the protocol according to POWELL, published in 1995 was used: first 20g previously mixed PDA (Potato Dextrose Agar) component was added to 5dl distilled water, then 50mg methionine, 0.5g biotin, 3.5g malt extract, 1g yeast extract, 0.4g tannic acid, 1mg thiamine and 25mg bromocresol green indicator was supplemented to it. The medium was cooked using a wet autoclave type RAIPA. This autoclave sterilizes according to the following programme: at a temperature of 12 C, at a pressure of 1.1bars and for 20 minutes. Then the sterilized medium was distributed in the Petri-dishes, and 10×10 mm medium squares with fungi mycelium tissue were transferred onto each of the dishes. 4 pairs of test could be run at one time in one Petri-dish. Samples were placed into a heatable/refrigeratable microbiological incubator type Heratherm IMH180S. Isolates were grown at a temperature of 25 °C and on the second week after the pairing tests were evaluated. Test strains that were essential for the execution of the tests (EU1-31) were provided by the Institute for Plant Protection, University of Debrecen, just as by the Institute of Forest Ecology, Nitra. As the first step of the analyses compatibility tests within the populations of each sampling test region were run. The results were processed in an Excel table, thus the presence of different VC groups at one test region became more visible. The distribution and percentage rate between the VC-groups of different test regions were visualized by pie charts. The results have been then compared to the results of previous analyses and any possible changes have been summarized.

## 2.4 Preparation of polymerase chain reaction analysis

The PCR analysis was carried out in a volume of 50µl that contained following components: 25µl 2XPCR Master Mix (Fermentas, K0171), 2µl genomic DNA (0.5–1µg), 2-2µl forward and reverse primer (10pmol/µl), 19µl sterilized, nuclease-free water (Fermentas, #R0581).

For the amplification of the ITS fragment following pair of primers was used: SR6R: 5'-AAG TAG AAG TCG TAA CAA GG-3' (SSU) 23 bp; just as LR1: 5'- GGT TGG TTT CTT TTC CT-3' (LSU) 17 bp (WHITE et al., 1990). The reaction environment was set according to the following protocol: initial denaturation at a temperature of 95°C for 3 minutes was followed by 5 cycles of further denaturation at 95°C for 1 minute each. This was followed by 1 minute annealing at 50°C and finally by the polymerisation for 1 minute at 72°C. Then 25 cycles of denaturation were run at 95°C for 1 minute each, followed by annealing at 50°C for 1 minute and finally by the polymerisation at 72°C for 1 minute. In the end of the process a polymerisation was run at 72°C for 15 minutes.

For the amplification of the translation elongation factor fragment the following pair of primers was used: EF1-728F: 5'- CAT CGA GAA GTT CGA GAA GG -3' 20 bp; just as EF1-986R: 5'- TAC TTG AAG GAA CCC TTA CC -3' 20 bp (DRUZHININA and KUBICEK, 2005). The reaction environment was set according to the following protocol: initial denaturation at a temperature of 95°C for 3 minutes was followed by 5 cycles of further denaturation at 95°C for 1 minute each, then annealing at 59°C for 1 minute and finally polimerisation at 72°C for 1 minute. Then 25 cycles of denaturation were run at 95 C for 1 minute each, followed by annealing at 59°C for 1 minute and finally by the polymerisation at 72°C for 1 minute. In the end of the process polymerisation was run at 72°C for 15 minutes. The PCR analysis was executed by the device of the MWG Biotech Inc. type Primus 25 (Milton Keynes, UK). Sequencing of the purified and amplified PCR-products was executed by the company MWG Biotech, Germany within the confines of a service purchase. The applied sequencing method is based on the method described by Sanger (SANGER et al., 1977), which was run with the application of devices developed by the company ABI. The reliability of the sequencing was warranted by the international quality assurance standard ISO (DIN EN ISO 9001:2000).

## **2.5 Details of microsatellite amplification**

In this research 6 different microsatellites were chosen and involved into the analyses which were described previously by BREUILLIN et al., (2006) and KUBISIAK et al., (2007). Amplification was carried out at a final volume of 50µl that contained 25µl 2XPCR Master Mix (ImmoMix, Bioline, 25020), 40-40pmol primers, just as 20-40ng DNA. This solution was filled to the required volume with nuclease-free water. Microsatellites were amplified using a Primus (MWG Biotech) thermocycler. Following PCR cycle was used for the amplification: initial denaturation at a temperature of 95°C for 3 minutes was followed by 5 cycles of further denaturation at 95°C for 1 minute each, then annealing at 50°C for 1 minute and finally polymerisation at 72°C for 1 minute. The temperature of the next 25 cycles of denaturation was lowered to 90°C and the temperature of the annealing varied between 53 and 56°C, depending on the given locus. Further settings were the same as those in case of the first 5 cycles. The final polymerisation was run at 72°C for 15 minutes. The analysis of fragments was run using an Origins electrophoresis device (Elchrom Scientific AG). Each PCR product was analysed first on a Spreadex\_ EL 500 gel (Elchrom Scientific AG) for 240 minutes at a temperature of 55°C. In order to keep the DNA bands sharp 1XTAE buffer was added that was enclosed to the gel product by the manufacturer. The gel was masked with EtBr (Sigma, USA) for 45 minutes, and it was then recorded using the BIO-RAD gel documentation system under UV light (k=250nm).

## **2.6 Software used for the phylogenetic analysis**

As the initial step of the phylogenetic analysis DNA sequences were merged using the software ClustalX. This software is in particular an application provided with a multiply sequence organizing function that is able to manage large number of nucleotide- and protein-sequences. The software identifies and analyses the analogy, homology of several sequences; thus the evolution relationship, differences or even identity between sequences can be made recognizable. After the sequences were merged with the software ClustalX a manual refinement was essential. Genedoc is also a multiply sequence merging and editing software that enables manual refinement. After merging the sequences the result data shall be graphically interpreted in order to visualize different evolution relationships between the analysed samples. For this purpose several software are available. In the present work the software Treeview was used first that can be used for the drawing and editing of phylogenetic

trees as well. The software Paup\*4.0 (SWOFFORD, 2000) is currently the primary phylogenetic tree maker software. This software manages data files from ClustalX, just as Genedoc and it can run several phylogenetic analyses, among other the analyses Maximum Parsimony and Maximum Likelihood that were used in the present work.

## **2.7 Microsatellite analysis**

For the evaluation of microsatellite data the software Popgene 1.31 (YEH et al., 1999) was used. The six studied microsatellite loci were analysed and the extent of heterozygosity was estimated (LEVENE, 1949; NEI, 1973). With this software allele frequency, effective number of alleles and genetic diversity were calculated for the entire population. The actual number of the observed alleles was determined with the software Popgene as well (KIMURA and CROW, 1964).

Studied samples were classified into 7 different groups according to their geographical origin, the number of polymorph loci and their percent distribution, the average number of alleles per locus, just as the actual number of loci, and Nei's gene diversity ( $h$ ) per locus were computed with the software. According to Nei's G-statistics the genetic difference between populations, just as Nei's normalized genetic identity and genetic distance were estimated (NEI, 1972; NEI, 1973; NEI, 1987).

The difference between populations was tested with the comparison of allele frequencies between the seven populations and Weir & Cockerham's  $\theta$  ( $F_{st}$ ) value (WEIR and COCKERHAM, 1984) was also calculated with the software Multilocus 1.3b. This software analysed  $\theta$ -values statistically by testing the null hypothesis  $\theta = 0$  (populations cannot be separated) with the randomly selected data of isolates ten thousand-times (AGAPOW and BURT, 2001).

The extent of gene flow between populations ( $N_m$ ), that is the number of migrant individuals within one generation was calculated using  $\theta$ -value according to the formula  $N_m = 0.5(1-\theta)/\theta$  (MCDERMOTT and MCDONALD, 1993).

The software Popgene 1.31 was used as well for the determination of the number of multilocus genotypes (MLG), just as for the calculation of standardized version of the index of association that corrects deviances in the number of samples. The null hypothesis of complete panmixia ( $r_D = 0$ ) was tested by comparing the data of randomly selected isolates ten thousand times (AGAPOW and BURT, 2001; GIRAUD et al., 2006).

The structure of the populations was analysed with the software BAPS 6.16 (Bayesian Analysis of Population Structure). According to the approach of Bayesian this software determines the most probable grouping of the individuals in the population. The software determines the most probable (posterior probability) number ( $k$  – cluster number) using the length data of microsatellite fragments and it defines the group for each individual (CORANDER et al., 2003; TANG et al., 2009).

### **3. MAIN STATEMENTS OF THE DISSERTATION**

#### **3.1 Results of the VCG analyses**

In the present research vegetative compatibility tests were run for altogether 168 different samples. Mainly samples of Hungarian and Slovakian origin were involved into the analysis. According to the relevant literature highest diversity between the vegetative compatibility groups was expected in these two production regions. In this research work vegetative compatibility tests for 44 samples originating from 13 chestnut growing regions in Hungary have been analysed. Production regions for Sopron, Zala, Baranya, the Danube bend and Northern Hungary were considered as separate groups. It is important to emphasize that VC tests were executed for the very first time in case of the subpopulations of Pálháza and Érsekvadkert. No previous literature data is available for these regions. In the region of Sopron VC groups EU-12 and EU-13 could be detected. In the production region Zala positive results were detected for VC groups EU-12, EU-13 and EU-16. The region of Baranya showed the same VC group results as that of Zala. Samples from the region Danube bend and Northern Hungary, Pálháza showed vegetative compatibility with the tester strain EU-12. In case of all Hungarian production regions the dominance of the VC group EU-12 could be stated. In the regions Zala and Baranya the VC group EU-16 could be detected in an extent of about 10% that is considered to be significant.

During the sample collection in Slovakia altogether 39 samples were collected from 9 sampling regions for which vegetative compatibility tests were run. The studied regions were the following: samples around Bratislava were classified in one group, then Nitra, Modrý Kameň, just as samples from the cemetery of Krná in the neighbourhood of Banská Bystrica, and those from the forest by Petrovce in Eastern-Slovakia directly close to the border to Ukraine were classified as different sampling groups. In case of the region of Bratislava VC groups EU-2, EU-12 and EU-13 could be identified. In the production region of Nitra VC groups EU-2, EU-12 and EU-16 showed positive results. The highest number of different VC

groups could be identified in these two studied regions. In case of the region of Modrý Kameň only VC groups EU-12 and EU-13 could be detected. Regarding the samples originating from the two sampling fields in the region of Krná in the neighbourhood of Banská Bystrica the strain EU-12 could be solely identified. In case of both two sampling fields from the region Petrovce in Eastern-Slovakia the group EU-12 could be identified. In general it can be summarized that strains EU-12 and EU-13 were dominant in the studied production regions. But regions in the Western-Slovakia (Bratislava and Nitra) showed higher variability and more vegetative compatibility groups could be detected there, however, in the region of Nitra the group EU-12 proved to be dominant, while in case of Bratislava it was EU-13.

During our field sample collection work infected chestnut bark samples were isolated and collected from 3 different production regions in Ukraine: from the surroundings of Serednje, just as Bobovyshe and from forests around Rostov'yatisya. In the previously executed VCG tests only the compatibility group EU-12 could be identified there. In case of the 4 sampling fields around Serednje tester strains EU-12 and EU-13 could be detected as well. These two VC strains could be identified in the samples originating from Bobovyshe and Rostov'yatisya.

Field studies were carried out in two different times in Romania. Samples were isolated from the chestnut production regions around Baia Mare on the 7<sup>th</sup> September 2011, while those from the forests around Tismana in Southern-Romania on the 2<sup>nd</sup> September 2012. Altogether 21 isolates were collected from the 13 sampling fields of these two production regions for which vegetative compatibility test were executed. The vegetative compatibility group EU-12 is dominant in the region of Baia Mare, in the territory of a Veresváz, Felsőtótfa and Kőbánya. In the production region of Tăuții-Măgherauș close to Baia Mare the presence of the vegetative compatibility group EU-13 could be detected beside the group EU-12. In case of the other studied Romanian region, Tismana all collected samples showed positive results with the EU-12 vegetative compatibility group.

After getting into contact with Professor Dr. Kiril Sotirovski successfully, samples were collected in the main chestnut production regions of Macedonia on the 4-5<sup>th</sup> October 2011 with his assistance. The collection of samples was started in the chestnut population near the city of Tetovo, 51km far from the capital Skopje. Then towards the south isolation was carried out in the surroundings of Vrutok and then near Osoj. After that sample collection was carried on in the chestnut forests of Volino and Radolistha, along the Lake Ohrid on the border between Albania and Macedonia. According to different monitoring results Macedonian populations showed compatibility in the highest extent with the tester strain EU-

12 (98%); while minimal presence of vegetative compatibility groups EU-1, EU-2, EU-10 and EU-22 was also detected. It has been stated from the analysis results that all samples showed compatibility with the tester strain EU-12.

Samples of Greek origin were provided by Professor Dr. Charikleia Perlerou. Samples from different chestnut production regions of the country represent the diversity of VC groups well. These samples were completed with the samples P5-1, P5-2, ME48-1 and ME 48-2 that were available at our Department. According to the results it has been clearly stated that all samples showed compatibility with the tester strain EU-12.

During the sample collection in Macedonia it has become possible with the assistance of Professor Dr. Kiril Sotirovski to continue the field work in the territory of Bulgaria. Samples were collected successfully from the chestnut population of Petrich that is in the south-western corner of the country, close to the triple border between Greece, Bulgaria and Macedonia. It was important to involve Bulgarian samples into the present research work, for in contrast to the detailed survey of Greek production regions the phytopathological state of Bulgarian chestnut populations is monitored far less. According to previous literature data 80% of the compatibility tests showed positive results with the EU-12 strain and confirmed the presence of compatibility groups EU-2 and EU-10, however, in a far lower extent. According to the results it has been clearly stated that all 5 samples showed compatibility with the tester strain EU-12.

For comparison purposes Portuguese samples could be used beside the involved samples of Carpathian Basin and Balkans origin. Portugal samples were provided by Dr. Helena Bragança researcher at the Unidade de Silvicultura e Produtos Florestais Instituto. Two samples were collected from the Azores, one from Alentejo in South-Western Portugal, one from the Northern Trás os Montes and one from Madeira island. At our request the samples were selected in order to represent Portuguese *Cryphonectria parasitica* populations as fine as possible. Positive results were found only in case of two samples during the analyses, while in case of the rest of the samples no compatibility was found with the available test material between EU-1 and EU-31. In case of 80.2% of the vegetative compatibility tests published in the literature the dominance of EU-11 strain was confirmed, while 7.1% showed compatibility with the tester strain EU-12. In case of 6.6% of the tests the presence of EU-66 could be confirmed, and the groups EU-1, EU-2, EU-28, EU-33, just as P-7 and P-9 were also detected. According to the results of the comparison of our analyses and literature data it can be stated that chestnut blight populations in Portugal show high diversity,

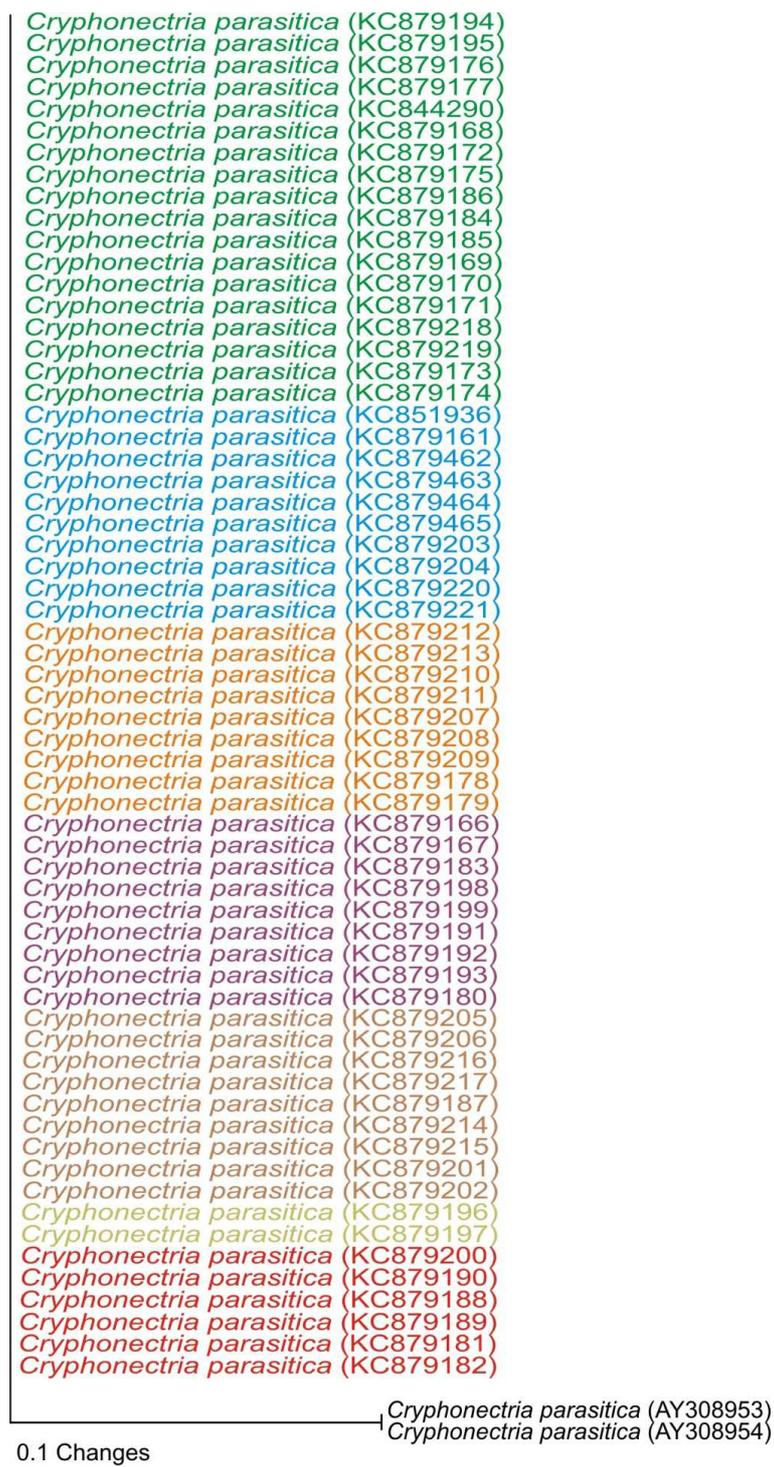
the presence of 9 different VC groups has been confirmed and their structure was different from that population in the Carpathian Basin.

### **3.2 Analysis of *tefl* fragments and obtained results**

*Tefl* fragments could be amplified and sequenced in case of 63 samples from the involved 95 isolates. Mixed sequences were found in several cases during the molecular biology lab analyses and no evaluable sequence result could be obtained after DNA isolation and the multiplied re-setting of PCR parameters. During the comparative analyses very few *tefl* sequences were available in data banks so for the final phylogenetic tree only two sequences could be used for comparison that were not deposited by us (<http://www.ncbi.nlm.nih.gov/>).

In the Parsimony test the software computed with 547 characters (bases) from which 539 characters were found to be constant, 6 characters were assessed as non-informative and only 2 characters were assessed as informative. The low number of informative characters suggests that the difference between the *tefl* fragments of *Cryphonectria parasitica* isolates of different geographical origin is low, so it is not suitable for the segregation within species. In order to verify the confidence of the comparison a bootstrap analysis was performed. High bootstrap values of the analysis confirmed the probability of branches' location and thus the drawn phylogenetic tree was confirmed to be correct. Distances between individual isolates (difference between bases, the length of branches) did not prove to be variant enough in order to consider the phylogenetic tree as well-established according to the results. On the basis of the drawn phylogenetic tree (*Figure 1.*) it can be stated that the collected and sequenced samples constitute a unit and do not show assessable difference. This result was expected, because it was clear already at the manual refinement of sequences that there was only minimal, only a few base pairs difference between the individual samples. In this context it can be stated that *tefl* sequences of our sequenced samples were practically identical. In contrast, samples of USA origin from the database of GeneBank (labelled black on the phylogenetic tree) built a separate group. In the literature it has already been referred to that the *tefl* fragment is a current and commonly used marker in population genetics and phylogenetical tests in case of filamentous fungi. However, in the present research work it has been proved that it is not suitable for the isolation of different populations within *Cryphonectria parasitica* species for it did not produce any evaluable difference between samples of different geographical origin. It can be considered as a result that the rather

incomplete *tefl* sequence set of the gene bank has been successfully completed with 63 *Cryphonectria parasitica* sequences.



**Figure 1.** Development of phylogenetic relationships on the basis of Parsimony analysis of *tefl* sequences (different colours refer to different locations of origin, samples from the database are labelled black)

### 3.3 Results of analysis of ITS fragments

In contrast to the observations regarding *tefl* fragments, the amplification of ITS fragments of *Cryphonectria parasitica* samples involved into the present research work was far more effective. In this case ITS fragments could be amplified and sequenced for 85 samples from the total 95 samples involved. Several ITS fragments of *Cryphonectria parasitica* were available in data banks for the comparative analyses, so as an extension of the comparison of deposited ITS sequences of other species of the genera *Cryphonectria* and *Endothia* were used for the analysis as well (<http://www.ncbi.nlm.nih.gov/>).

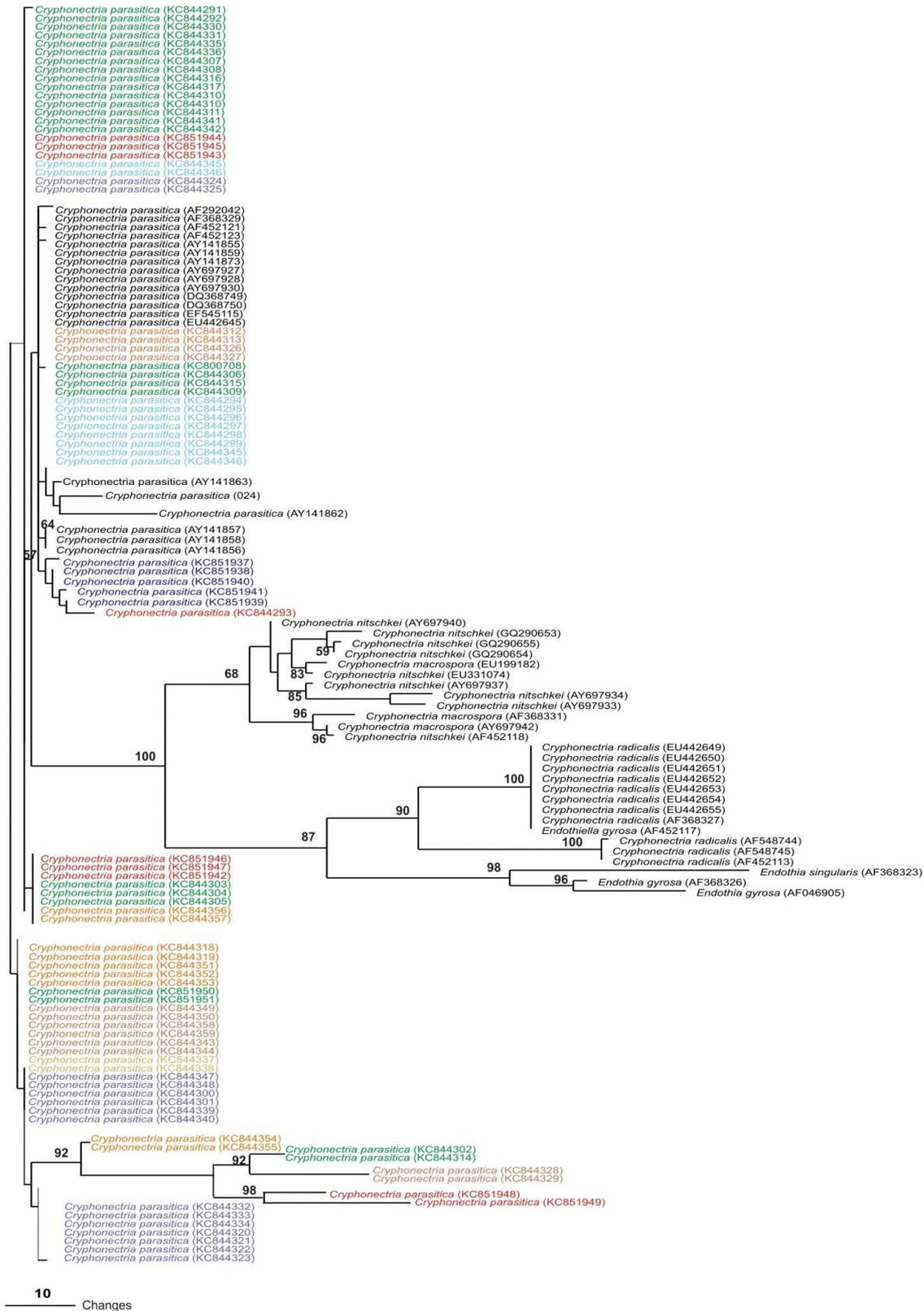
Isolates that had been successfully amplified and sequenced were deposited in the data bank used by us as well (GeneBank). After the processing of the provided data, the data bank labelled isolates with an identification code. Sequences from the databank were included in the analysis for the drawing of the phylogenetic tree in order to extend relationships within and between species. 45 samples were downloaded from the databank. 19 deposited sequences of the species *Cryphonectria parasitica* from the genus *Cryphonectria* were available in the GeneBank data base, the length of the ITS fragment of which ranged from 525 to 638 pairs of bases. ITS sequences of samples of the following origin have been downloaded from the database (the number of isolates is provided in brackets): USA (7), Japan (6), Portugal (3), China (2) and Azerbaijan (1). The species *Cryphonectria nitschkei* was involved into the analysis with 9 isolates with fragment lengths of 507-669 base pairs. 5 isolates of these originated from Japan, while the origin of the rest 4 isolated was unknown. In case of the species *Cryphonectria macrospora* 3 isolates were involved into the analyses: 1 sample from Russia, 2 samples from Japan with fragment lengths of 509-591 base pairs. The species *Cryphonectria naterciae* was involved into the analysis with 7 isolates with fragment lengths of 531-532 base pairs the origin of which was in case of each sample Portugal. In case of *Cryphonectria radicalis* 3 isolates with fragment lengths of 535-544 base pairs were analysed (1 from Italy and 2 from Switzerland). In case of the species *Endothiella gryosa* 1 isolate was available from Portugal with fragment length of 535 base pairs. Also 1 isolate was involved in case of the species *Endothia singularis* that originated from the USA and had a fragment length of 749 base pairs. In case of *Endothia gyrosa* 2 isolates were analysed, the origin for which was the USA and the fragment length ranged between 450 and 475 base pairs.

The phylogenetic tree drawn on the basis of the Parsimony analysis of ITS fragments is represented on *Figure 2*. The software analysed 547 characters (bases) in the analysis from

which 299 were assessed to be constant, 76 as non-informative and altogether 172 as informative. The large number of informative characters – in contrast to the results of *tef1* fragments – suggests that reliability of ITS fragments is far better for the separation of *Cryphonectria parasitica* isolates of different geographical origin.

Distinct groups can be identified in the phylogenetic tree drawn from the results (Figure 2.). Groups were formed typically according to the separation of different species. Independently from their sampling origin (own collection or data bank), *Cryphonectria parasitica* isolates formed a distinct group and could be separated from the close relative *Cryphonectria*, *Endothia* and *Endothiella* species. This result proves that the chosen ITS fragment can be successfully used for the separation of different species of the genera *Cryphonectria* and *Endothia*. In contrast, the separation of subpopulations within species did not prove to be that clear. Even if far more complex representation can be achieved in the phylogenetic tree so regarding the location of *Cryphonectria parasitica* isolates of different geographical origin, than in case of *tef1* fragments, unambiguous groups were not formed where isolates only from a given geographical region would be included. Location of samples of Hungarian origin gives good example for that on the phylogenetic tree: isolates labelled with green are separated into 5 different groups and formed groups with almost all involved samples of different geographical origin. It is important to consider samples from Portugal that are labelled dark blue on the phylogenetic tree. These samples formed a separate group within the analysed populations that confirms the results of microsatellite analysis. Clear separation was expected previously in case of samples of Greek origin, but contrarily the available 9 samples were grouped into several different groups. It is important to notice that populations in the region of Nagymaros and Pálháza were treated with hypovirulent strains of Greek origin in the past years and the group of these Hungarian isolates contained these samples from Greece as well. On the basis of the presence of individuals having similar genetic characters as the isolates of Greek origin it can be assumed that these are of external origin and they subsisted in the population of phytopathogens.

In case of the species *Cryphonectria parasitica* the use of ITS fragments for the separation of subpopulations within species cannot be considered as suitable, however, in contrast to the results obtained in case of *tef1* fragment it proved to be far more applicable.



**Figure 1.** Development of phylogenetic relationships on the basis of Parsimony analysis of ITS sequences (different colours refer to different locations of origin, samples from the database are labelled black) Numbers on the lines refer to the likelihood of branches

### 3.4 Results of microsatellite analyses

In order to achieve results as exact, just as to characterize populations of different geographical origin as detailed as possible in the population genetic analyses with microsatellites, the whole population was divided into further groups according to the geographical origin of the samples. Groups 1-4 included samples from the Carpathian Basin (Hungarian, Slovakian, Ukrainian and Romanian), samples from Macedonia and Bulgaria formed group 5, Greek samples formed group 6 and group 7 included samples of Portuguese origin.

According to the differences in the size of the 6 microsatellite markers used in the tests, the analysed 95 isolates could be separated into 71 different genotypes. The number of alleles ( $n_a$ ) varied between 2.66 and 4.00 in case of groups 1-5, while in case of groups 6 and 7 in the range of 1.50-2.00.

Genetic diversity within the population showed differences between the analysed groups. The lower the extent of genetic diversity within the population is, the uniform the population is. In case of groups 1-5 quite high values (0.44-0.61) were obtained. According to the results of the VC tests in the present research work – that are going to be detailed later – it has become clear that the samples from the Carpathian Basin show high heterogeneity that is confirmed by the high value of genetic diversity as well. In contrast, in case of the groups 6 and 7 that included Greek and Portuguese samples the values of Nei's genetic diversity ranged between 0.17 and 0.29 (*Table 10.*). In case of the samples of Greek and Portuguese origin low genetic diversity can be explained by the rather closed character of populations (e.g. Azores, Madeira).

Standardized version of the index of association ( $r_D$ ) calculated for all involved individuals ignoring the data of clones (i.e. only the data for individuals of different genotypes) differed significantly ( $r_D=0.038$ ;  $p<0.01$ ) from the value zero that suggests that the analysed populations were highly different from each other. The highest value was obtained in case of the population no. 2 ( $r_D=0.134$ ;  $p<0.01$ ). In this population both Hungarian and Slovakian samples were involved. Index of association values differed significantly from zero value in case of the populations 1-4 ( $r_D$ : 0.073–0.134;  $p<0.1$ ) suggested the possibility of separation for further groups within each population. For the groups 6 and 7 that covered Greek and Portuguese samples the index value was zero. According to the analysis the lowest extent of genetic variability could be assumed for these samples.

On the basis of the comparison of allele frequencies of populations Weir&Cockerham's fixation index ( $\theta$ ) values were calculated using F-statistics. F-statistics indicate the genetic variance of populations using indexes derived from each other. Fixation index can range between 0 and 1 where in case of values close to 0 there is no difference assumed between populations, while in case of values close to 1 very significant difference can be assumed. According to this interval very significant ( $\theta > 0.25$ ) difference was found in 12 cases for the analysed populations. The population no. 7 that included Portuguese samples showed typically significant differences in all comparisons. Significant differences could be stated in 5 further cases ( $\theta$ : 0.15-0.25) which were related to the population no. 6 that were the Greek samples. In the 4 rest cases only moderate ( $\theta$ : 0.05-0.15) separation could be detected. In case of populations 1-5 that included higher number of individuals this difference was often significant.

Gene flow means the changes in different populations, e.g. genetic change in the population induced by migration. Gene flow value ( $N_m$ ) can range between 0 and 1 where in case of the value 0 there is no gene flow between populations consequently the genetic differentiation is high, while in case of values close to 1 the extent of gene flow is high and genetic differentiation is low at the same time. According to the results of the calculation of gene flow following statements can be made for the different populations analysed in the present research work. Like in case of Weir&Cockerham's fixation index ( $\theta$ ) values the population 7 can be distinguished: the extent of gene flow was an order of magnitude lower ( $N_m$ : 0.028-0.096) in case of the Portuguese samples. By such low extent of gene flow the genetic differentiation is usually very high and the genetic difference is high in contrast to the other analysed populations. Among the seven analysed population gene flow showed the highest values for the populations 1 and 2 ( $N_m$ : 0.156-0.353). These values suggest that genetic differentiation is of low extent in these populations and the genetic difference in contrast to the other populations analysed is rather low.

In order to compare the genetic patterns of the collected different subpopulations values of Nei's genetic identity (I) and genetic distance (D) have been calculated. The value of genetic identity (I) is referred to one locus in case of n alleles and between two populations. The closer the value of I is to 0, the less common alleles can be identified in both populations. If I=1, the frequency of alleles is the same for the analysed locus and if I=0, allele frequencies are absolutely different on the given locus of the two analysed populations. According to the results it can be stated that the relative genetic identity of populations showed the lowest extent in case of the population no. 7, so this population could be definitely separated from

the others. In case of populations 1, 2 and 3 Nei's genetic identity values were high, so partial genetic identity can be assumed for these populations.

Values of genetic distance between the analysed populations ( $D$ ) can be calculated from the allele frequency values of each population. In case of this index standard genetic distance ( $D_S$ ) and minimum genetic distance ( $D_M$ ) values shall be distinguished. For the calculation of standard genetic distance it is assumed that genetic drift and mutation are balanced in case of all alleles. If  $D=0$ , allele frequencies are the same. The closer the value of  $D$  is to the maximal value, which is 1, the more different the allele frequencies of the two populations are. The larger the genetic difference between populations is, the less effective the gene flow is. According to the obtained results it can be stated that the genetic distance of populations from each other was the largest in case of the populations no. 6 and 7, so these two populations could be clearly distinguished from the others. This statement is confirmed by the results of genetic identity analyses as well. In case of populations 1, 2, 3, 4 and 5 Nei's genetic distance showed low values, therefore allele frequencies can be considered as the same in case of these populations.

On the basis of Nei's genetic distance calculated from microsatellite results phylogenetic tree was drawn using the UPGMA method. The drawn phylogenetic tree did not show unambiguous relationship between the geographical location of sample collection and the genetic distance between the samples. Samples originating from the Carpathian Basin that were the core of the present research work could be classified into several groups (clades). In contrast, the population no. 7 (Portuguese samples) could be separated clearly from any other groups of different geographical origin.

The software BAPS determined the highest likelihood of grouping of individuals within a population using the Bayesian method. This software determines the most likely (posterior likelihood) number ( $k$  – number of clusters) of populations from the data of molecular markers (e.g. DNA sequences, microsatellite fragment length) and it provides reference to the group membership of each individual. Samples collected from 29 different locations were classified into 16 different groups by the BAPS analysis. The probability of the separation of these 16 groups was very high (0.9131). Some groups of distant geographical origin (e.g. Greece, Portugal) could be clearly distinguished. According to the results the *Cryphonectria parasitica* population of the Carpathian Basin could be separated to several groups. There was no relationship found between the similarity of populations and geographical locations. The similarity of populations of more distant geographical origin can

be explained by the human contribution to the spread of the disease, e.g. its introduction with propagation material or young plants.

#### 4. NEW SCIENTIFIC RESULTS OF THE DISSERTATION

1. In the vegetative compatibility analyses of *Cryphonectria parasitica* isolates from chestnut production regions in the Carpathian Basin the tester strains EU-12 and EU-13 could be detected in the 4 sampling fields around Serednje. These two VC groups could be detected in the samples from the surroundings of Bobovyshe and from the forests near Rostov'yatisya as well. According to the previous respective literature data only the compatibility group EU-12 could be detected in these regions before. In case of the production region of Tăuții-Măgherăuș beside Baia Mare the vegetative compatibility group EU-13 could be also detected beside the presence of the group EU-12.
2. Using *tefl* sequences of chestnut blight samples analysed in the present research work we attempted to determine the genetic distance between the populations of different production regions. The phylogenetic tree drawn from the obtained results has clearly confirmed that the *tefl* genetic marker is not suitable for the exact determination of relative genetic differentiation of chestnut blight *Cryphonectria parasitica* phytopathogen fungus species populations of different geographical origin and for the separation of populations. *Tefl* sequences of 63 *Cryphonectria parasitica* isolates have been uploaded into the database of the molecular gene bank (GenBank) used in this present research as well.
3. Similar to the *tefl* genetic marker, comparative analyses was executed with ITS sequences as well. ITS sequences of species of the genera *Cryphonectria*, *Endothiella* and *Endothia*, beside those of *Cryphonectria parasitica* downloaded from the database of the molecular gene bank (GenBank) were involved into the analyses of sequences. According to the drawn phylogenetic tree based on the obtained results confirmed that the ITS genetic marker is applicable to separate the members of the genera *Cryphonectria* just as *Endothiella* and *Endothia*, but it doesn't provide exact and reliable results for the determination of relative genetic differentiation of chestnut blight *Cryphonectria parasitica* phytopathogen fungus species populations of different

geographical origin and for the separation of populations. After the analyses ITS sequences of 85 *Cryphonectria parasitica* isolates have been successfully uploaded into the database of the molecular gene bank (GenBank) used in this present research work as well.

4. Among the first 6 microsatellites from the genome of the fungus species *Cryphonectria parasitica* have been used in order to determine the genetic distance between populations of different production regions. In contrast to the analyses using the genetic markers *tefl* and ITS, the basis of this comparison was the fragment length provided by gel electrophoresis. Thus the analysis of microsatellites seems to be the most suitable method – among the three genetic markers analysed in the present research work – for the separation of populations within the species *Cryphonectria parasitica* on genetic basis.
  
5. Damage of the phytopathogen chestnut blight fungus *Cryphonectria parasitica* was detected for the first time on sessile oak host plants by analysing the samples collected in the forest located in the surroundings of the settlement Bobovyshche, Ukraine. The presence of the pathogen was analysed by classical mycological methods first, using growing of cultures on special medium then by the visual analysis of cultures and conidia. After that the pathogen was identified using molecular biology analyses. For this purpose the ITS gene marker was used. During the nucleotide BLAST analysis of the resulted sequences the analysed sequence proved to be 100% homologous with the pathogen *Cryphonectria parasitica*.

## 5. RESULTS APPLICABLE IN THE PRACTICE

1. Beside the strain EU-12 the presence of the vegetative compatibility group EU-13 could be verified in the vegetative compatibility studies in case of samples collected near to the Ukrainian Serednje, Bobovyshche and Rostov'yatisya, just as from the production area of Tăuții-Măgherăuș near to Baia Mare in Romania. These results may promote the effective use of hypovirulent strains within the confines of biological plant protection. Vaccines compatible with the VC groups mentioned above shall be applied in potential treatments. Repeating previous studied it has become obvious that VC groups other than those that were previously present in the given territory can turn up in a short time; therefore the continuous monitoring of populations is of high importance.
2. By the executed phylogenetic analyses sequences were obtained and uploaded to the GenBank database, thus made public, the sequence orders can be downloaded. Therefore if the conventional phytopathogenic identification of a pathogen would be hindered, these results can be used as basis for comparison for molecular biology, just as ITS and *tefl* marker analyses. Data obtained by molecular biology analyses extend the basic research of the pathogen, as well as they can be used in both further research work and field plant protection measurements.
3. The phylogenetic tree obtained by the analysis of microsatellites in order to execute the molecular biology characterization of the pathogen described the genetic distance of samples from the Carpathian Basin and the Balkans adequately. Results confirmed that the studied populations were in close relationship, which can support the determination of spreading direction of the pathogen. In order to execute coordinated protection measurements against the pathogen the study of the details of the spreading pathogen, just as its direction is of basic importance.

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## 7. LIST OF PUBLICATIONS



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Candidate: Gábor Görcsös

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Doctoral School: Hankóczy Jenő Doctoral School of Crop Production, Horticulture and Food Sciences

### List of publications related to the dissertation

#### Foreign language scientific article(s) in Hungarian journal(s) (1)

1. **Görcsös, G.**, Irinyi, L., Radócz, L., Tarcali, G., Sándor, E.: Diversity of *Cryphonectria parasitica* populations from the Carpathian Basin.  
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