Acta Microbiologica et Immunologica Hungarica, 62 (3), pp. 247–266 (2015)
DOI: 10.1556/030.62.2015.3.3

DIVERSITY OF *CRYPHONECTRIA PARASITICA* POPULATIONS FROM THE CARPATHIAN BASIN

GÁBOR GÖRCSÖS[1], LÁSZLÓ IRINYI[1‡], LÁSZLÓ RADÓCZ[1], GÁBOR TARCALI[1] and ERZSÉBET SÁNDOR[2]*

[1]Faculty of Agricultural and Food Sciences and Environmental Management, Institute of Plant Protection, University of Debrecen, Debrecen, Hungary
[2]Faculty of Agricultural and Food Sciences and Environmental Management Institute of Food Science, University of Debrecen, Debrecen, Hungary
[‡]Present address: Molecular Mycology Research Laboratory, Centre for Infectious Diseases and Microbiology, University of Sydney, Westmead Millennium Institute for Medical Research, Sydney, Australia

(Received: 14 January 2015; accepted: 16 April 2015)

The ascomycetous fungus *Cryphonectria parasitica* (Murr.) is one of the most important fungal pathogens of chestnut (*Castanea sativa* Mill.), causing chestnut blight. It is originated from Eastern Asia and was introduced into North America, and later into Europe. Almost 100 *C. parasitica* isolates were collected mainly from the Carpathian Basin to study their diversity and phylogenetic relationships. Three nuclear molecular markers were tested: (i) internal transcribed spacers (ITS), (ii) translation elongation factor 1 subunit alpha *(tef1),* and (iii) six microsatellite loci. The ITS region proved to be highly polymorphic but this kind of variability did not reflect the geographical regions of the isolates, while the *tef1* sequences were identical in all isolates. Microsatellite haplotype diversity was relatively high (0.923–0.984) calculated for the fungal populations of the Carpathian Basin. This may have resulted from the multiple introductions from diverse origins of the populations, close to the first site of observation in Northern Italy. The BAPS analysis of the microsatellite markers occasionally grouped together samples from remote geographical origin, suggesting human assistance in the introduction of new *C. parasitica* haplotypes from distinct areas.

Keywords: *Cryphonectria parasitica,* population structure, phylogenic markers, microsatellite, BAPS analysis

Introduction

The causal agent of chestnut blight, a heterothallic ascomycetous fungus, *Cryphonectria parasitica* (Murr.) Barr (syn. *Endothia parasitica* [Murr.] And.) is

*Corresponding author; E-mail: karaffa@agr.unideb.hu

one of the most important fungal pathogens of chestnut (*Castanea sativa* Mill.) in Europe and North America. It originated from Eastern Asia and was introduced into North America at the beginning of the 20th century where it devastated the American chestnut (*Castanea dentata,* Fagaceae, Fagales) causing serious damage in orchards and in forests [1]. In the middle of the last century, the pathogen was introduced into Europe, firstly reported in Italy [2] and has infested the European chestnut (*Castanea sativa* Mill) populations. In Central Europe, the pathogen was firstly identified in Austria by Donaubauer [3]. In Hungary, the first symptoms of the disease were reported by Körtvély in 1969 [4], then in Slovakia by Juhásová [5], in Romania by Florea and Popa [6] and in Ukraine by Radócz [7]. Nowadays the chestnut blight became an important and widespread disease in Hungary. The disease kills the infected tree branches and causes a rapid death of the entire tree resulting in environmental and economic concerns.

In this study, we investigated phylogenetic relationships of *C. parasitica* isolates collected from different European growing sites mainly from the Carpathian Basin.

Many studies using different molecular markers and other traits such as vegetative compatibility were published to assess population genetic parameters and genetic diversity among isolates from different geographical regions [8–15]. Translation elongation factor 1 subunit alpha (EF1α) 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 [16]. Its coding gene, *tef1* is well suited for determining phylogenetic relationships [17], due to its universal occurrence and its presence as a single copy within the genome [18]. 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 used a primer pair which facilitates the PCR amplification of the large intron of *tef1* gene [19]. So far, the translation elongation factor has not been used to investigate phylogenetic relationships among *C. parasitica*.

Ribosomal DNA (rDNA) has long been used as a potential marker for phylogenetic studies (reviewed by Avise, [20]). The rRNA genes are organized in clusters of tandemly repeated units, each of which consists of coding regions (18S, 5.8S, and 28S) [21] and 2 internal transcribed spacers (ITS) and intergenic spacer (formerly called the "Non-Transcibed Spacer", NTS) region. While the coding regions are evolutionarily conserved and have been utilized for phylogenetic inferences for major phyla (reviewed by Hillis and Dixon [22]), the two 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 [23–29]. In this study, we used the complete inter-

nal transcribed spacer regions since many studies were published using ITS sequences for phylogenetic analysis in the *Cryphonectria* genus [30, 31].

Microsatellite loci, also called simple sequence repeats (SSR), are widely used as genetic markers because of their ubiquity, ease to score, co-dominance, reproducibility, assumed neutrality and high level of polymorphism [32]. Microsatellites are tandemly repeated specific DNA sequences of 1 to 10 bases up to about 100 times [33]. The lengths of microsatellites tend to be highly polymorphic between individuals due to variation in the number of repeat units of different alleles. Like all genetic loci, SSRs are subject to point mutations but slipped-strand mispairing at meiosis or during DNA replication also may occur and can change the number of repeat units [34, 35]. Microsatellites are powerful markers for genetics and population biology analyses because they have co-dominant alleles and are amplified by specific primers. They are more polymorphic than other amplifiable markers [36]. Their mutation rates are thought to vary from 10^{6} to 10^{3} per locus per gamete per generation depending on organism and locus [37, 38]. Microsatellites have been evaluated extensively in many fields of biology including the population genetic studies of *C. parasitica*. Breuillin et al. [10] identified high genetic diversity in *C. parasitica* populations of different geographical regions of France using microsatellite loci. Kubisiak et al. [39] reported 53 polymorphic microsatellite loci for population genetic studies of *C. parasitica*. Other studies used different molecular methods such as DNA fingerprinting and RFLP for genetic population studies mostly to retrace the *C. parasitica*'s history of invasion into North America and Europe during the twentieth century [9, 40, 41].

Materials and Methods

Isolates

Almost 100 *C. parasitica* isolates were studied from different parts of European countries but mainly from the Middle-Europe and the Carpathian Basin (Table I and Fig. 1). Bark samples were collected from chestnut trees located in geographically different areas. In each site, bark samples were collected from the margin of cankers from at least 10 evenly distributed, blighted trunks. Only one bark sample was collected from each trunk to avoid sampling clones. Individual samples were surface disinfected in 70% ethanol, rinsed in sterile water then placed on PDA (40 g potato dextrose agar, Scharlau, in 1 l distilled water). Plates were incubated at 24 °C in the dark for 7 days and all cultures were checked for typical *C. parasitica* characteristics.

Table I. Origin of *Cryphonectria parasitica* isolates

Sample name[1]	Location[2]	Country	Collection date[2]	Population ID[3]	BAPS Group[4]	Accession number of	
						tef1	ITS
AGF 2	Ágfalva	Hungary	9 May 2012	1	1		KC844291
AGF 4	Ágfalva	Hungary	9 May 2012	1	1		KC844292
DOBR 1	Döbröce	Hungary	10 May 2012	1	1	KC879169	KC844303
DOBR 3	Döbröce	Hungary	10 May 2012	1	1	KC879170	KC844304
DOBR 5	Döbröce	Hungary	10 May 2012	1	1	KC879171	KC844305
IHB 1	Iharosberény	Hungary	10 May 2012	1	1		
IHB 3	Iharosberény	Hungary	10 May 2012	1	1		
KAD 1	Kadarkút	Hungary	7 August 2012	1	1	KC879176	
KAD 3	Kadarkút	Hungary	7 August 2012	1	1	KC879177	
PÉCS 1	Pécs	Hungary	22 September 2012	1	2	KC879194	KC844335
PÉCS 3	Pécs	Hungary	22 September 2012	1	2	KC879195	KC844336
BF 1	Sopron	Hungary	9 May 2012	1	1		KC844316
BF 3	Sopron	Hungary	9 May 2012	1	1		KC844317
FBR 1	Sopron	Hungary	9 May 2012	1	1		KC844310
FBR 3	Sopron	Hungary	9 May 2012	1	1		KC844311
PRINC 1	Sopron	Hungary	9 May 2012	1	1		KC844341
PRINC 3	Sopron	Hungary	9 May 2012	1	1		KC844342
ZENG 2	Zengővárkony	Hungary	22 September 2012	1	4	KC879218	
ZENG 3	Zengővárkony	Hungary	22 September 2012	1	4	KC879219	
BRAT 1	Bratislava	Slovakia	18 January 2012	1	6	KC879166	KC844300
BRAT 3	Bratislava	Slovakia	18 January 2012	1	6	KC879167	KC844301
MOD 1	Modra	Slovakia	18 January 2012	1	3		KC844324
MOD 4	Modra	Slovakia	18 January 2012	1	3	KC879183	KC844325
PAR 1	Párovské Háje	Slovakia	18 January 2012	1	9	KC879191	KC844332
PAR 3	Párovské Háje	Slovakia	18 January 2012	1	9	KC879192	KC844333
PAR 5	Párovské Háje	Slovakia	18 January 2012	1	9	KC879193	KC844334
SVE 1	Svätý Jur	Slovakia	18 January 2012	1	5		KC844347
SVE 3	Svätý Jur	Slovakia	18 January 2012	1	5		KC844348
ÉRSEK 1	Érsekvadkert	Hungary	13 May 2011	2	6	KC879173	KC844307
ÉRSEK 3	Érsekvadkert	Hungary	13 May 2011	2	6	KC879174	KC844308
B 1	Nagymaros	Hungary	13 May 2011	2	7	KC844290	KC800708

Table I. (cont.)

Sample name[1]	Location[2]	Country	Collection date[2]	Population ID[3]	BAPS Group[4]	Accession number of	
						tef1	ITS
C 1	Nagymaros	Hungary	13 May 2011	2	7	KC879168	KC844302
E 3	Nagymaros	Hungary	13 May 2011	2	7	KC879172	KC844306
F 4	Nagymaros	Hungary	13 May 2011	2	7		KC844309
J 2	Nagymaros	Hungary	13 May 2011	2	7	KC879175	KC844314
MV 1/4	Nagymaros	Hungary	13 May 2011	2	7	KC879184	KC851950
MV 1/6	Nagymaros	Hungary	13 May 2011	2	7	KC879185	KC851951
N 2	Nagymaros	Hungary	13 May 2011	2	7	KC879186	KC844315
KRNA 1	Krná	Slovakia	3 August 2012	2	8		
KRNA 5	Krná	Slovakia	3 August 2012	2	8		
MDK 1	Modrý Kameň	Slovakia	8 December 2011	2	8		KC844320
MDK 2	Modrý Kameň	Slovakia	8 December 2011	2	8		KC844321
MDK 4	Modrý Kameň	Slovakia	8 December 2011	2	8	KC879180	KC844322
MDK 6	Modrý Kameň	Slovakia	8 December 2011	2	8		KC844323
PAL 2	Pálháza	Hungary	18 May 2012	3	1		KC844330
PAL 3	Pálháza	Hungary	18 May 2012	3	1		KC844331
PETROVCE 1	Petrovce	Slovakia	19 September 2012	3	9	KC879198	KC844339
PETROVCE 3	Petrovce	Slovakia	19 September 2012	3	9	KC879199	KC844340
BOB 1–1	Bobovyshche	Ukraine	23 April 2011	3	10	KC851936	KC844294
BOB 1–3	Bobovyshche	Ukraine	23 April 2011	3	10	KC879161	KC844295
BOB 2–2T	Bobovyshche	Ukraine	23 April 2011	3	10	KC879162	KC844296
BOB 2–4T	Bobovyshche	Ukraine	23 April 2011	3	10	KC879163	KC844297
BOB 3–1	Bobovyshche	Ukraine	23 April 2011	3	10	KC879164	KC844298
BOB 3–3	Bobovyshche	Ukraine	23 April 2011	3	10	KC879165	KC844299
RO 4	Rostovjatica	Ukraine	23 April 2011	3	14	KC879203	KC844345
RO 6	Rostovjatica	Ukraine	23 April 2011	3	14	KC879204	KC844346
SER 1	Serednje	Ukraine	23 April 2011	3	11	KC879220	
SER 3	Serednje	Ukraine	23 April 2011	3	11	KC879221	
FEL 1T	Nagybánya	Romania	7 September 2011	4	12		KC844312
FEL 4	Nagybánya	Romania	7 September 2011	4	12		KC844313
KOBA 2	Nagybánya	Romania	7 September 2011	4	12	KC879178	KC844318
KOBA 4T	Nagybánya	Romania	7 September 2011	4	12	KC879179	KC844319

Table I. (cont.)

Sample name[1]	Location[2]	Country	Collection date[2]	Population ID[3]	BAPS Group[4]	Accession number of	
						tef1	ITS
TG 1	Nagybánya	Romania	7 September 2011	4	12	KC879207	KC844351
TG 2	Nagybánya	Romania	7 September 2011	4	12	KC879208	KC844352
TG 4T	Nagybánya	Romania	7 September 2011	4	12	KC879209	KC844353
VEVI 1	Nagybánya	Romania	7 September 2011	4	12	KC879212	KC844356
VEVI 2	Nagybánya	Romania	7 September 2011	4	12	KC879213	KC844357
TIS 3	Tismana	Romania	2 September 2012	4	2	KC879210	KC844354
TIS 5	Tismana	Romania	2 September 2012	4	2	KC879211	KC844355
PET 1	Petrich	Bulgaria	5 October 2011	5	14	KC879196	KC844337
PET 3	Petrich	Bulgaria	5 October 2011	5	14	KC879197	KC844338
OSOJ 2	Osoj	Macedonia	4 October 2011	5	13	KC879187	KC844328
OSOJ 7	Osoj	Macedonia	4 October 2011	5	13		KC844329
RAD 1	Radolishta	Macedonia	5 October 2011	5	12	KC879201	KC844343
RAD 5	Radolishta	Macedonia	5 October 2011	5	12	KC879202	KC844344
TET 3	Tetovo	Macedonia	4 October 2011	5	12	KC879205	KC844349
TET 9	Tetovo	Macedonia	4 October 2011	5	12	KC879206	KC844350
VOL 1	Volino	Macedonia	4 October 2011	5	12	KC879214	KC844326
VOL 4	Volino	Macedonia	4 October 2011	5	12	KC879215	KC844327
VRUT 1	Vrutok	Macedonia	4 October 2011	5	12	KC879216	KC844358
VRUT 3	Vrutok	Macedonia	4 October 2011	5	12	KC879217	KC844359
ARK 14	n.a.	Greece	n.a.	6	15		KC844293
HOR 10 hpv	n.a.	Greece	n.a.	6	15		KC851945
KAV 5 hpv	n.a.	Greece	n.a.	6	15		KC851944
ME48–1	n.a.	Greece	n.a.	6	15	KC879181	KC851948
ME48–2	n.a.	Greece	n.a.	6	15	KC879182	KC851949
P 73	n.a.	Greece	n.a.	6	15	KC879190	KC851943
P5–1	n.a.	Greece	n.a.	6	15	KC879188	KC851946
P5–2	n.a.	Greece	n.a.	6	15	KC879189	KC851947
PIR 1	n.a.	Greece	n.a.	6	15	KC879200	KC851942

Table I. (cont.)

Sample name[1]	Location[2]	Country	Collection date[2]	Population ID[3]	BAPS Group[4]	Accession number of	
						tef1	ITS
C0006	Alentejo	Portugal	n.a.	7	16		KC851938
C0009	Madeira	Portugal	n.a.	7	16		KC851940
C0008	Terceira	Portugal	n.a.	7	16		KC851939
C0722	Terceira	Portugal	n.a.	7	16		KC851941
C0003	Trás os Montes	Portugal	n.a.	7	16		KC851937

[1]Host of the isolates were *Castanea sativa* except the ones with a "T" ending (BOB 2–2T, BOB 2–4T, TG 4T, FEL 1T, KOBA 4T). Those were isolated from *Quercus petraea.*
[2]n.a.: not available.
[3]Number of population: geographical locations are indicated in Fig. 1.
[4]BAPS Group: according to the results of the BAPS analysis.

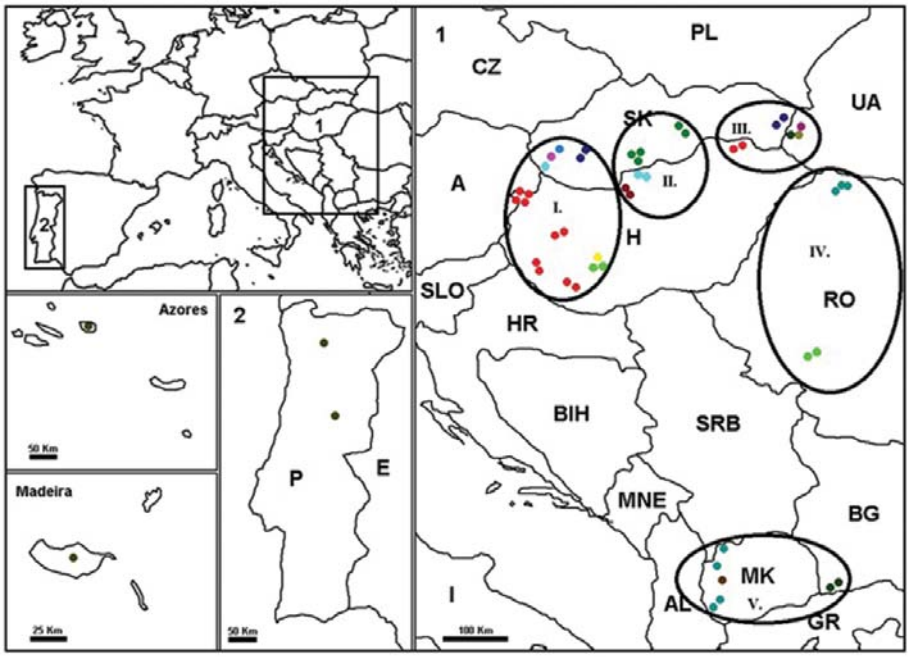

Figure 1. Geographical location of the *Cryphonectria parasitica* populations sampled in the Carpathian Basin and in Europe. Each isolate is represented by one dot. I–V indicates population numbers of the isolates (Table I). The colours of the dots correspond with the result of BAPS analysis

Culture morphology and growth

Isolates were grown on PDA (40 g l^{-1}, Scharlab) in 90 mm diameter Petri dishes 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 four weeks.

DNA extraction

For molecular works, the cultures were grown in 100 ml of malt broth (MB, containing 2% malt extract, Scharlab) 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 50ml 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 and sequencing of ITS and tef1 sequences

Amplifications of 50 µl PCR reaction containing 25 µl 2 × PCR Master Mix (ImmoMix, Bioline, 25020), 40–40 pmol of each primer, 20–40 ng of genomic DNA and nuclease free water were run. SR6R and LR1 primer pair [42] (Integrated DNA Technologies, Inc.) were used to amplify the full length of ITS region, 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 of the *tef1* gene was amplified by the EF1-728F and EF1-986R primer pair [19] 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 DNA sequences obtained were aligned first with ClustalX [43] automatically then manually checked for ambiguities and adjusted when necessary using GeneDoc [44]. Single gaps were treated as missing data and multistate characters as uncertain. In the phylogenetic analyses, *tef1* and ITS fragments of other *C. parasitica* isolates downloaded from GenBank (NCBI) were also included. Parsimony analyses [45–47] were performed using PAUP 4.0 [48] and consisted of heuristic searches with 1000 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 1000 bootstrap replications. Phylogenetic trees were drawn by TreeView [49].

Microsatellite amplification and analysis

In this study, six microsatellites (Table II) were selected as previously described by Breuillin et al. [10] and Kubisiak et al. [39]. Amplifications were carried out in 50 µl reactions, which 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. Microsatellites were amplified in a Primus (MWG Biotech) thermocycler using the following thermal profile: 3 min initial

Table II. List of microsatellite loci with primer sequences and annealing temperatures used for the amplification

Locus	Primers	Annealing temperature
CpSI085	Forward 5' AGGCCTGCTTCTTTTTGGAT 3' Reverse 5' CGGGTCTATATGGTGGCTTC 3'	55 °C
CpSI102	Forward 5' GCTCCGAGGACTTTGATGAG 3' Reverse 5' TCATCACCACCAACACCATT 3'	55 °C
CpSI108	Forward 5' CGGAACTACCTGCTCTTTGC 3' Reverse 5' GCGATCCGCATTCCTGTAT 3'	56 °C
CPG6	Forward 5' ATCATCACGACGCAATGGTA 3' Reverse 5' TCCGGGCATTCAGCAMAT 3'	53 °C
CPE5	Forward 5' TGTCAACAACGGTCAACACC 3' Reverse 5' CGGAGAGGAGAACTCTGAGAC 3'	56 °C
CpSI014	Forward 5' TCGGAGGCTTTATTGTCGTT 3' Reverse 5' TGGGTGTATTTGCTCGGTAA 3'	53 °C

denaturing at 95 °C, followed by 5 cycles of 1 min at 95 °C, 1 min annealing (Table II) 1 min at 53–56 °C (depending on the primer sequences) and 25 cycles of 1 min at 90 °C, 1 min annealing at 53–56 °C, 1 min at 72 °C and 15 min final extension at 72 °C. Fragment analyses were completed by Origins electrophoresis system produced by Elchrom Scientific AG (Switzerland). Each PCR product was analyzed on Spreadex EL 500 Gel (Elchrom Scientific AG, Switzerland), run at 55 °C for 240 min in a 1 X TAE buffer. The gel was stained with EtBr (Sigma, USA) for 45 min, and photographed under UV light (k = 250 nm) using Bio-Rad gel documentation system.

Microsatellite fragment length analysis

The Popgene version 1.31 software [50] was used to compute basic population parameters such as "observed number of alleles" (n_a), "effective number of alleles" (n_e), and "Nei's gene diversity" (h) in the total sample and in each population. Nei's [51] genetic identity is the normalized identity of genes between two populations and varies between 0 (the compared populations are different), and 1 (the compared populations are identical). Multilocus 1.3b was used to calculate the number of different multilocus genotypes (MLG) and the standardized version of the index of association r_D [52]. The r_D index was calculated to test for genetic recombination. This index is a measure of multilocus linkage disequilibrium, which gives information on whether two different individuals sharing the same allele at one locus are more likely to share an allele at another locus. The null hypothesis of complete panmixia ($r_D = 0$) was tested by comparing the observed data set to 100 randomized data sets in which infinite recombination was simulated upon randomly shuffling the alleles among individuals, independently for each locus.

Population differentiation was tested by comparing allele frequencies among the six populations using Weir and Cockerham's θ (F_{st}) value [53]. The θ-value was estimated under the null hypothesis of non-differentiation among subpopulations, when θ = 0. Statistical analysis was performed by comparing the calculated θ-values to those of data sets in which the isolates have been randomized across populations 10,000 times using the Multilocus 1.3b (Multi Locus Sequence Typing) [52]. Gene flow (N_m – equivalent to the number of migrants per generation) was determined using the equation $N_m = 0.5(1 - \theta)/ \theta$ [54].

The structure of the populations were analyzed with BAPS 6.16 (Bayesian Analysis of Population Structure) program (http://www.helsinki.fi/bsg/software/BAPS/), applying Bayesian approach. This program was used to determine the most probable number of groups of *C. parasitica* isolates, based on posterior

probability, and to define the group for each isolate [55, 56]. The most probable cluster number was tested in 10 repetitions between 1 and 50 values. The allele frequencies of the isolates were tested on the defined cluster number with 100 repetitions.

Results

Phylogenetic analyses of tef1 and ITS sequences

PCR amplifications resulted in single fragments for both *tef1* (~350 bp) and ITS region (~560 bp). No size variation was observed among the amplified *tef1* and ITS fragments of the different isolates. The parsimony analysis of the ITS fragment revealed 299 constant sites, with 172 parsimony-informative sites, and 76 parsimony-uninformative sites among all isolates. The *C. parasitica* was clearly separated from other species in the *Cryphonectriaceae* family and the isolates were grouped in several clades which indicated the high variability in the ITS region (Fig. 2). However, no correlation was found between the clades and the geographical regions. The isolates of the same regions were not grouped in the same clade. Moreover, isolates with different geographical origins were randomly mixed in the same clade (Fig. 2, Table I). The ITS sequences downloaded from the GenBank were also found in different clades, sometimes together with isolates from Hungary. The robustness of the tree was supported by relatively high bootstrap values. *Tef1* sequences were edited to 320 bp for the alignment. The *tef1* sequences of all studied isolates proved to be perfectly identical, including the two *tef1* sequences from South Africa (AY308953, AY308954). No sequence variation was found in the *tef1* sequences of *C. parasitica* isolates collected from Europe.

Microsatellite analysis

Microsatellite fragment length analysis included 82 samples collected from the Carpathian Basin, especially from southern Romania, Macedonia and Bulgaria. Further 14 isolates were provided by different European laboratories. Six microsatellites of a total of 96 isolates were amplified and their lengths were determined. Seven different populations with similar geographical origin were grouped together for further analysis. Isolates from the Carpathian Basin were divided into four populations: (I) Western, (II) Central-Northern, (III) North-Eastern, and (IV) Eastern (Fig. 1). All populations contained more than one mul-

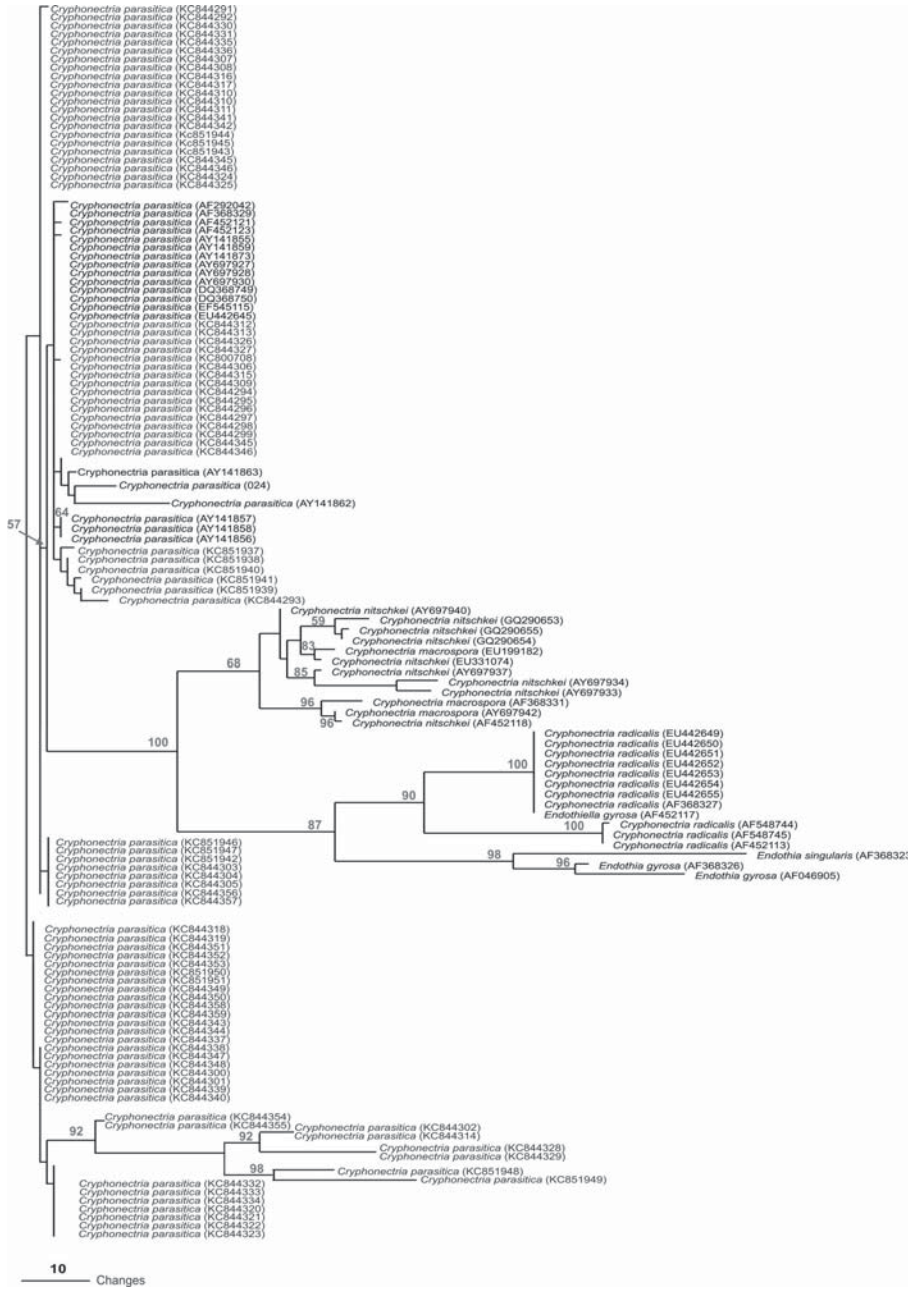

Figure 2. One of the most parsimonious trees of ITS sequences of *Cryphonectria parasitica* isolates

Table III. Genetic diversity indexes of *Cryphonectria parasitica* populations

Population[a]	n[b]	n_a[c]	n_e[d]	h[e]	No. of distinct MLG[f]	Max. no. of repeated MLG[g]	Genotypic diversity	r_D[h]
1	28	4.00 (1.55)	2.38 (0.88)	0.52 (0.20)	23	3	0.984	0.073**
2	16	3.33 (1.63)	2.58 (1.40)	0.49 (0.29)	13	2	0.975	0.134**
3	14	3.50 (0.84)	2.92 (0.94)	0.61 (0.19)	8	3	0.923	0.130*
4	11	3.00 (0.63)	2.29 (0.38)	0.55 (0.07)	9	2	0.963	0.085*
5	12	2.66 (0.81)	2.00 (0.83)	0.44 (0.17)	9	2	0.954	0.067
6	9	2.00 (0.63)	1.50 (0.19)	0.29 (0.20)	7	2	0.944	0
7	5	1.50 (0.83)	1.37 (0.58)	0.17 (0.27)	4	2	0.900	0
Total	95	5.67 (1.63)	3.25 (0.83)	0.67 (0.12)	71	2	0.999	0.038**

[a] Population numbers according to Fig. 1.
[b]Sample size.
[c]Observed number of alleles.
[d]Effective number of alleles.
[e]Nei's gene diversity.
[f]Number of distinct multilocus genotypes.
[g]Maximum number of repeated multilocus genotypes.
[h]Standardized index of association (clone corrected).
Standard deviation between brackets.
*$p<0.01$
**$p<0.001$

tilocus genotype (MLG) (Table III). The studied alleles proved to be suitable to measure genetic diversity among the isolates since the genetic diversity did not change significantly including more than three alleles in the analysis (data not shown). Fragment size analysis of the six microsatellite loci produced 71 haplotypes among 95 isolates (Table III). The effective number of alleles (n_e: 2.00–2.58) and Nei's gene diversity (h: 0.44–0.61) were similar in the populations with more than 10 isolates (Table III).

The genotypic diversity calculated both for the total samples (0.999) and for the individual populations (0.900–0.984) was similar, independently of the population size (Table III).

Table IV. F statistics of *Cryphonectria parasitica* populations. Weir and Cockerham's θ (F_{st}) (rows), and N_m (columns) were calculated with Multilocus 1.3b; $N_m = 0.5 (1 – θ)/θ$. Light grey background indicates moderate (θ: 0.05–0.15), dark grey big (θ: 0.15–0.25), black very big (θ: > 0.25) differences between populations

N_m / θ	1. population	2. population	3. population	4. population	5. population	6. population	7. population
1. population		0.242**	0.124**	0.207*	**0.293***	**0.282****	**0.387****
2. population	0.156		0.167**	0.263**	**0.403****	0.238	**0.341**
3. population	0.353	0.249		0.130	**0.263**	0.185	**0.350**
4. population	0.191	0.140	0.334		0.067	0.207	**0.444**
5. population	0.120	0.074	0.140	0.696		**0.336**	**0.561**
6. population	0.127	0.160	0.220	0.191	0.090		**0.619**
7. population	0.028	0.096	0.092	0.062	0.039	0.030	

*p<0.01
**p<0.001

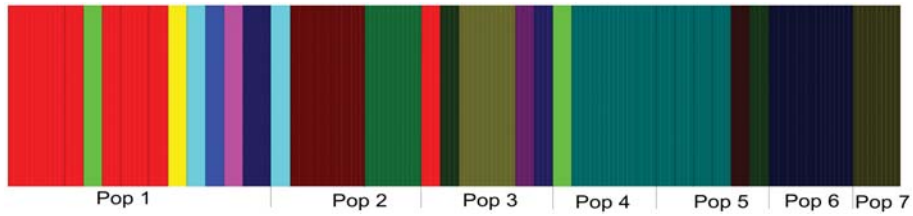

Figure 3. Bayesian assignment of individuals into different clusters assigned by BAPS program (http://www.helsinki.fi/bsg/software/BAPS/). The bar-plot with different colours shows the assignment of individuals into different clusters

The clone-corrected standardized index of association (r_D), computed on the total sample was significantly different from zero ($r_D = 0.038$, $p < 0.01$, Table III), indicating genetic differentiation between populations. The estimate of linkage disequilibrium varied between 0.073 and 0.134 at $p < 0.01$. This may suggest the possible differentiation within the populations (Table III).

The F statistics (Weir and Cockerham's θ), and gene flow (N_m values) indicated very high (θ: >0.25), high (θ: 0.15–0.25) and moderate (θ: 0.05–0.15) differentiation between populations with significant differences in populations with many isolates (Table IV). *C. parasitica* isolates from Portugal (population 7) markedly differed from all the other isolates from the Balkans and Central Europe. However, only a moderate difference (θ: 0.124–0.167) could be observed between populations from the northern part (populations 2 and 3) or eastern part (populations 3 and 4) of the Carpathian Basin. Similar moderate differences were

also calculated between geographically distinct populations (populations 4 and 6, Fig. 1).

The BAPS analysis created 16 groups with 0.9131 probability (Fig. 3) based on the microsatellite diversity of the samples originated from twenty-three different sampling sites. Samples from Portugal and Greece markedly separated from other isolates. However, *C. parasitica* samples from the Carpathian Basin formed several groups, but there was no connection between the groups and the geographical regions (Fig. 1). Occasionally samples from remote geographical origin grouped together (in populations I and III, I and IV, IV and V). However, samples from different hosts (*Castanea sativa* and *Quercus petraea*) always grouped together on the same sampling sites (Table I and Figs 1 and 3).

Discussion

The nuclear ribosomal DNA has long been used as a potential genetic marker for phylogenetic studies [20, 57]. In many fungal taxonomy studies, the ITS region was suitable for resolving relationships at genus and species level [58–64]. In this study, ITS sequences were used to estimate the phylogenetic relationships at intraspecies level among *C. parasitica* isolates collected from different geographical regions of Europe. The ITS region proved to be highly polymorphic but this kind of variability did not reflect the geographical regions of the isolates. The translation elongation factor coding gene proved to be a useful gene to resolve phylogenetic relationships at species level, as well as in deeper divergences in many fungi [17, 19, 65]. However, in this study, the *tef1* sequences were identical in all isolates supporting that these sequences remained conservative within *C. parasitica* species. The *tef1* sequence was not suitable for investigating phylogenetic relationships within *C. parasitica* isolates collected from different regions of Europe.

The diversity of *C. parasitica* populations has been investigated extensively. It was reported to be higher in its native range, in eastern Asia, than in recently established populations in Europe and North America [66]. High vegetative compatibility diversity was found in northern Italy, where chestnut blight was first reported in Europe in 1938 [67], moreover in the neighbouring southern part of Switzerland [68], in Bosnia-Herzegovina [69] and in the southern part of France [70]. However lower diversity was observed in the newly infected areas e.g. in Germany [71], Portugal and northern Switzerland [8]. The diversity of the *C. parasitica* populations in the Carpathian Basin populations ($h = 0.49–0.61$) was higher, than calculated for the populations in North America ($H_e = 0.41$), but lower than in Asia ($H_e = 0.73$) [72]. Microsatellite haplotypes were found to be

highly diverse in Hungary [7], France [10], Slovenia and Croatia [73] which corresponds well with the relatively high diversity of vegetative compatibility type in that area. This may have resulted from the multiple introductions from diverse origins of the European populations, close to the first site of observation in Northern Italy [66]. We calculated moderate differences between geographically distinct populations of Romania and Greece. Moreover the BAPS analysis occasionally grouped together samples from remote geographical origin, suggesting human assistance in the introduction of new *C. parasitica* haplotypes from distinct areas (e.g. by infected propagation materials).

Acknowledgement

E. Sándor was supported by the "Research Grant" of the University of Debrecen.

Conflict of Interest

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

References

1. Anagnostakis, S. L.: Chestnut blight: The classical problem of an introduced pathogen. Mycologia **79,** 23–37 (1987).
2. Braghi, A.: Il cranco del castagno causato da *Endothia parasitica.* Ital Agric **7,** 406–412 (1946).
3. Donaubauer, E.: Unterschungen über den die variation der Krankheitsanfälligkeit verschiedener Pappeln. Mitt. FBVA. Brunn. 1964, pp. 70–120.
4. Körtvély, A.: A gesztenye endotiás kéregelhalása. Növényvédelem **6,** 358–361 (1969).
5. Juhásová, G.: A summary of knowledge of fungal diseases of Spanish chestnut in Slovakia. Forestry **38,** 449–460 (1976).
6. Florea, S., Popa, I.: Diseases of edible chestnut reported in the fruit growing area of Baia Mare. In Cercetarea ştiinţifică in slujba producţiei pomicole1969–1989, Bucureşti, Romania, 1989, pp. 365–372.

7. Radócz, L.: Study of subpopulations of the chestnut blight *(Cryphonectria parasitica)* fungus in the Carpathian-Basin. For Snow Landsc Res **76,** 368–372 (2001).
8. Hoegger, P. J., Rigling, D., Holdenrieder, O., Heiniger, U.: Genetic structure of newly established populations of *Cryphonectria parasitica*. Mycol Res **104,** 1108–1116 (2000).
9. Marra, R. E., Cortesi, P., Bissegger, M., Milgroom, M. G.: Mixed mating in natural populations of the chestnut blight fungus *Cryphonectria parastica*. Heredity **93,** 189–195 (2004).
10. Breuillin, F., Dutech, C., Robin, C.: Genetic diversity of the chestnut blight fungus *Cryphonectria parasitica* in four French populations assessed by microsatellite markers. Mycol Res **110,** 288–296 (2006).
11. Milgroom, M. G., Sotirovski, K., Spica, D., Davis, J. E., Brewer, M. T., Milev, M., Cortesi, P.: Clonal population structure of the chestnut blight fungus in expanding ranges in southeastern Europe. Mol Ecol **17,** 4446–4458 (2008).
12. Prospero, S., Rigling, D.: Invasion genetics of the chestnut blight fungus *Cryphonectria parasitica* in Switzerland. Phytopathology **102,** 73–82 (2012).
13. Prospero, S., Lutz, A., Tavadze, B. A., Supatashvili, B., Rigling, D.: Discovery of a new gene pool and a high genetic diversity of the chestnut blight fungus *Cryphonectria parasitica* in Caucasian Georgia. Infect Genet Evol **20,** 131–139 (2013).
14. Jezic, M., Krstin, L., Rigling, D., Curkovic-Perica, M.: High diversity in populations of the introduced plant pathogen, *Cryphonectria parasitica*, due to encounters between genetically divergent genotypes. Mol Ecol **21,** 87–99 (2012).
15. Peters, F. S., Bußkamp, J., Prospero, S., Rigling, D., Metzler, B.: Genetic diversification of the chestnut blight fungus *Cryphonectria parasitica* and its associated hypovirus in Germany. Fungal Biol **118,** 193–210 (2014).
16. Moldave, K.: Eukaryotic protein synthesis. Annu Rev Biochem **54,** 1109–1149 (1985).
17. Roger, A. J., Sandblom, O., Doolittle, W. F., Philippe, H.: An evaluation of elongation factor 1α as a phylogenetic marker for eukaryotes. Mol Biol Evol **16,** 218–233 (1999).
18. Baldauf, S. L., Doolittle, W. F.: Origin and evolution of slime molds (Mycetozoa). Proc Natl Acad Sci U S A **94,** 12007–12012 (1997).
19. Druzhinina, I., Kubicek, C. P.: Species concepts and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species cluster? Journal of Zhejiang University Science **6,** 100–112 (2005).
20. Avise, J. C.: Molecular markers, natural history, and evolution. 2nd Edition. Sinauer Associates, Sunderland, Massachusetts, 2004, pp. 660–684.
21. Gerbi, S. A.: Evolution of ribosomal DNA. In Macintyre, R. J. (ed.): Molecular Evolutionary Genetics. Plenum, New York, 1985, pp. 419–517.
22. Hillis, D. M., Dixon, M. T.: Ribosomal DNA: Molecular evolution and phylogenetic inference. Q Rev Biol **66,** 411–453 (1991).
23. Baldwin, B. G.: Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: An example from the Compositae. Mol Phylogenet Evol **1,** 3–16 (1992).
24. Schlötterer, C., Hauser, M., von Haeseler, A., Tautz, D.: Comparative evolutionary analysis of rDNA ITS regions in *Drosophila*. Mol Biol Evol **11,** 513–522 (1994).
25. Mai, J. C., Coleman, A. W.: The internal transcribed spacer 2 exhibits a common secondary structure in green algae and flowering plants. J Mol Evol **44,** 258–271 (1997).

26. Weekers, P. H. H., Jonckheere, F. J., de, Dumont, H. J.: Phylogenetic relationships inferred from ribosomal ITS sequences and biogeographic patterns in representative of the genus *Calopteryx* (Insecta: Odonata) of the West Mediterranean and adjacent west European zone. Mol Phylogenet Evol **20,** 89–99 (2001).
27. Oliverio, M., Cervelli, M., Mariottini, P.: ITS2 rRNA evolution and its congruence with the phylogeny of muricid neogastropods (Caenogastropoda, Muricoidea). Mol Phylogenet Evol **25,** 63–69 (2002).
28. Chen, C. A., Wallace, C. C., Wolstenholme, J.: Analysis of mitochondrial 12S RNA gene supports a two-clade hypothesis of the evolutionary history of scleractinian corals. Mol Phylogenet Evol **23,** 137–149 (2002).
29. Chen, C. A., Yu, J. K., Wei, N. W.: Strategies for amplification by polymerase chain reaction of the complete sequence of nuclear large subunit ribosomal RNA-encoding gene in corals. Mar Biotech **6,** 558–570 (2000).
30. Braganca, H., Rigling, D., Diogo, E., Capelo, J., Phillips, A., Tenreiro, R.: *Cryphonectria natarciae*: A new species in the *Crpyhonectria-Endothia* complex and diagnostic molecular markers based on microsatellite-primed PCR. Fungal Biol **115,** 852–861 (2011).
31. Gryzenhout, M., Wingfield, B., Wingfield, M.: New taxonomic concepts for the important forest pathogen *Cryphonectria parasitica* and related fungi. FEMS Microbiol Lett **258,** 161–172 (2006).
32. Jarne, P., Lagoda, P. J. L.: Microsatellites, from molecules to populations and back. Trends Ecol Evol. **11,** 424–429 (1996).
33. Tautz, D.: Notes on the definition and nomenclature of tandemly repetitive DNA sequences. In Pena, S. D. J., Chakraborty, R., Epplen, J. T., Jeffreys, A. J. (eds): DNA Fingerprinting: State of Science. Birkhauser, Basel, 1993, pp. 21–28.
34. Lai, Y., Sun, F.: The relationship between microsatellite slippage mutation rate and the number of repeat units. Mol Biol Evol **20,** 2123–2131 (2003).
35. Levinson, G., Gutman, G. A.: Slipped-strand mispairing: A major mechanism for DNA sequence evolution. Mol Evol **4,** 203–221 (1987).
36. Selkoe, K. A., Toonen, R. J.: Microsatellites for ecologists: A practical guide to using and evaluating microsatellite markers. Ecol Lett **9,** 615–629 (2006).
37. Weber, J. L., Wong, C.: Mutation of human short tandem repeats. Hum Mol Genet **2,** 1123–1128 (1993).
38. Schug, M. D., Mackay, T. F. C., Aquadro, C. F.: Low mutation rates of microsatellite loci in *Drosophila melanogaster*. Nature Genet **15,** 99–102 (1997).
39. Kubisiak, T. L., Dutech, C., Milgroom, M. G.: Fifty-three polymorphic microsatellite loci in the chestnut blight fungus, *Cryphonectria parasitica*. Mol Ecol Notes **7,** 428–432 (2007).
40. Milgroom, M. G., Lipari, S. E., Powell, W. A.: DNA fingerprinting and analysis of population-structure in the Chestnut blight fungus, *Cryphonectria parasitica*. Genetics **131,** 297–306 (1992).
41. Milgroom, M. G., Wang, K. R., Zhou, Y., Lipari, S. E., Kaneko, S.: Intercontinental population structure of the chestnut blight fungus, *Cryphonectria parasitica*. Mycologia **88,** 179–190 (1996).
42. Vilgalys, R., Hester, M.: Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J Bacteriol **20,** 4238–4246 (1990).

43. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., Higgins, D. G.: The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res **24,** 4876–4882 (1997).
44. Nicholas, K. B., Nicholas, Jr. H. B., Deerfield, D. W. I.: GeneDoc: Analysis and visualization of genetic variation. Embnew News **4,** 14 (1997).
45. Kluge, A. G., Farris, J. S.: Quantitative phyletics and the evolution of anurans. Syst Zool **18,** 1–32 (1969).
46. Farris, J. S.: Estimating phylogenetic trees from distances matrixes. Am Nat **106,** 645–668 (1970).
47. Fitch, W. M.: Toward defining the course of evolution: Minimum change for a specific tree topology. Syst Zool **20,** 406–416 (1971).
48. Swofford, D. L.: PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4b10. Sinauer Associates, Sunderland, MA, 2002.
49. Page, R. D. M.: TREEVIEW: An application to display phylogenetic trees on personal computers. Comput Appl Biosci **12,** 357–358 (1996).
50. Yeh, F. C., Boyle, T.: Popgene: Microsoft Window-Based Freeware for Population Genetic Analysis. Version 1.31. Alberta, Canada: University of Alberta; http://www.ualberta.ca/~fyeh/popgene download.html, 1999.
51. Nei, M.: Genetic distance between populations. Amer Nat **106,** 283–292 (1972).
52. Agapow, P. M., Burt, A.: Indices of multilocus linkage disequilibrium. Mol Ecol Not **1,** 101–102 (2001).
53. Weir, B. S., Cockerham, C. C.: Estimating F-statistics for the analysis of population structure. Evolution **38,** 1358–1370 (1984).
54. McDermott, J. M., McDonald, B. A.: Gene flow in plant pathosystems. Annu Rev Phytopathol **31,** 353–373 (1993).
55. Corander, J., Waldmann, P., Sillanpää M. J.: Bayesian analysis of genetic differentiation between populations. Genetics **163,** 367–374 (2003).
56. Tang, F., Barbacioru, C., Wang, Y., Nordman, E., Lee, C., Xu, N., Wang, X., Bodeau, J., Tuch, B. B., Siddiqui, A., Lao, K., Surani, M. A.: mRNA-Seq whole-transcriptome analysis of a single cell. Nat Methods **6,** 377–382 (2009).
57. White, T. J., Bruns, T., Lee, S., Taylor, J.: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In Innis, M. A., Gelfand, D. H., Sninsky, J. J., White, T. J. (eds): PCR Protocols. A Guide to Methods and Applications. Academic Press, Inc., New York, 1990, pp. 315–322.
58. Gottlieb, A. M., Lichtwardt, R. W.: Molecular variation within and among species of Harpellales. Mycologia **93,** 66–81 (2001).
59. Nugent, K. G., Saville, B. J.: Forensic analysis of hallucinogenic fungi: A DNA-based approach. Forensic Sci Int **140,** 147–157 (2004).
60. Yli-Mattila, T., Mach, R. L., Alekhina, I. A., Bulat, S. A., Koskinen, S., Kullnig-Gradinger, C. M., Kubicek, C. P., Klemsdal, S. S.: Phylogenetic relationships of *Fusarium langsethiae* to *Fusarium poae* and *Fusarium sporotrichioides* as inferred by IGS, ITS, *β-tubulin* sequences and UP-PCR hybridization analysis. Int J Food Microbiol **95,** 267–285 (2004).
61. Voglmayr, H., Yule, C. M.: *Polyancora globosa* gen. sp. nov., an aeroaquatic fungus from Malaysian peat swamp forests. Mycol Res **110,** 1242–1252 (2006).

62. Morocko, I., Fatehi, J.: Molecular characterization of strawberry pathogen *Gnomonia fragariae* and its genetic relatedness to other *Gnomonia* species and members of Diaporthales. Mycol Res **111,** 603–614 (2007).
63. Padamsee, M., Matheny, P. B., Dentinger, B. T. M., McLaughlin, D.: The mushroom family Psathyrellaceae: Evidence for large-scalepolyphyly of the genus *Psathyrella*. Mol Phylogenet Evol **46,** 415–429 (2008).
64. Takamatsu, S., Hirata, T., Sato, Y., Nomura, Y.: Phylogenetic relationships of *Microsphaera* and *Erysiphe* section *Erysiphe* (powdery mildews) inferred from the rDNA ITS sequences. Mycoscience **40,** 259–268 (1999).
65. Irinyi, L., Kövics, G. J., Sándor, E.: Taxonomic re-evaluation of *Phoma*-like soybean pathogenic fungi. Mycol Res **113,** 249–260 (2009).
66. Liu, Y. C., Milgroom, M. G.: High diversity of vegetative compatibility types in *Cryphonectria parasitica* in Japan and China. Mycologia, **99,** 279–284 (2007).
67. Cortesi, P., Milgroom, M. G., Bisiach, M.: Distribution and diversity of vegetative compatibility in subpopulations of *Cryphonectria parasitica* in Italy. Mycol Res **100,** 1087–1093 (1996).
68. Heiniger, U., Rigling, D.: Biological control of chestnut blight in Europe. Annu. Rev. Phytopathol **32,** 581–599 (1994).
69. Trestic, T., Uscuplic, M., Colinas, C., Rolland, G., Giraud, A., Robin, C.: Vegetative compatibility type diversity of *Cryphonectria parasitica* populations in Bosnia-Herzegovina, Spain and France. For Snow Landsc Res **76,** 391–396 (2001).
70. Robin, C., Heiniger, U.: Chestnut blight in Europe: Diversity of *Cryphonectria parasitica,* hypovirulence and biocontrol. For Snow Landsc Res **76,** 361–367 (2001).
71. Seemann, D., Bouffier, V., Kehr, R., Wulf, A., Schröder, T., Unger, J.: Die Ess – kastanie (*Castanea sativa* Mill.) in Deutschland und ihre Gefährdung durch den Kastanienrindenkrebs (*Cryphonectria parasitica* [Murr.] Barr). Nachr.bl. Dtsch. Pflanzen – schutzd. **53,** 49–60 (2001).
72. Dutech, C., Barrès, B., Bridier, J., Robin, C., Milgroom, M. G., Ravigné, V.: The chestnut blight fungus world tour: Successive introduction events from diverse origins in an invasive plant fungal pathogen. Mol Ecol **21,** 3931–3946 (2012).
73. Jezic, M., Krstin, L., Rigling, D., Curkovic-Perica, M.: High diversity in populations of the introduced plant pathogen, *Cryphonectria parasitica,* due to encounters between genetically divergent genotypes. Mol Ecol **21,** 87–99 (2012).