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EXPERIMENTAL ARTICLES

Molecular Genetic Identification of the Phytopathogenic Fungus *Cryphonectria parasitica*

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Abstract—Mycological analysis of samples of sweet chestnut (*Castanea sativa*) collected in the northern Turkish zone of its habitat was carried out. A total of 300 strains of micromycetes belonging to 13 taxa were isolated in pure cultures. The phytopathogenic fungus *Cryphonectria parasitica*, the causal agent of chestnut blight, was identified among these strains. A technique for detection of *C. parasitica* in pure culture and host tissues using species-specific PCR markers was developed and tested. This technique can be used as an express method for detection of chestnut blight.

Key words: Cryphonectria parasitica, PCR-based diagnostics.

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The ascomycete *Cryphonectria parasitica* (Murrill) M.E. Barr 1978 (=*Endothia parasitica* (Murrill) P.J. Anderson et H.W. Anderson) is the pathogen responsible for chestnut blight (cancer or cryphonecrosis), the most harmful chestnut disease [1, 2]. The infectious agent *C. parasitica* was first reported in the United States in 1904. By the 1950s, it had virtually eliminated American chestnut (*Castanea dentata*), a forest-forming species of the eastern United States, which is considered the worst botanical catastrophe in human history [3, 4]. In Europe, where sweet chestnut (*C. sativa*) is the main host plant of this pathogen, *C. parasitica* was first discovered in Italy in 1938. Over several decades, this fungus colonized almost completely the entire chestnut habitat, i.e., Italy, France, Spain, Greece, Hungary, Austria, Belgium, Switzerland, what are now the former Yugoslavia and Soviet Union (the Black Sea coast), and Turkey [5]. At present, only scattered crops in northern Europe and Great Britain remain blight-free [5, 6]. Asian chestnut species (*C. mollissima* and *C. crenata*) are also susceptible to cryphonecrosis; however, they rarely die from this disease [7]. It is assumed that they have coevolved with this fungus and, being tolerant enough, represent a natural reservoir of the infection. In particular, it is believed that the introduction of *C. parasitica* into North America and Europe was due to the introduction of Asian chestnuts, the subsequent rapid extinction of American chestnut probably resulting from its low resistance to the fungus [4, 7].

Attempts to save sweet chestnut from this fate in its natural and restored habitats in Europe and the Caucasus, which represent an important scientific problem and practical task aimed at forest protection, have aroused considerable scientific interest in *C. parasitica*. Intraspecific polymorphism, determinants, and molecular biological mechanisms of the *C. parasitica* hypovirulence, as well as the genetic, physiological, and biochemical aspects of the host–parasite relationships, are problems that scientists around the world are struggling to solve [8–14]. At the same time, precise identification of the pathogen is required for efficient disease control, which is of particular importance at the early stages of the pathological process. Polymerase chain reaction (PCR) is presently the most sensitive technique for molecular, genetic, and clinical diagnostics. The advantages and possibilities of this technique allow us to achieve maximum sensitivity and specificity and, therefore, to detect individual fragments of genetic material reliably and precisely, as well as to identify the organism in question even in the presence of the DNA of other organisms.

The goal of this work was to develop a method for identification of *C. parasitica* in chestnut tissues by PCR that would allow specific diagnostics of chestnut infestation at any stage of the pathological process, even if no distinct symptoms of chestnut blight are observed.

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MATERIALS AND METHODS

Isolation and identification of micromycetes. Microscopic fungi were isolated from the samples of bark, sprouts, fruits, and leaves of sweet chestnut collected in 2005–2008 in the Zonguldak and Bartyn Provinces of Turkey. The samples were surface-sterilized with 70% ethanol for 40 s. Then they were washed twice with sterile water and placed on the surface of potato–dextrose agar (PDA) supplemented with lactic acid (4 ml/l medium) to prevent bacterial growth. The plates were incubated at room temperature for 3–14 days. Any mycelium that grew from the edge of the plant sample was plated on individual petri dishes with PDA for subsequent identification. The species identification of pure cultures was carried out on the basis of their cultural and morphological properties according to published recommendations [15, 16]. The strains selected for molecular biological analysis were deposited in the All-Russian Collection of Microorganisms (VKM).

Molecular identification of *C. parasitica*. To obtain DNA preparations of micromycetes, 100–150-μl fragments of nutrient medium with mycelium were collected from the surface of petri dishes and placed in Eppendorf test tubes. DNA was extracted using a lysing buffer based on guanidine thiocyanate and "glass milk" sorbent (SiO_2) with a SILICA M sample preparation kit (Biokom Co., Russia). For plant tissue analysis, fragments of smooth bark of healthy and infected chestnut trees were used. Portions (100 to 150 μl; bulk volume) of bark material were chopped with sterile pincers, a scalpel, and scissors and placed in Eppendorf test tubes. The tubes were then supplemented with 300 μl of one of the following buffers (pH 8): (1) CTAB (cetyl trimethylammonium bromide), 2.7%; EDTA, 18 mM; NaCl, 1.3 M; PVP (polyvinyl pyrrolidone), 0.9%; Tween-20, 0.9%; and sorbitol, 182 mM; (2) CTAB, 2.7%; EDTA, 18 mM; NaCl, 1.3 M; PVP, 0.9%; Tween-20, 0.9%; and PEG, 1.4%; (3) CTAB, 2.7%; EDTA, 18 mM; NaCl, 1.3 M; PVP, 0.9%; Tween-20, 0.9%; sucrose, 273 mM; and mannose, 182 mM; and (4) CTAB, 2%; EDTA, 20 mM; NaCl, 1.4 M; $NaHSO_3$, 0.1%; and Tris–HCl, 10 mM. The samples were homogenized with a homogenizer pestle, supplemented with 300 μl of lysing buffer (SILICA M sample preparation kit; Biokom, Russia), and thoroughly agitated. The subsequent DNA isolation procedures were performed according to the kit instructions.

PCR analysis was performed with the PCR-Core kit (Biokom Co., Russia) and the following oligonucleotide primers synthesized by Syntol (Russia) and selected for amplification of the species-specific gene fragments of *C. parasitica*:

MF1-F: 5'-GAAGCCTGGTGTCTCTTCCAC-3';
MF1-R: 5'-GTGGAAGAGACACCAAGCCTC-3';
MF2-F: 5'-ATGCCTTCCAACACCCAGAC-3';
MF2-R: 5'-GACAACGCAGTAGGAGTAGCC-3'.

Amplification was performed in a Tercyc OMC2 programmable thermostat (DNA-Technology, Russia). The PCR cycle parameters were as follows: 94°C for 3 min, 1 cycle; 94°C for 40 s; 62°C for 30 s; 72°C for 20 s; 35 cycles; and 72°C for 3 min, 1 cycle. To detect the obtained PCR products, agarose gel electrophoresis was carried out using the molecular weight marker Gene Ruler 100 bp DNA ladder (Fermentas, Lithuania).

RESULTS AND DISCUSSION

A total of 75 samples collected in the Zonguldak and Bartyn Provinces of Turkey were subjected to mycological analysis. A total of 300 strains of micromycetes belonging to 13 taxa were isolated and identified, including 45 strains that were deposited in the All-Russian Collection of Microorganisms (VKM) (table). Some of the isolated strains were represented by the fungi associated with various chestnut diseases, such as *Cryphonectria parasitica, Cryptodiaporthe castanea, Discella castanea, Phomopsis castaneae, Phyllosticta castaneae*, and *Monochaetia concentrica* [1, 15]. *Alternaria alternata* belongs to phytopathogenic fungi with a wide distribution area that includes chestnut as well. The micromycete *Pestalotiopsis guepinii* was isolated from virtually all samples. Its representatives have not been previously detected on chestnut; however, there are indications that this species causes hazel and walnut diseases in Turkey [17]. The following species were isolated from a few samples: *Aplosporella obscura, Ascotricha chartarum, Seimatosporium lichenicola, S. pestalozzioides*, and *Truncatella angustata*. This group of fungi usually infects various plants causing leaf spot disease and is not typical for chestnut.

In culture, the pathogen that causes chestnut blight, *C. parasitica*, produced only an anamorphic stage (*Endothiella* st. *Cryphonectria parasitica*), the characteristic trait of which is the formation of yellow to orange yellow, spherical or pulvinate aggregated pseudostromatic conidiomates with one ostiole. Conidiophores are branched, septated, hyaline, smooth, and up to 60 μm long; they are formed from the cells lining the conidiomatal cavity. Conidiogenous cells are enteroblastic and phialidic. Conidia (3–5 × 1–1.5 μm) are hyaline, non-septate, smooth-walled, and ellipsoidal to bacilliform. It should be noted that there were significant differences between the isolated strains in their macromorphological properties and growth rates, which, most probably, is due to the presence of hypovirulent morphotypes (VKM FW-3138, VKM FW-3139, VKM FW-3140, VKM FW-3141, VKM FW-3143, VKM FW-3144, VKM FW-3149, VKM FW-3150, and VKM FW-3151). It is known that these morphotypes produce poor mycelium and exhibit reduced spore formation and lack of orange pigmentation [16, 18, 19]. The isolated strains were used for

Taxonomic diversity of the micromycetes isolated from *Castanea sativa*

Species	Isolation sources	VKM strain number
Alternaria alternata (Fr. 1832) Keissl. 1912	Bark	FW-3038
Aplosporella obscura Pass. 1885	Bark	F-3899
Ascotricha chartarum Berk. 1838	Bark	
Cryptodiaporthe castanea (Tul. et C. Tul. 1863) Wehm. 1933	Fruit	F-3893
Discella castanea (Sacc. 1884) Arx 1970	Bark	F-3894
Endothiella st. *Cryphonectria parasitica* (Murrill 1906) M.E. Barr 1978	Bark	F-3897, F-3901, F-3904, FW-2986, FW-3131–FW-3151
Monochaetia concentrica (Berk. et Broome 1874) Sacc. et D. Sacc. 1906	Bark	F-4073
Pestalotiopsis guepinii (Desm. 1840) Steyaert 1949	Bark, leaves, fruits	F-3892, F-3902, F-3903
Phomopsis castaneae Woron. 1915	Bark	F-3898
Phyllosticta castaneae Ellis et Everh. 1894	Leaves, fruits	F-3900, FW-2983, FW-2984, FW-2993, FW-2994
Seimatosporium lichenicola (Corda 1839) Shoemaker et E. Müll. 1964	Leaf	F-3928
Seimatosporium pestalozzioides (Sacc. 1877) B. Sutton 1975	Bark	F-4074
Truncatella angustata (Pers. 1801) S. Hughes 1958	Leaf	F-3929
Sordaria sp.	Bark	FW-3952, FW-3953, FW-3954

DNA extraction and subsequent PCR analysis for *C. parasitica* molecular identification.

The genes *Mf1/1*, *Mf2/1* (*Vir1*), and *Mf2/2* (*Vir2*) encoding the precursor proteins of sex pheromones of *C. parasitica* were used as PCR markers for the detection of this organism [20]. The presumption was that, on one side, these genome elements a priori exhibit genetic conservatism, while, on the other hand, they contain unique sequences that do not occur in the DNA of other fungi. The selected primer pair MF1-F,R flanks a fragment (189 bp) of the *Mf1/1* gene; the MF2-F,R primer pair flanks a fragment (66 bp) of the *Mf2/1* or *Mf2/2* genes.

Using the obtained DNA of the isolated micromycetes as a DNA template for PCR, we demonstrated that amplification with the resultant production of targeted fragments occurred only in the presence of the DNA of *C. parasitica*. It was true for all 25 studied strains isolated in the course of this study (table), as well as for strain VKM F-123 deposited in the VKM culture collection in 1962. No PCR products were obtained when using the DNA of other fungi (Fig. 1a). Thus, it was found that the selected PCR conditions allows us to specifically detect *C. parasitica* in pure culture and to perform genetic identification of this organism.

It should be noted that, at all stages of our work, we used PCR analysis of a highly conservative 18S rRNA gene fragment (which served as a universal eukaryotic PCR marker) to control the quality of DNA isolation. The presence of target amplicons (445 bp) in the obtained PCR products indicated the satisfactory quality of the obtained DNA preparation (Fig. 1c). It was demonstrated that the quality of the DNA preparations obtained from the tissues of infected chestnuts using the standard sample preparation procedure was not high enough for PCR analysis. Problems in DNA isolation from necrotic chestnut tissues may be explained by the specificity of the biological material. In particular, isolation of high-quality DNA was possibly hampered due to the effect of some organic compounds (gum, etc.) formed in plant tissues as a result of pathogen–host relationships or, due to the same reasons, PCR inhibitors were present in the obtained DNA preparations. The applied technique was optimized by using lysing buffers during sample preparation; these buffers contained antioxidant compositions, which, as the results of control PCR analyses have demonstrated (Fig. 2b), allowed us to obtain high-quality DNA preparations from any chestnut tissues.

The final stage of our study included PCR analysis of the DNA preparations from the bark samples taken from healthy and infected chestnuts, using the MF1 and MF2 primers, species-specific for *C. parasitica*. The DNA isolated from the pure culture of *C. parasitica* VKM F-3901 served as a positive control for PCR amplification and subsequent electrophoretic analysis of its products.

This analysis allowed us to reveal the presence of the *C. parasitica* DNA in a number of plant samples (Fig. 2a), including bark samples taken directly from cankers on blight-infected branches, as well as from adjacent, seemingly undamaged, tissues. No molecu-

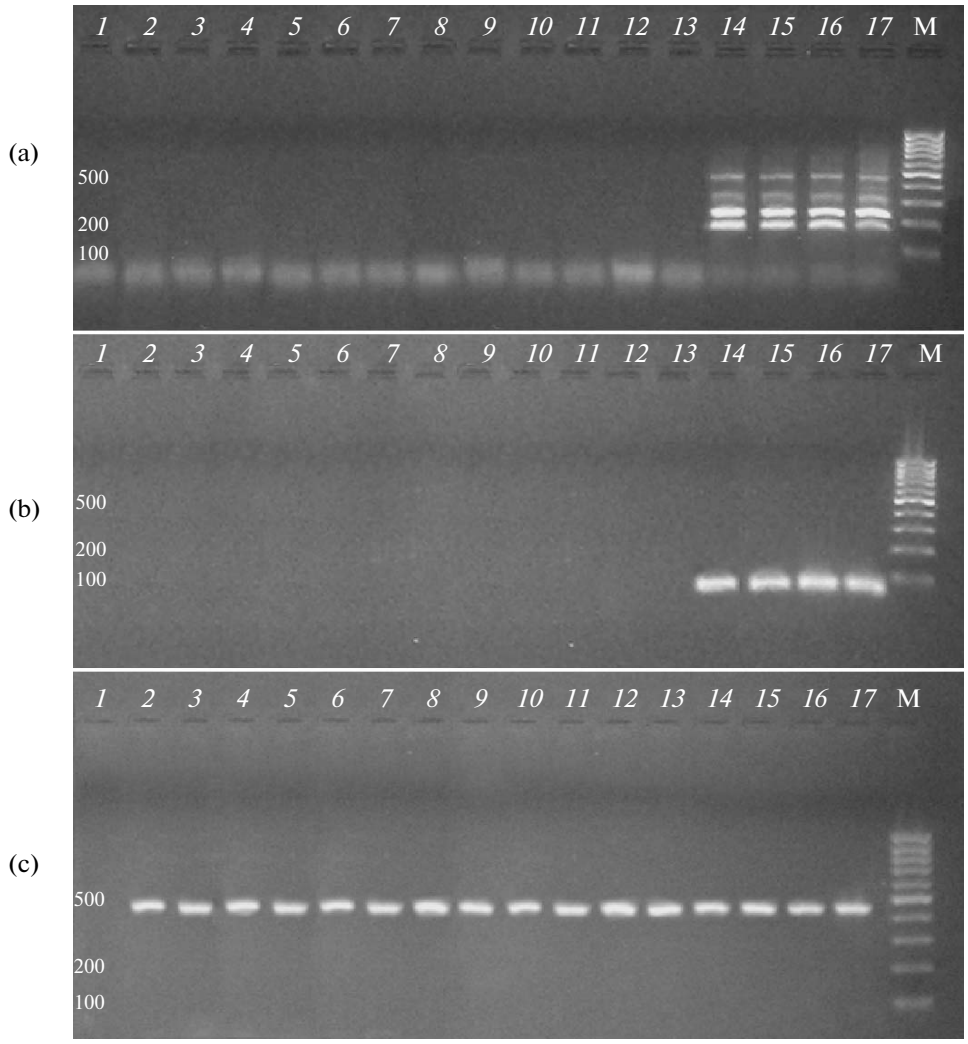

Fig. 1. Electrophoretogram of the PCR products obtained by amplification of the DNA of some phytopathogenic fungi using the species-specific primers MF1 (a) and MF2 (b) targeting *C. parasitica*, as well as the universal primers homologous to the 18S rRNA gene fragment of eukaryotes (c): negative control (water), *1*; DNA of *Discella castanea*, *2*; DNA of *Phomopsis castaneae*, *3*; DNA of *Cryptodiaporthe castanea, 4*; DNA of *Phyllosticta castaneae, 5*; DNA of *Pestalotiopsis guepinii, 6*; DNA of *Alternaria alternate, 7*; DNA of *Monochaetia concentrica, 8*; DNA of *Aplosporella obscura, 9*; DNA of *Ascotricha chartarum, 10*; DNA of *Seimatosporium lichenicola, 11*; DNA of *Seimatosporium pestalozzioides, 12*; DNA of *Truncatella angustata, 13*; and DNA of *Cryphonectria parasitica* (strains VKM F-3897, F-3901, F-3904, and F-123, respectively), *14–17*; M, DNA molecular weight marker, bp.

lar markers of the *C. parasitica* genome were detected in the DNA of healthy chestnut trees (Fig. 2a).

Hence, we have developed a technique that allows determination of *C. parasitica* in sweet chestnut tissues irrespective of the possible presence of other microscopic fungi in samples. It should be noted that phytopathogenic analysis based on the assaying of the indirect symptoms of the disease, as well as the associated procedures of mycological analysis [3, 7, 21], remains virtually the sole method for diagnostics of plant diseases caused by microscopic fungi. At the same time, determination of specific pathogens is traditionally affected by the following factors:

—similarity of external symptoms of some diseases (for example, chestnut blight may be confused with chestnut ink disease caused by *Phytophthora cambivora* [3, 5, 16]);

—differences in the aggressiveness, morphology, and effect of different forms and strains of one species, which is typical of *C. parasitica* as well [3, 16];

—"blurring" of the symptoms and signs due to simultaneous infection with multiple pathogens; and

—the effect of saprotrophic epiphytic fungi, etc.

Mycological analysis is, in turn, a time-consuming procedure: it takes up to several weeks from inoculation of plant tissue specimens to identification of fungi species. In addition, this method is associated with a number of methodical difficulties, when, for instance, macromorphological properties (growth rate, myce-

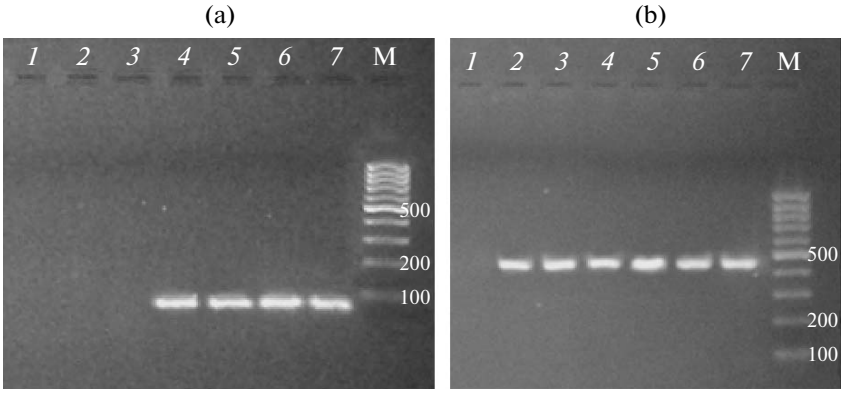

Fig. 2. Electrophoretogram of the PCR products obtained by amplification of the DNA of *C. parasitica* (target, gene *Mf2* (a)) and the eukaryotic genome (target, 18S rRNA gene (b)): *1*, negative control (water); *2–3*, DNA isolated from the tissues of healthy chestnut trees; *4–6*, DNA isolated from the tissues of infected chestnut trees; and *7*, positive control (DNA of *Cryphonectria parasitica*); M, DNA molecular weight marker, bp.

lium pigmentation, development of the mycelium) of the hypovirulent *C. parasitica* strains are similar to those of *P. guepinii* strains. Representatives of the latter are always isolated in pure cultures simultaneously with *C. parasitica*.

The developed technique for PCR detection of chestnut blight has a significant advantage over the methods of phytopathogenic analysis due to its efficiency and the possibility of immediate pathogen detection. It can be used for disease monitoring in natural populations and artificial plantations, as well as for confirmation, updating of the results of phytopathogenic analysis, infection control of planting and inoculative material, etc.

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