ISSN 1022-7954, Russian Journal of Genetics, 2007, Vol. 43, No. 3, pp. 255–258. © Pleiades Publishing, Inc., 2007.
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GENETICS
OF MICROORGANISMS

Heteroplasmosis in *Phytophthora infestans*

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Received October 5, 2006

Abstract—PCR and monozoospore plating was used to demonstrate a simultaneous presence of the mitochondria with mitochondrial DNA of haplotypes Ia and IIa in the mycelium of several *Phytophthora infestans* strains.

DOI: 10.1134/S1022795407030052

INTRODUCTION

The haplotype of mitochondrial DNA (mtDNA) is an independent genetic marker used in population studies of the phytopathogenic oomycete *Phytophthora infestans* (Mont.) de Bary, the agent of late blight disease of potato and tomato. Initially, the analysis of mtDNA restriction fragments was a rather laborious procedure [1]; however, this marker became one of the most popular in the studies of *P. infestans* after Griffith and Shaw [2] had developed a simple and quick method for determining mtDNA haplotypes (PCR–RFLP method). The type of mtDNA is used in the comparative analysis of strains and populations. The strains from populations of Europe and Asia [3–5], North and South Americas [6–8], Africa [9, 10], and Australia were analyzed according to this trait. In several works, the type of mtDNA was used for the isolation of clonal lines [10, 11] and certification of the isolates used [12]. Analysis of the mtDNA types detected in the plant remnants dating back to the century before last made it possible to infer the distribution of *P. infestans* genotypes in the past [13]. Now the complete nucleotide sequences of various mtDNA haplotypes are determined and available in the Internet (GenBank [14]), thereby facilitating the design of new tester systems based on mtDNA structure.

The essence of PCR–RFLP method is amplification of an mtDNA fragment and the subsequent restriction of the PCR product. This method allows four haplotypes—Ia, Ib, IIa, and IIb—to be distinguished between; for this purpose, it is necessary to perform two tests for each isolate. Only the strains displaying haplotypes Ia and IIa are recorded in Russia during the last 12 years; therefore, one PCR–RFLP test, distinguishing between types I and II, is sufficient to determine the haplotype of an isolate ([5, 15, 18] and our unpublished data).

The main distinction of haplotype II from haplotype I is the presence of the insert with a size of 1881 bp (Fig. 1). The bands corresponding to both mtDNA haplotypes were detected in electrophoretic patterns of the restriction products of a number of Russian isolates; moreover, they differed considerably in the luminescence intensity. It was assumed that these isolates contained genetically different mitochondria in their mycelia. i.e., they were heteroplasmons.

Heteroplasmic isolates have been found in several fungi. For example, the appearance of heteroplasmons after sexual process was reported for *Neurospora crassa* [16]. However, we failed to find in literature any references to the research into *P. infestans* heteroplasmons. The goal of this work was to prove a heteroplasmic nature of the *P. infestans* isolate studied using monozoospore plating and analysis of mtDNA haplotypes.

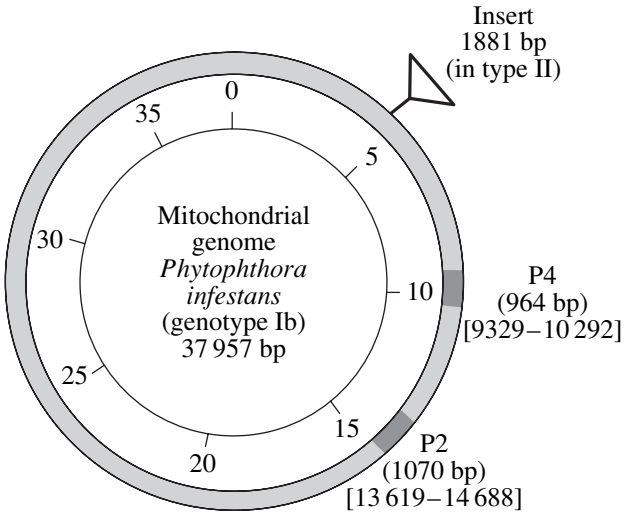

Fig. 1. A scheme of the mitochondrial genome of *Phytophthora infestans.*

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5195                                              gcagcc caaatatctc gaaatcttat
5221 aatttcactt ggagaatcta ttgatgttgt tgttgtaaat ggaatagtcg gtattattga
5281 acgcattata actccaccta tagggggtaa acccattgaa cttaatgcca tagaacctaa
5341 aataccaacc ccctcataaa ctagtcttcg tctatttaaa gaaattctag aatcaatttc
5401 cctattaaac tcatcaactc tttgttgaag ctgttcttca ttaactaatc gaatttgatt
5461 taaatattca tcataattat ctacttgagt atttaaaatt gaaaaaattt aattattatt
5521 ataaactacc attgattttc tcatattttg aataaagtct cttataacta attcatctat
5581 atctaaatta gatctagtat ataaaaatat attacctatt cgatataagt cttcttctga
5641 tatatttaaa tcattcgaag atcgaattaa taaatcatgt aaatatgtcc taacttgtaa
5701 tgaactattt cttattagta ataaactaat ttcctgatta ttttgataat ttaaaatgtt
5761 ttgcattaac tctatacgtt cttcagatga agtcctatta ttaatattaa taacaccttc
5821 actatctaaa acttcagtat ttcccatttg ttctacccat ggaatcctat atcgagttga
5881 atgtaataat tgatgcgcca ttgc
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Fig. 2. Nucleotide sequence of the fragment (5195–5904, type IIa) amplified by the primers VSFor and VSRev (according to GenBank; positions of primers are underlined).

MATERIALS AND METHODS

Isolates. Isolate 3MOBTL139 from the collection of the Chair of Mycology and Algology, Biological Faculty, Moscow State University, isolated into a pure culture from the leaves of a tomato plant grown in the Odintsovsky raion, Moscow oblast, Russia, was used in the work. The isolates were cultivated in liquid or agar pea medium (160 g of frozen green peas are boiled in 1 l of distilled water for 10 min, filtered through several layers of gauze, and sterilized by autoclaving) or agar oatmeal medium [17].

Production of monozoospore isolates. The parental strain was cultivated in agar oatmeal medium. After 7 days of cultivation, an agar slab with a size of 10×40 mm with an intense asexual spore formation was cut off and placed into a 15-ml plastic tube filled with 7 ml of sterile distilled water. The tube was shaken rigorously for 1 min and placed into a refrigerator (3°C). Zoospores were released after 3–5 h; the liquid with actively swimming zoospores was taken with a microsampler trying to prevent shaking of the tube and admixing of the sediment, formed of the agar medium, fungal hyphae, and empty envelopes of zoosporangia.

The zoospores were placed into a 100 ml of liquid pea medium diluted twofold; the concentration of zoospores was preliminary adjusted to 100 spores per flask. After 3 days of exposure to 18°C, part of the flask content (approximately 25 ml) was poured into a sterile Petri dish. The germinated zoospores detected by examination with a binocular microscope were taken with a sterile micropipette with cut-off tips (to increase their diameter). The samples microcolonies were placed into Petri dishes with agar oat medium (one per petri dish).

PCR primers. Nucleotide sequence of the insert that distinguishes between haplotypes I and II was extracted from the GenBank [14]. The primers to its fragment were selected using the Oligo software. The fragment with a size of 709 bp (Fig. 2) was chosen for the work; the flanking 20-nt-long regions, designated VSFor and VSRev (table), were selected as the primers. Primers F4 and R4 [8] (table) were used to amplify P4 fragment.

DNA isolation and the conditions for amplification and restriction. Total DNA was isolated from the dehydrated mycelium ground in liquid nitrogen by phenol–chloroform deproteination upon an alkaline treatment of the mycelium [8].

The amplification was conducted in a Biometra T1 device. The mode for amplifying P4 region comprised $1 \times$ (90 s at 90°C), $30 \times$ (30 s at 90°C, 30 s at 52°C, and 90 s at 72°C), and $1 \times$ (180 s at 72°C). The reaction mixture contained 0.2 U of *Taq* DNA polymerase, $1 \times$ 2.5 mM $MgCl_2$–*Taq* buffer, 0.2 mM of each dNTP, 30 pM primer (table), 5 ng of DNA sample, and deionized water to 20 μl. The PCR product was hydrolyzed

The primers used for amplification of the mtDNA polymorphic regions (for genotype IIa)

Locus	Primer	Length and position of primer	Length of PCR product
P4	F4: 5'-TGGTCATCCAGAGGTTTATGTT-3'	22; 11290–11311	964
	R4: 5'-CCGATACCGATACCAGCACCAA-3'	22; 12253–12232	
Insert	VSFor: 5'-GCAGCCCAAATATCTCGAAA-3'	20; 5195–5214	709
	VSRev: 5'-GCAATGGCGCATCAATTATT-3'	20; 5904–5885	

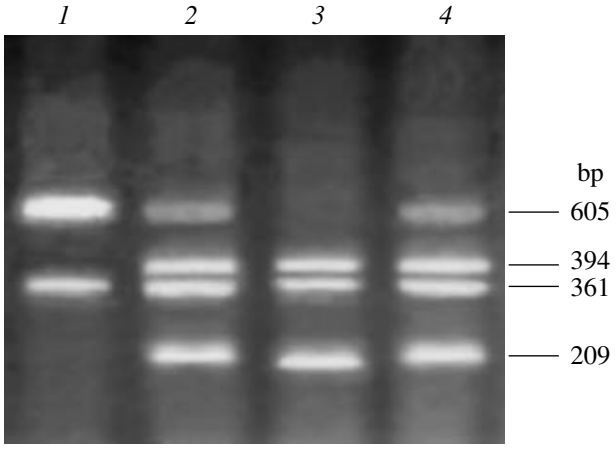

Fig. 3. Electrophoretic pattern of the restriction products of homo- and heteroplasmic isolates: (*1*) control (homoplasmon IIa); (*2*) strain 3MOBTL139 (heteroplasmon Ia + IIa); (*3*) isolate K9 (homoplasmon Ia); and (*4*) isolate K11 (heteroplasmon Ia + IIa).

for 2 h at a temperature of 37°C. the restriction mixture (20 μl) contained 2 μl of 10 × *Eco*RI, 2 μl of 10 × restriction buffer, 6 μl of deionized water, and 10 μl of PCR product.

The following mode was used for amplification of the insert: 1 × (60 s at 92°C), 30 × (25 s at 92°C, 25 s at 52°C, and 60 s at 72°C) and 1 × (180 s at 72°C). The composition of the restriction mixture was analogous to that used for amplification of P4 fragment.

The amplification and restriction products were separated in 0.8 and 1.5% agarose gels prepared using the Tris–borate buffer supplemented with ethidium bromide.

RESULTS AND DISCUSSION

According to the PCR–RFLP test, the isolate 3MOBTL139 had haplotype Ia; however, the electrophoretic pattern contained a weakly luminescing additional band corresponding in its size to haplotype IIa (Fig. 3). The amplification with the primers VSFor and VSRev demonstrated the presence of the insert. To confirm a heteroplasmic nature of this strain, we used two successive monozoospore platings. The first plating yielded 12 monozoospore isolates, all carrying the insert. The isolate displaying the least luminescence intensity of the PCR product in electrophoretic pattern was chosen for the second plating. In turn, 15 monozoospore isolates were recovered from it. The subsequent PCR assay using the primers VSFor and VSRev detected differences in the concentration of PCR product (Fig. 4); note that the isolate K9 (lane *9*) contained no insert. Presumably, the parental zoospore of this isolate had the mitochondria of only Ia haplotype.

All the monozoospore isolates and the parental strain were rechecked by PCR–RFLP test, which confirmed that their mtDNA was of haplotype Ia. However, the clones with a high concentration of the amplified insert displayed a weakly luminescing band corresponding to IIa haplotype of mtDNA (Fig. 3).

The results of our work suggest a new view on the studies utilizing mtDNA haplotypes as genetic markers. As a rule, the standard tests allow the mtDNA haplotype dominating in the mycelium of an isolate to be determined.

The free availability of the complete sequence of mitochondrial genome induced the development of new methods for comparative analysis of strains and populations utilizing amplification of particular mtDNA regions. Interpretation of the results of such analysis may be hindered if the mycelium of a strain studied contains genetically different mitochondria.

On the other hand, the mitochondrial genome is involved in a number of vital functions; therefore, the heteroplasmic state may increase the adaptation potential of the organism. Our preliminary studies demonstrate a wide distribution of heteroplasmons in natural populations. Of the isolates recovered from the late blight–infected samples collected in 2003–2005 in various parts of the Moscow oblast, the Republic of Mordovia and Mari El, over 90% were heteroplasmic. Presumably, such a high concentration of heteroplasmons in field populations is connected with high genetic diversity of *P. infestans* in the natural populations of the European Russia.

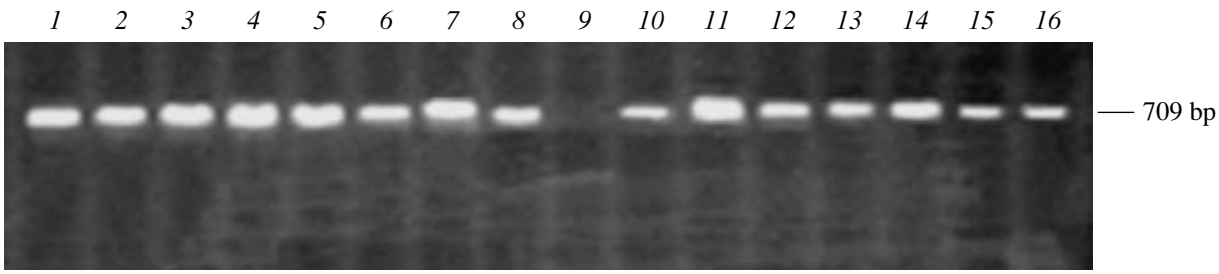

Fig. 4. Electrophoretic pattern of the PCR amplification product of the insert: (*1–15*) the monozoospore isolates descending from 3MOBTL139 and (*16*) isolate 3MOBTL139.

ACKNOWLEDGMENTS

We thank Yu.T. Dyakov and I.V. Elanskaya for their advices and discussion of the work and V.P. Apryshko and M.A. Pobedinskaya, for assistance in isolation and analysis of the strains.

This work was supported by the International Science and Technology Center (project no. 3440).

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