

# Molecular Identification of the Species Composition of Russian Isolates of Pathogens, Causing Early Blight of Potato and Tomato

SERGEY N. ELANSKY<sup>1</sup>, MARINA A. POBEDINSKAYA<sup>1</sup>, LYUDMILA YU. KOKAEVA<sup>1</sup>,  
NATALIA V. STATSYUK<sup>2</sup> & ALINA V. ALEXANDROVA<sup>1</sup>

<sup>1</sup>Lomonosov Moscow State University, Moscow, 119899 Russia; e-mail: elansky@yahoo.com

<sup>2</sup>All-Russian Research Institute of Phytopathology, Bolshie Vyazemy, Moscow region, 143050 Russia

## SUMMARY

A comparative study of the genome structure has been carried out for seven large-spore and 25 small-spore strains, isolated in 2007-2010 from infected tomato and potato plants, growing in seven distant regions of the European part of Russia and Russian Far East. To make a comparison, a sequence of ribosomal genes and intergenic regions of the nuclear rDNA has been selected. The tested strains have been divided into three groups. The first one includes isolates, which were morphologically determined as *A. alternata*, *A. tenuissima*, and *A. arborescens*. These isolates do not have any differences in the structure of the studied DNA region. The second group includes large-spore isolates, classified as *A. solani*. Some strains of this group differ from others in small single-nucleotide substitutions. No any differences between large-spore strains, isolated from potato and tomato, have been observed. The third group includes the only *A. infectoria* isolate, collected in 2010 in the Kostroma region.

## KEYWORDS

early blight, potato protection, *Alternaria solani*, *Alternaria alternata*

## INTRODUCTION

Early blight is a dangerous potato and tomato disease, typical in the whole area of cultivation of these crops. This disease is able to result in the significant yield decrease under favorable weather conditions.

Among early blight agents in Russia, there can be some species from the genus *Alternaria*, including one large-spore species *Alternaria solani* Sorauer and several small-spore species, such as *A. alternata* (Fr.) Keissl., *A. tenuissima* (Kunze) Wiltshire, *A. infectoria* E.G. Simmons, and *A. arborescens* E.G. Simmons (Orina *et al.*, 2010).

Different species can have their own biological features and differ in such important characteristics as the aggressiveness, virulence toward different potato and tomato cultivars, fungicide resistance, toxigenicity, optimum growth temperature, and winter survivability (Ivanyuk *et al.*, 2005).

The identification of species by their morphological characteristics is connected with some problems, related to the dependence of the morphology of conidiogenic structures on the medium composition, temperature, lighting mode, etc. Large-spore strains often do not form any conidial fruiting on a nutrient medium. Therefore, morphology-independent features, such as those, based on the genome structure analysis, are especially important for this type of studies.

The purpose of our study was to investigate the species composition of early blight agents, isolated on the territory of Russia, using both morphological and molecular markers.

## MATERIALS AND METHODS

### *Samples*

In our study we analyzed early blight agents, isolated in 2007-2010 from infected potato and tomato plants in the Leningrad, Moscow, Astrakhan, and Kostroma regions, Mariy El Republic, Tatarstan (Fig. 1), the Stavropol Territory, and Primorye. Isolates of *A. solani* and *A. arborescens* from the Primorye and some strains of small-spore species from the Leningrad region were kindly provided by our colleagues from the Laboratory of Mycology and Phytopathology of the All-Russian Research Institute of Plant Protection.



Figure 1. Location of sampling sites in the European part of Russia. 1, Leningrad region; 2, Moscow region; 3, Kostroma region; 4, Mariy El Republic; 5, Tatarstan; 6, the Stavropol Territory; 7, Astrakhan region.

### Strain isolation

Isolation of strains into pure culture was carried out using wet chambers. After the appearance of fruiting on the surface of an infected sample, the sample was microscopied; using a sharp sterile preparation needle, we transferred conidia onto wort agar medium, supplemented with penicillin (1000 µg/ml), and incubated until the colony diameter reached 4-5 cm. Then a piece of mycelium from the edge of the colony was transferred onto a Petri dish with nutrient medium.

### Species identification

Identification of species was performed according to morphological criteria (Simmons, 2007). Isolates were grown on Petri dishes with potato carrot agar (PCA) under fluorescent lamps at 25°C. Colonies were microscopied after 7-10 days of incubation to register the features of the formation of conidial chains and the morphology of spores.

### DNA isolation

DNA isolation from the studied *Alternaria* isolates was carried out by the chlorophorm deproteinization using a CTAB buffer.

### PCR reaction

The amplification was carried out in a Biometra T1 thermocycler using the following scheme: initial denaturation at 95°C for 3 min, then the denaturation at 94°C for 40 s, the annealing temperature shown in Table 1 for 40 s, elongation at 72°C for 60 s, and a final elongation at 72°C for 3 min. The number of amplification cycles was 25. To amplify specific DNA regions, we used primers listed in Table 1.

The final volume of a PCR reaction mixture was 25 µl and included 2.5 µl of 10x PCR buffer (Helicon, Russia), 2 µl of 25 mM MgCl<sub>2</sub>, 0,5 µl of Taq polymerase (5 U/ µl), 2 µl of dNTP mix (0,2 mM of each dNTP), 0,4 µl of each primer, and 1 µl of sample DNA. The rest of volume represented deionized water.

Table 1. List of primers used in the study

Name	DNA sequence	Reference	Annealing temperature, °C
<b>ITS5-ITS4 region of nuclear ribosomal genes</b>			
ITS5	5'-GGAAGTAAAAGTCGTAACAAGG-3'	White et al., 1990	
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	White et al., 1990	58
<b>Large subunit region of mitochondrial ribosomal genes (mt LSU)</b>			
ML1	5'-GTACTTTTGCATAATGGGTCAGC-3'	White et al., 1990	
MLR1	5'-GCCCTTCCGAGAGCAAATAC-3'	Peever et al., 2004	52
<b>Reverse primer for small-spore species; is used together with the ITS 5 primer</b>			
MR	5' – GACCTTGGCTGATAGAGAGTG	Designed by authors of this study	50

### Sequencing

After the electrophoretic separation in 1.2% agarose (TBE buffer), the band with the PCR product was cut out of the gel, and the DNA fragment was isolated using a “Cytokin” kit. DNA sequencing was performed by the Eurogene company with the use of a BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing kit; the subsequent analysis of the reaction products was carried out using an Applied Biosystems 3730 xl DNA sequencer. The primers used for the amplification of the studied region, were also used as the sequencing primers. The reading of each sequence was carried out twice using the forward and reverse primers. All DNA sequences, obtained during the sequencing, were combined into a database, used for the reconstruction of taxonomic relations between studied species. The reconstruction was carried out by a maximum-likelihood method using a Mega software.

## RESULTS AND DISCUSSION

To identify the species composition using the genome structure, we took DNA samples from 7 large-spore and 25 small-spore isolates, collected from potato and tomato plants in different regions of Russia. For the comparative study we selected a nuclear rDNA sequence, confined by ITS5 and ITS4 primers (Fig. 2). This region is well studied in many species and is widely used for the analysis of taxonomic relations.

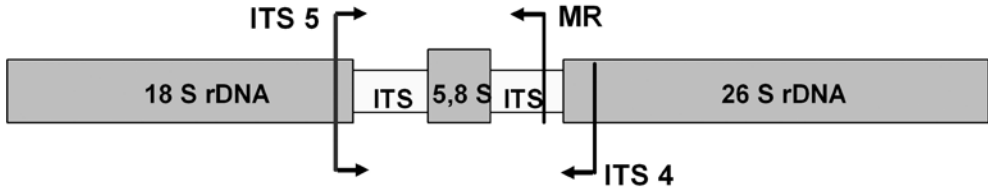


Figure 2. The arrangement of genes and intergene sequences in the nuclear rDNA region and the localization of ITS5, ITS4, and MR primers within this region.

As a result of our study, we determined nucleotide sequences of the studied region, which lengths were equal to 595 bp (small-spore isolates), 60 bp (*A. solani*), and 627 bp (*A. infectoria*), including the sequences of ITS5 and ITS4 primers.

According to the obtained results, the studied strains were divided into three groups (Fig. 3).

The first group included all studied small-spore isolates, excepting *A. infectoria* and including typical strains of *A. arborescens* and *A. tenuissima* (the sequences were taken from the Genbank).

The second group contained all studied large-spore isolates, including typical strains of *A. tomatophyla* and *A. solani*. However, *A. tomatophyla* strain significantly differed from other strains of this group. It seems that the studied large-spore strains, isolated from tomato plants, represent rather *A. solani* than *A. tomatophyla* (it is also confirmed by the results of the morphological diagnostics), though this supposition contradicts the opinion of Simmons (Simmons, 2007), who postulates the absence of *A. solani* on tomato plants. *A. solani* strains, isolated from tomato and potato plants on the Far East, demonstrated some minor differences comparing to other samples.

The third group included one *A. infectoria* isolate from the Kostroma region, one typical isolate from US, kindly provided by our colleagues from the Laboratory of Mycology and Phytopathology of the All-Russian Research Institute of Plant Protection, and also the sequence of strain from the Genbank.

The studied isolates were also identified by their morphological characteristics. As a result of this part of our study, we identified strains of four small-spore (*A. alternata*, *A. tenuissima*, *A. arborescens*, and *A. infectoria*) and one large-spore (*A. solani*) species (Fig. 3). According to the genome structure, *A. solani* and *A. infectoria* were referred to the corresponding separate groups. Morphological species *A. alternata*, *A. tenuissima*, and *A. arborescens* did not differ in their genome structure and, therefore, were included into the same group with the typical *A. arborescens* and *A. tenuissima*.

The dendrogram, based on the data of the mtLSU sequencing, allowed us to divide the analyzed isolates into two groups. The first group consisted of small-spore strains, including *A. infectoria*; the second group consisted of *A. solani*. Within the groups, the strains were almost identical, whereas the groups significantly differed from each other. The typical *A. alternata* and *A. infectoria* strains were included into the group, containing small-spore strains.

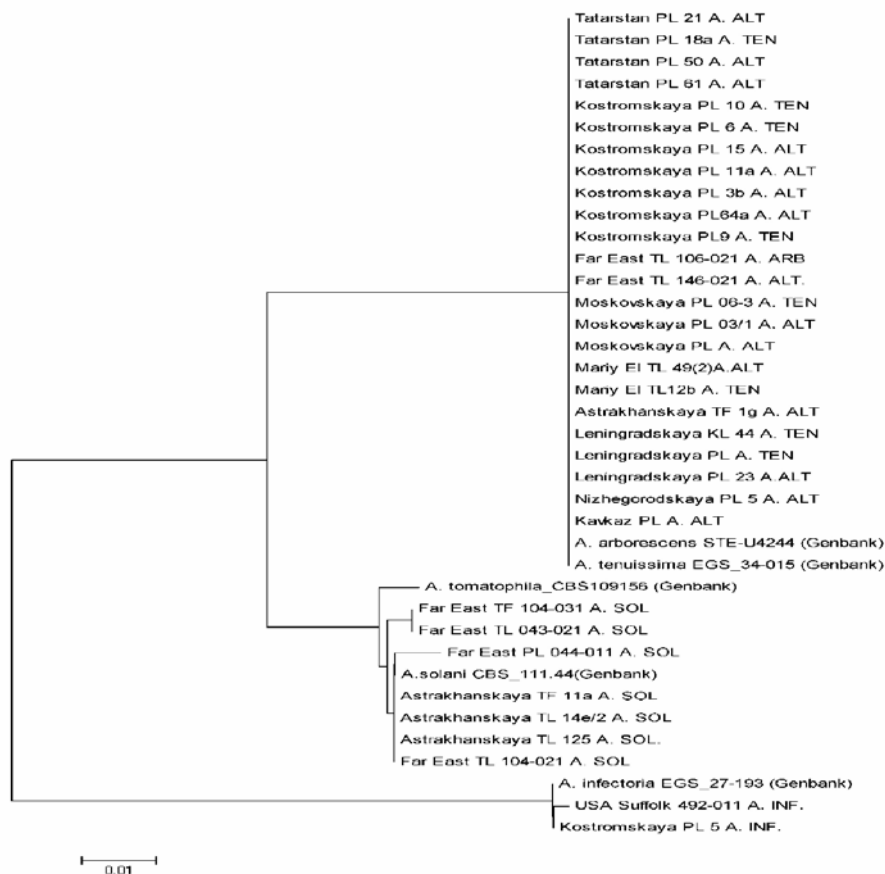


Figure 3. Dendrogram, obtained by the maximum-likelihood method for the structure of the ITS5-ITS4 region. Species identification according to the morphological study: A.ALT, *A. alternate*; A.TEN, *A. tenuissima*; A.ARB, *A. arborescens*; A.SOL, *A. solani*; A.INF, *A. infectoria*. Plant parts, from which the strains were isolated: PL, potato leaves; TL, tomato leaves; TF, tomato fruits.

Thus, the analysis of the *Alternaria* genome showed the studied strains are divided into three clades: *A. solani*, *A. infectoria*, and small-spore strains. The revealed differences between the sequences of these species, confined by ITS5 and ITS4 primers, allowed us to design a specific MR primer for the selective amplification of the corresponding genome regions of small-spore *Alternaria* species (Table 1).

The amplification of DNA of a small-spore strain using the ITS5-MR primer pair resulted in a specific 505-bp PCR product (including primer sequences). At the same time, the amplification of DNA of *A. infectoria*, *A. solani*, and other fungi, often isolated from potato leaves (genera *Bipolaris*, *Fusarium*, etc.), did not result in the formation of a PCR product (Fig. 4).

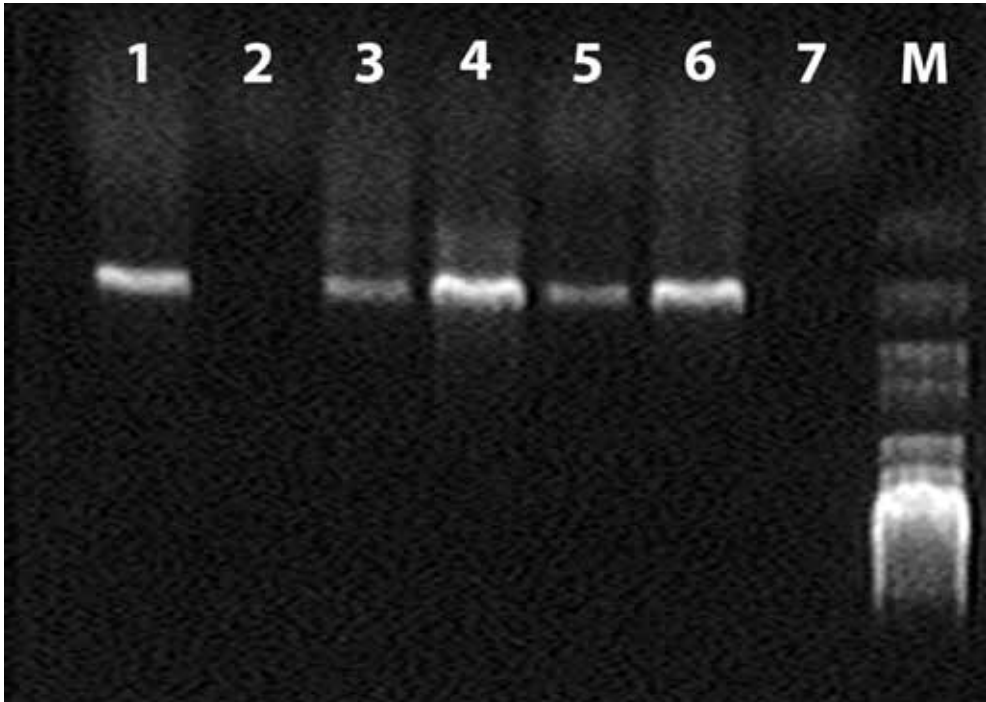


Figure 4. Specific amplification of *Alternaria* genome fragments using ITS5/MR primer pair. 1, control (*A. alternate*); 2, *A. solani*; 3–6, various small-spore isolates; 7, *A. infectoria*; M, 1-kb molecular weight marker.

The designed primer can be used for the specific amplification of DNA of small-spore *Alternaria* species, providing their successful identification in the case of any problems with their morphological identification.

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