

MOLECULAR VARIABILITY OF EGYPTIAN AND HUNGARIAN *BOTRYTIS CINEREA* ISOLATES

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Abstract: An attempt was made to evaluate the variation between Hungarian and Egyptian isolates of *Botrytis cinerea* on the basis of molecular studies. Sequences from the minisatellites MSB1 and the widely used molecular marker in phylogenetic works, the translation elongation factor 1 subunit alpha (EF-1 α =*tef1*), were identified. The phylogenetic analysis was conducted with PAUP*4.0b by parsimony analysis. Topological robustness in parsimony analysis was estimated using 1000 bootstrap replicates. Our results show that two of the three studied Egyptian isolates of *B. cinerea* have been proved to be similar to the Hungarian ones on the basis of both minisatellites and *tef1* sequences while the remaining one was completely different. The similarity between Hungarian and Egyptian isolates might be due to phylogenetic relationship or conidial dispersal between both countries.

Key words: minisatellite, translation elongation factor, *Botrytis*

Introduction

The genus *Botrytis* contains 22 species and a large number of host-specific pathogens, such *B. fabae* Sardina on Leguminosae (oligophagous) and *B. aclada* Fresenius on *Allium* spp. [3] *B. cinerea* Pers.:Fr. (the asexual stage of the teleomorph: *Botryotinia fuckeliana*), is a polyphagous, haploid, heterothallic Eucosmomyces fungus with a genome size of 30 Mb. However, it is an important pathogen that can attack more than 200 species in the field [3,11], greenhouse [5,15], and during storage [7,8,12]. One of its most notable hosts may be grapevine. Under unique ecological circumstances, noble rot can develop on *Botrytis* infected grapes, which is beneficial for wine production [3]. This fungus can infect at every development stage and can be found on every part of plants. Its detection at early stages is difficult because *Botrytis* species can be found in a latent stage on the host plant [1], so making epidemic studies are difficult.

B. cinerea is a morphologically and genetically highly variable species which is much more due to sexual reproduction than heterokaryosis or aneuploidy [2].

The objective of present work was to characterize the variability among *B. cinerea* isolates from Egypt as well as from Hungary on the base of molecular markers.

We have studied two phylogenetic markers, MSB1 minisatellite which has been found and identified in the intron of the ATP synthase gene of *B. cinerea* [6] and *tef1*, which is widely used for phylogenetic studies of different fungi [13].

MSB1 minisatellite

One of the fungal minisatellites named MSB1 was isolated in the intron of ATP synthase of *B. cinerea* by Giraud et al. [6]. It has 37bp-repeat units different from almost all the minisatellites analyzed till now. It is an AT-rich minisatellite, but does not carry the core sequence GGGCAGGAXG. It can be found at only one locus in the genome, which makes suitable for population studies. It has been found in the ATP synthase gene of all the *Botrytis* species analysed, but the repeat unit is absent from all of the other genus scored, especially *Sclerotinia*, which is the closest to *Botrytis* [6].

Translation elongation factor

Ribosomal RNA genes (rRNA) have been widely used in phylogenetic studies of fungi. Translation elongation factor 1 subunit alpha (EF-1 α =*tef1*) 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 [9]. It is an essential component of the protein synthesis process in eukaryotes and archaeobacteria. Complexed with GTP, it carries the aminoacyl-tRNA to the A site of the ribosome-mRNA-peptidyl-tRNA complex; upon hydrolysis of GTP it leaves the ribosome as EF-1 α -GDP.

Simultaneously, elongation factor 1 α (EF-1 α) is a highly conserved ubiquitous protein that has been suggested to have desirable properties for phylogenetic inference [13]. EF-1 α is well-suited for determining phylogenetic relationships, due to its universal occurrence and presence typically as a single copy within the genome [1]. 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 have used primer pair which facilitates the PCR amplification of the large intron of *tef1* gene [4] (Fig. 1).

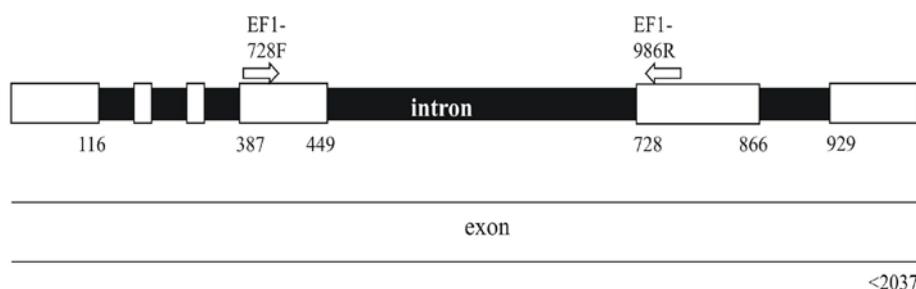


Fig. 1: Schematic structure of *tef1* gene in *Phoma* spp. and location of primers for phylogenetic analyses [4]

Materials and Methods

Three Egyptian isolates of *B. cinerea* from different host plants, bean, pea, and strawberry (Table 1) and compared with previously described Hungarian isolates [14, 18] which were involved in the present study. Individual isolates were obtained by single spore isolation.

Table 1: Data of Egyptian *Botrytis cinerea* isolates*

Name of isolates	Host plant	Year of isolation
Egypt 1	<i>Phaseolus vulgaris</i>	2005
Egypt 2	<i>Pisum sativum</i>	2005
Egypt 4	<i>Fragaria moschata</i>	2006

*Isolates can be found in the Culture Collection of the Plant Protection Department of University of Debrecen, Hungary

Total genomic DNA was extracted from aerial mycelium by MagNA Lsyer (Roche) and isolated using the E.Z.N.A.[®] TM Fungal DNA Isolation Kit (Omega Bio-tek, Inc., USA) following the manufacture's instructions.

MSB1F and MSB1R primers [6] were used in the amplification of ATP synthase, containing the MSB1 minisatellite. The following amplification protocol was used: 3 min. initial denaturing at 95°C, followed by 5 cycles of 1 min. at 95°C, 1 min. annealing at 60°C, 2 min. at 72°C and 25 cycles of 1 min. at 90°C, 1 min. annealing at 60°C, 2 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 [4] with the following programme: 3 min. initial denaturing at 95°C, followed by 5 cycles of 1 min. at 95°C, 1 min. annealing at 59°C, 1 min. at 72°C and 25 cycles of 1 min. at 90°C, 1 min. annealing at 59°C, 1 min. at 72°C and 15 min. final extension at 72°C. PCR Master Mix 2X (Fermentas) was used in all amplification reactions. Purified amplification products were sequenced by MWG Biotech Company in Germany.

The obtained DNA sequences were aligned first with ClustalX [17] and manually adjusted using Genedoc [10]. Single gaps were treated either as missing data or as the fifth base and multistate characters were treated as uncertain ones.

Phylogenetic analyses were performed in PAUP*4.0b [16]. The following settings were used: heuristic search with tree bisection-reconnection (TBR), with random addition of sequences with 1000 replicates. Stability of clades was assessed with 1000 bootstrap replications.

Results and Discussion

MSB1 minisatellite

In the PCR reaction, the MSB1 minisatellite containing variant repeat units were amplified from the Egyptian isolates (Fig. 2). Fig. 4 shows the parsimony tree based on MSB1 sequences.

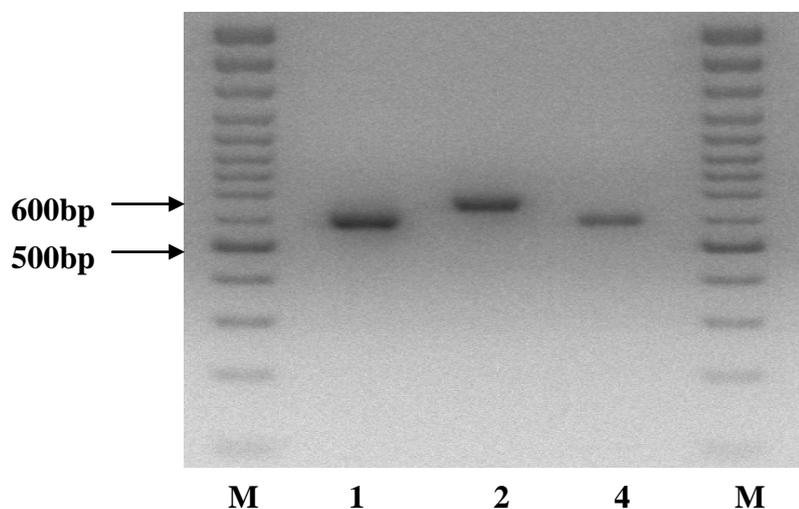


Fig. 2: Amplified MSB1 sequences of three Egyptian *Botrytis cinerea* isolates. M= 100bp DNA Ladder, 1=Egypt 1, 2=Egypt 2, 4=Egypt 4

Results obtained from phylogenetic tree and bootstrap analysis indicate, that the minisatellite sequence at of one of the Egyptian isolates (Egypt 2) differs from both the nine studied Hungarian isolates (previously analyzed [14, 18]), and the other two Egyptian ones (Egypt 1 and Egypt 4, Fig. 4). However, Egypt 1 and Egypt 4 isolates are similar to the Hungarian allele 4 (Fig. 4).

tef1

We amplified and sequenced a 0.3 kb fragment of the large intron of the *tef1* gene from all Egyptian isolates of *B. cinerea* species (Fig. 3).

According to phylogenetic tree based on the *tef1* sequences Egypt 2 isolate is also well separated from all previously studied Hungarian isolates (Fig. 5).

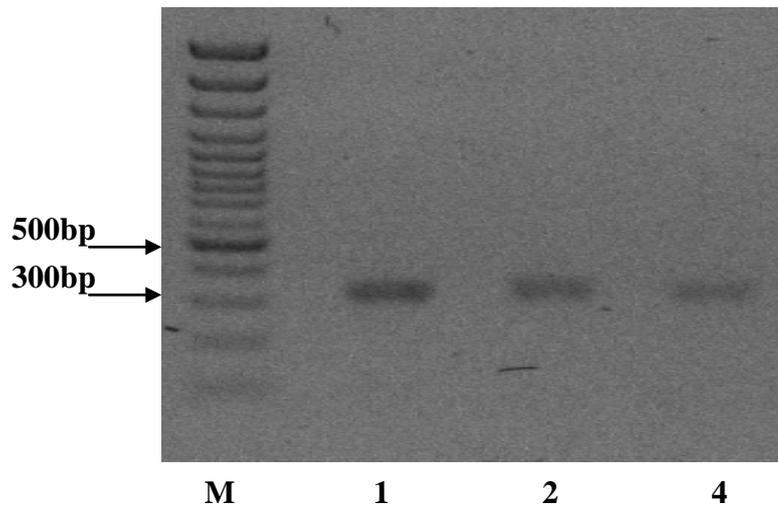


Fig. 3: Amplified *tef1* sequences of three Egyptian *Botrytis cinerea* isolates. M= 100bp DNA Ladder, 1=Egypt 1, 2=Egypt 2, 4=Egypt 4

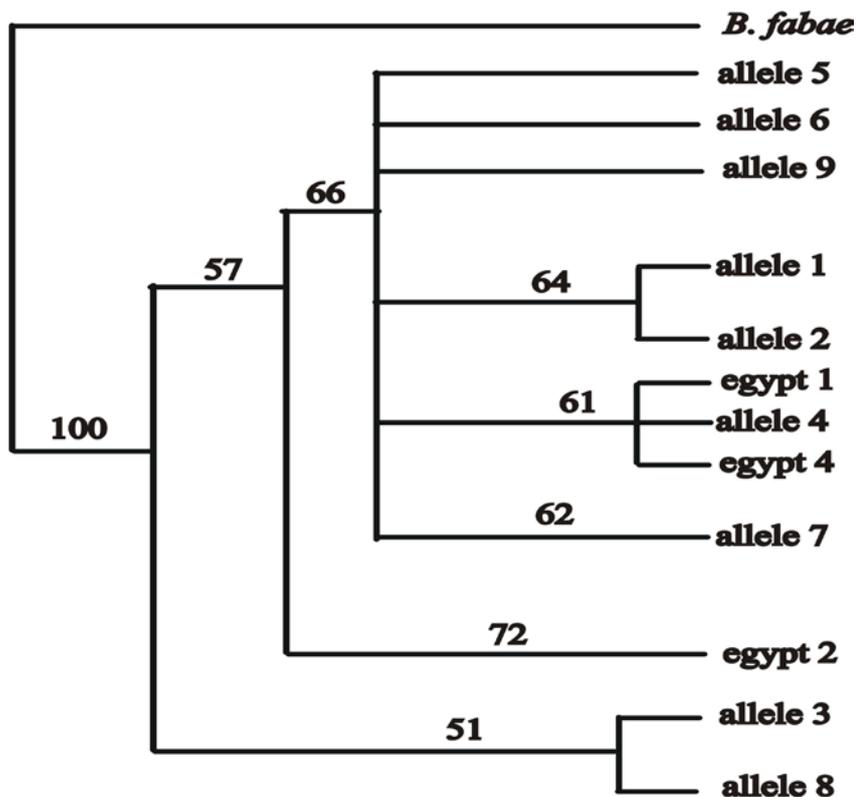


Fig. 4: Phylogenetic relationships of *Botrytis cinera* isolates inferred by the parsimony analysis of MSB1 variant repeat sequences. The numbers above the lines represent the bootstrap (bootstrap=1000) values. Allele 1-9 represents the different sequences of *B. cinerea* isolates, previously described in Hungary [14, 18].

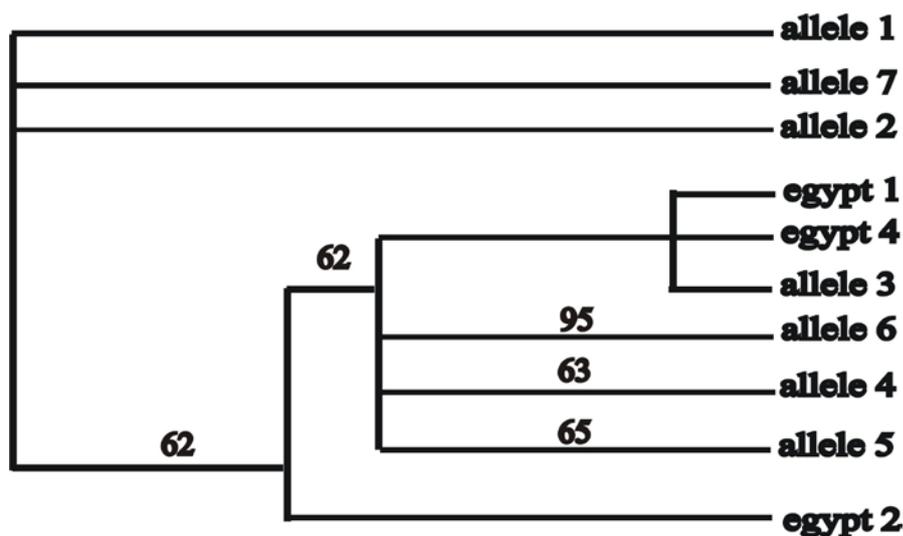


Fig. 5: Phylogenetic relationships of *Botrytis cinera* isolates inferred by the parsimony analysis of *tefl* sequences. The numbers above the lines represent the bootstrap (bootstrap=1000) values. Allele 1-7 represents the different sequences of *B. cinerea* isolates, (isolates previously described in Hungary [14, 18])

Conclusions

The present result confirmed that two of Egyptian isolates (Egypt 1 and Egypt 4) have the same molecular characteristic as the Hungarian isolates as to MSB1 and *tefl*. Such results can attribute from either phylogenetic relationship or conidial dispersal between both countries by wind transfer or human activities. On the other hand, Egypt 2 isolate represent a unique characteristic, which has never been described in Hungary [14, 18], or in France [6].

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VARIABILITATEA MOLECULARĂ A UNOR IZOLATE DE *BOTRYTIS CINEREA* DIN EGIPT ȘI UNGARIA

(Rezumat)

A fost efectuat un studiu privind variabilitatea unor izolate de *Botrytis cinerea* din Egipt și Ungaria pe baza investigațiilor moleculare. Au fost identificate secvențele din minisateliții MSB1 și markerul molecular cel mai răspândit pentru lucrările de filogenie, precum și factorul de elongare subunitatea alfa 1 (EF-1 α = *tefl*). Analiza filogenetică a fost realizată prin investigarea parsimonială (minimală) cu echipamentul PAUP*4.0b. Vigoarea topologică în analiza parsimonială a fost apreciată folosind 1000 de replici. Rezultatele au arătat că două dintre cele trei izolate egiptene de *B. cinerea* s-au dovedit similare cu cele din Ungaria pe baza minisateliților și a secvențelor *tefl*, unul singur fiind complet diferit. Asemănarea dintre izolatele din Ungaria și cele din Egipt se datorează relațiilor filogenetice sau dispersării conidiilor între cele două țări.

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