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ORIGINAL ARTICLE

A UNIVERSAL METHOD FOR THE IDENTIFICATION OF GENES ENCODING AMATOXINS AND PHALLOTOXINS IN POISONOUS MUSHROOMS

*Agata Wołoszyn, Roman Kotłowski**

Gdansk University of Technology,
Department of Molecular Biotechnology and Microbiology, Gdansk, Poland

ABSTRACT

Background. As the currently known diagnostic DNA targets amplified in the PCR assays for detection of poisonous mushrooms have their counterparts in edible species, there is a need to design PCR primers specific to the genes encoding amanitins and phallotoxins, which occur only in poisonous mushrooms.
Objective. The aim of the study was testing of PCR-based method for detection of all genes encoding hepatotoxic cyclic peptides - amanitins and phallotoxins present in the most dangerous poisonous mushrooms.
Material and Methods. Degenerate primers in the PCR were designed on the basis of amanitins (n=13) and phallotoxins (n=5) genes in 18 species of poisonous mushrooms deposited to Genbank of the National Center for Biotechnology Information.
Results. The specificity of the PCR assays was confirmed against 9 species of edible mushrooms, death cap - *Amanita phalloides* and panther cap - *Amanita pantherina*.
Conclusions. Designed two couples of PCR-primers specific to amanitins and phallotoxins genes can be recommended for detection of *Amanita phalloides* and other mushroom species producing hepatotoxic cyclic peptides - amanitins and phallotoxins.

Key words: *poisonous mushrooms, amanitins, phallotoxins, PCR, Amanita phalloides*

STRESZCZENIE

Wprowadzenie. Ponieważ, obecnie znane diagnostyczne cele molekularne w genomach trujących grzybów kapeluszowych amplifikowane metodą PCR mają swoje odpowiedniki u grzybów jadalnych, istnieje potrzeba zastosowania specyficznych sekwencji starterowych wobec amanityn i fallotoksyn, występujących jedynie u grzybów trujących.
Cel. Celem prowadzonych badań było sprawdzenie przydatności sekwencji starterowych do reakcji PCR specyficznych wobec wszystkich aktualnie poznanych genów kodujących hepatotoksyczne cykliczne peptydy - amanityny oraz fallotoksyny trujących grzybów kapeluszowych.
Materiał i Metody. Sekwencje oligonokleotydowe starterów do reakcji PCR zaprojektowane zostały w oparciu o zdeponowane w Genbanku geny amanityn (n=13) oraz fallotoksyn (n=5).
Wyniki. Specyficzność opracowanych testów PCR potwierdzono wobec 9 gatunków grzybów jadalnych oraz muchomora sromotnikowego - *Amanita phalloides*, jak i muchomora plamistego - *Amanita pantherina*.
Wnioski. Zastosowane sekwencje starterowe do wykrywania genów kodujących amanityny i fallotoksyny metodą PCR, mogą być wykorzystane do wykrywania muchomora sromotnikowego oraz innych gatunków zdolnych do syntezy amanityn oraz fallotoksyn.

Słowa kluczowe: *grzyby trujące*, *amanityny, fallotoksyny, PCR, Amanita phalloides*

INTRODUCTION

Gathering of edible mushrooms, although associated with a high risk of mistaking with poisonous mushroom species, is a popular pastime for many people especially from Eastern-European countries. However, recently in Poland significant decline in the number of mushroom poisoning cases has been observed, indicating that more careful and proper criteria are taken into consideration during identification of edible mushroom species. Based on the information from the National Institute of Public Health in Warsaw, in the range between 1995 and 2016 about 8-times decrease in the number of mushroom poisoning cases was registered in Poland (Figure 1) [10] calculated based on nonlinear regression graph using Past3 software [4].

* **Corresponding author:** Roman Kotłowski, Gdansk University of Technology, Department of Molecular Biotechnology and Microbiology, Narutowicza 11/12, 80-233 Gdansk, Poland; Tel.:+48 58 3472383; Fax: +48 58 3471822; e-mail: romkotlo@pg.gda.pl.

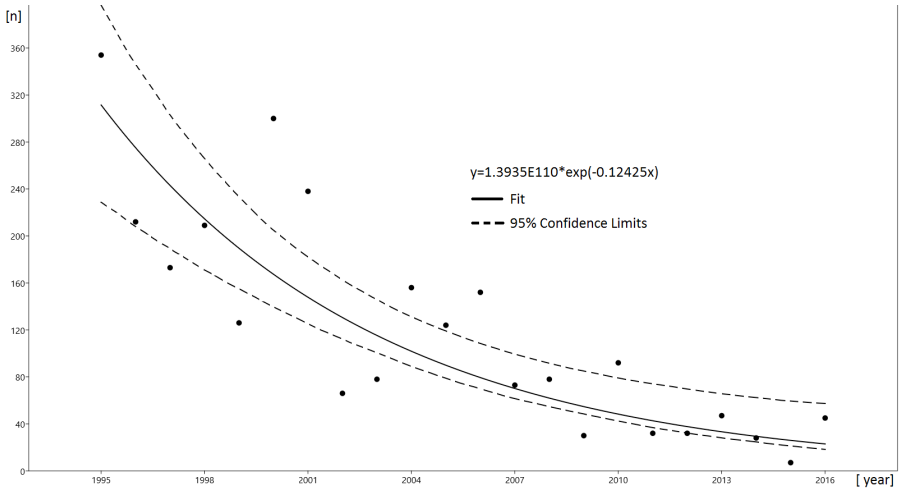

Figure 1 Annual numbers of mushroom poisoning cases in Poland registered in the range between 1995-2016. Data extracted from the National Institute of Public Health-National Institute of Hygiene in Warsaw [10]. Nonlinear regression graph created in Past 3 software [4].

Usually, the poisonings resulted from mistaking death cap *Amanita phalloides* for the edible green-cracking russula *Russula virescens*, the white forms of deathcap for the field mushroom *Agaricus campestris*, and poisonous sulphur knight *Tricholoma sulphureum* for the yellow knight *Tricholoma equestre*. Identification of the microscopic spores of poisonous mushrooms obtained from clinical samples is usually associated with certain difficulties [6, 9], and so routine diagnostics increasingly often use genetic tests based on amplification of specific regions of the DNA characteristic of the *Amanita phalloides* [8], or *A. phalloides*, *A. virosa* and *A. verna* simultaneously in a single PCR assay [2]. While the sequences amplified in the PCR assays have their counterparts in edible species, sequences of PCR primers used in this study were specific to the genes encoding amanitins and phallotoxins which occur only in poisonous mushrooms [3]. This minimizes the risk of non-specific amplification of edible fungi DNA. The aim of the study was development of PCR-based test for detection of all genes encoding amatoxins and phallotoxins present in some poisonous mushrooms.

MATERIAL AND METHODS

The research material used in this study came from the forests near the city of Gdansk and were collected from previously frozen *Amanita phalloides* mushrooms. Species of edible mushrooms: fresh portobello *Agaricus bisporus*, dried bolete *Boletus badius*, marinated *Pholiota nameko*, marinated chanterelle *Cantharellus cibarius*, dried *Leccinum aurantiacum*, dried birch bolete *Leccinum scabrum*, fresh parasol mushroom *Macrolepiota procera* or fresh yellow knight *Tricholoma equestre*, and larch bolete *Suillus grevillei* were purchased in shops or in the marketplace. The diagram of mushroom DNA extraction is shown in Figure 2.

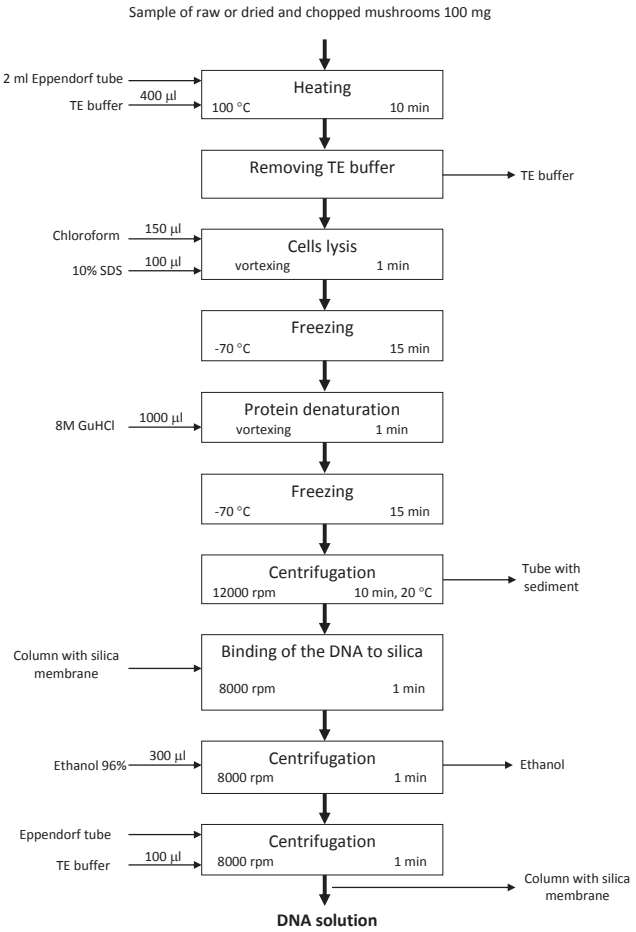

Figure 2. DNA extraction procedure from raw and dried mushrooms.

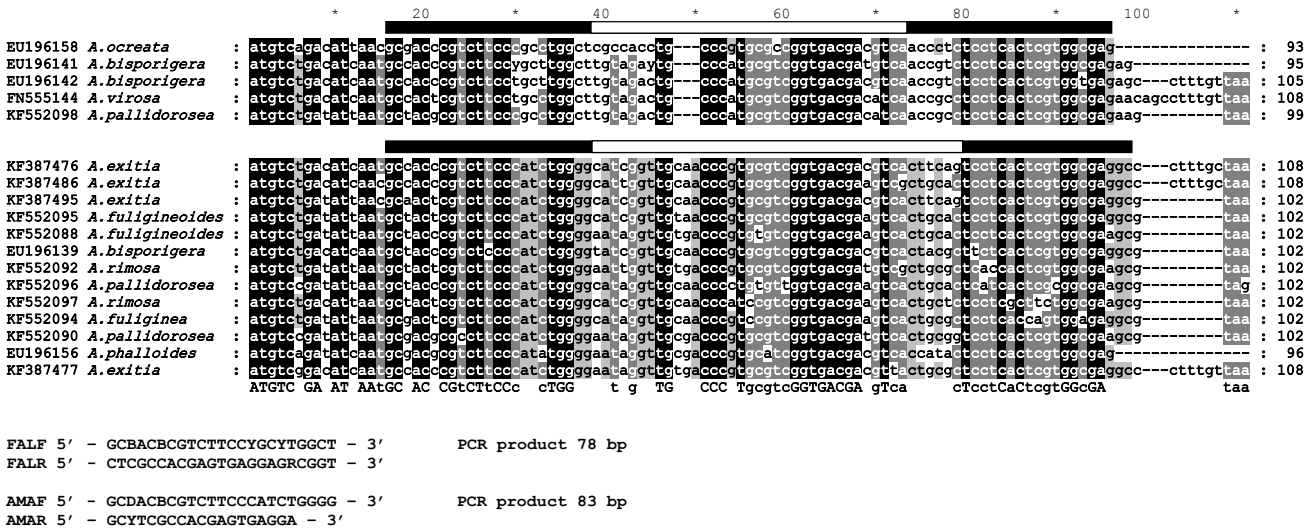

Figure 3. Multiple alignment of DNA sequences for known genes encoding phallotoxins and amatoxins. Black lines indicate DNA amplification products for phallotoxins and amatoxins, respectively

The *A. phalloides* identification was performed by PCR using oligonucleotide primers specific for the introns of the gene encoding aldehyde-3-phosphate dehydrogenase [8]. For the purposes of the specific PCR we carried out a comparison of all amanitins and phallotoxin genes available in Genbank (Figure 3), and on this basis we selected evolutionarily conserved regions for the amplification of toxin-encoding genes. Temperature-time profile of the PCR reaction consisted of 30 cycles, consisting of denaturation at 94°C for 1 min, annealing of primer sequences from the template DNA at 68°C for 1 min and the DNA elongation at 72°C for 1 min. The PCR partition was carried out in TBE buffer (10.5 g Tris-HCl, 5.5 g boric acid, 0.45 g EDTA in 1 litre of distilled water) in 3% agarose gel containing ethidium bromide.

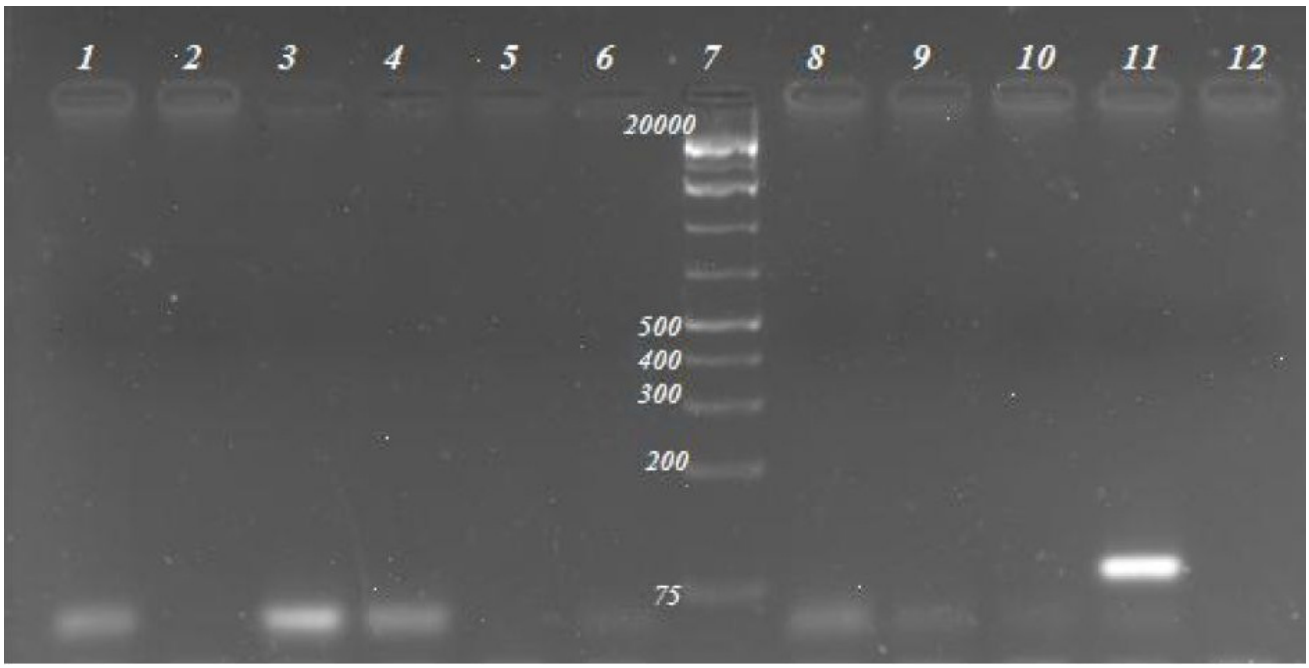

Figure 4. Results of DNA amplification for genes encoding amatoxins using primers AMAF and AMAR. Testing specific ity of oligonucleotide primers. Gel lines: 1- fresh Brown Champignon, 2- dried *Xerocomus badius*, 3- marinated *Pholiota nameko*, 4- marinated *Cantharellus cibarius*, 5- dried *Leccinum aurantiacum*, 6- dried *Leccinum scabrum*, 7- DNA ladder 75-20000 bp, GeneRuler 1kb Plus DNA Ladder, 8- fresh *Macrolepiota procera*, 9- fresh *Tricholoma equestre*, 10- dried *Suillus grevillei*, 11- fresh *Amanita phalloides*, 12- negative control.

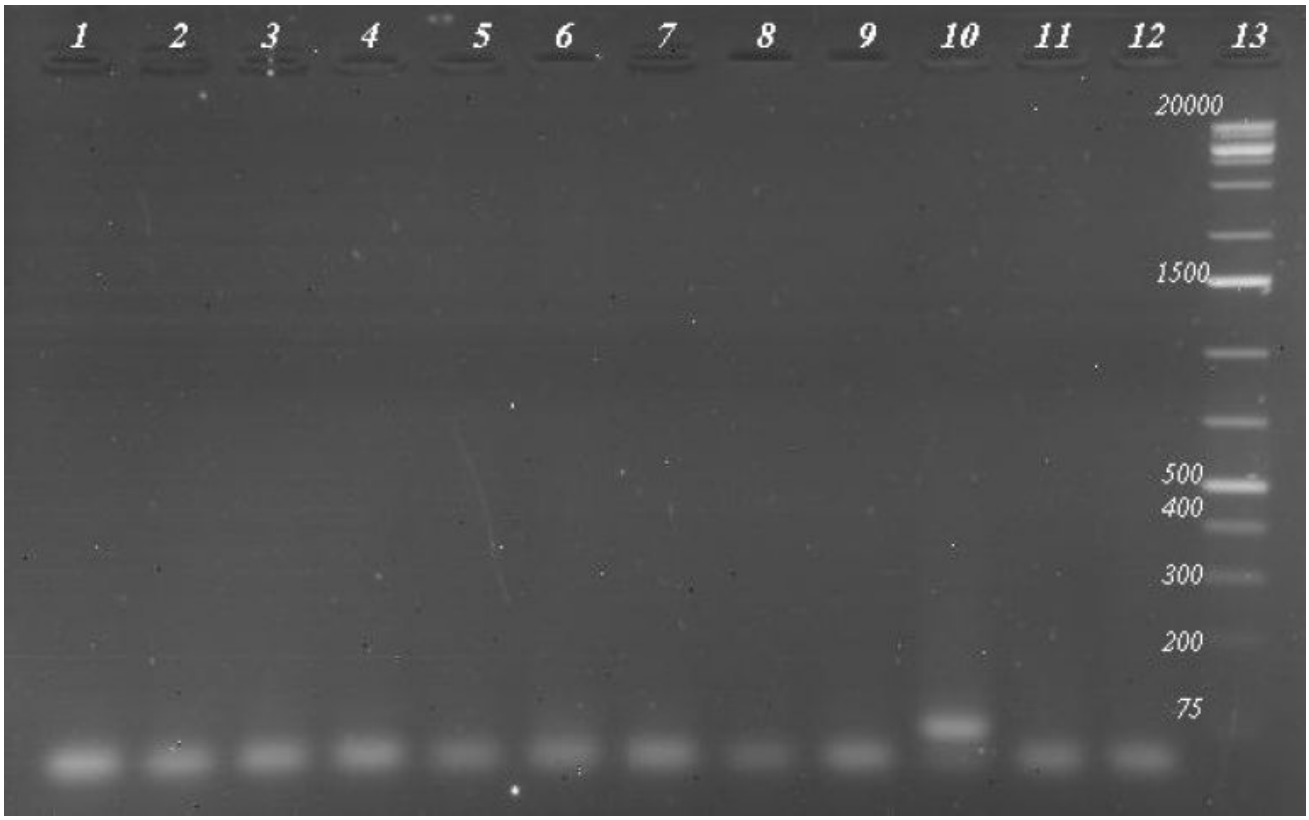

Figure 5. Results of DNA amplification for genes encoding amatoxins using primers FALF and FALR. Testing specificity of oligonucleotide primers. Gel lines: Gel lines: 1- fresh Brown Champignon *Agaricus bisporus*, 2- dried *Xerocomus badius*, 3- marinated *Pholiota nameko*, 4- marinated *Cantharellus cibarius*, 5- dried *Leccinum aurantiacum*, 6- dried *Leccinum scabrum*, 7- dried *Suillus grevillei*, 8- fresh *Macrolepiota procera*, 9- fresh *Tricholoma equestre*, 10- fresh *Amanita phalloides*, 11- fresh *Amanita pantherina*, 12- negative control. 13- DNA ladder 75-20000 bp, GeneRuler 1kb Plus DNA Ladder.

RESULTS

In the study we tested primers [7] specific for the genes encoding amanitins and phallotoxins (Figure 3). Figures 4 and 5 show the results of the amplification of amatoxins and phallotoxins, and the lack of amplification for nine DNA samples of edible mushrooms. The results of electrophoresis confirmed the specificity of the primers AMAF and AMAR for the genes encoding amanitins, because only for well no. 11 did we obtain a band of the right length of 83 bp. This means that only in the case of the gene encoding the toxin specific primers specifically hybridized with the template DNA, allowing the amplification of the selected DNA fragment. Previously extracted DNA was also subjected to PCR using specific primers for phallotoxins – FALF and FALR. The performed agarose electrophoresis confirmed that the PCR product was specific for phallotoxins, because only in the case of well no. 10 did we obtain a band of the right length of 78 bp.

DISCUSSION

Among published PCR-based tests for detection of poisonous mushrooms generally two molecular targets like *rrn* operon containing highly specific ITS regions [2] and *gpd* gene intron sequences [8] were described. Interestingly, *Vilgalys* et al., (1990) [12] have found that rDNA-region of plant pathogen basidiomycete *Thanatephorus praticola* is repeated about 59 times in one genome. Based on this information we can speculate about possibility of increasing sensitivity of DNA detection using ITS sequences in the PCR test for other *Basidiomycetes*, especially *Amanita phalloides* and its white varieties, often related with severe poisonings.

Amanitins poisonings are associated with acute clinical symptoms resulting from the impairment of many organs, sometimes leading to liver transplantation or even to patient death. The identification of mushrooms species by microscopic analysis of spores does not always bring satisfactory results for clinical samples. That is why methods based on the analysis of nucleic acids may be a valuable complementation to standard diagnostic methods.

Degenerate primers specific for the genes encoding amanitins and phallotoxins [7], made it possible to identify the species *A. phalloides* using a PCR method. Based on the comparative analysis of genes fragments encoding amanitins and phallotoxins we can speculate, that presented oligonucleotide primers will allow for

specific amplification of genes coding for amanitins and fallotoxins regardless of the mushroom species producing these toxins. Moreover, we excluded the possibility of obtaining a positive result for selected species of mushrooms which do not produce amatoxins and phallotoxins. The method of DNA extraction used in this paper may be applied for the isolation of nucleic acids from fresh and dried samples. In the comparison of presented DNA extraction method to the method developed for DNA extraction from plants [1] and later adapted for mushroom samples [5, 11], there is no need to use liquid nitrogen and all stages in the DNA extraction and purification procedures can be performed using disposable materials in order to avoid contamination.

CONCLUSIONS

Presented in this paper two couples of PCR-primers specific to amanitins and phallotoxins genes can be recommended for detection of *Amanita phalloides* and other poisonous mushroom species producing hepatotoxic cyclic peptides.

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