Journal of Animal Environment

(E-ISSN 2717-1396, ISSN 2717-1388)

Original Research Paper

IN-SILICO IDENTIFICATION OF A CANDIDATE PKS-BASED DNA PROBE FOR THE DETECTION OF OCHRATOXIN-ASSOCIATED STRAINS OF ASPERGILLUS SEQUENCES

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ABSTRACT

Ochratoxin A (OTA) is an important mycotoxin associated with several filamentous fungi and represents a concern in food and agricultural commodities. Rapid and reliable identification of OTA-associated fungal strains is therefore important for the early assessment of potential contamination. Molecular approaches targeting genes involved in OTA biosynthesis may provide an alternative to conventional microbiological identification methods. The present study investigated the feasibility of identifying a candidate DNA probe targeting a polyketide synthase (PKS)-associated sequence through an in-silico bioinformatics workflow. The OTA biosynthetic pathway was initially examined using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and candidate biosynthetic genes, including PKS, non-ribosomal peptide synthetase (NRPS), and chloroperoxidase, were considered. PKS was selected as the primary target because of its involvement in the biosynthetic pathway and the availability of sequence information. Candidate PKS sequences and domains, including ketosynthase (KS), C-methyltransferase (C-MeT), and acyltransferase (AT), were examined, and six candidate regions were selected for primer design. Primer characteristics were evaluated using PrimerStat. The predicted melting temperatures (Tm) of the six candidates ranged from 57.00 to 64.78°C. Among the candidates, SEQ3 showed a predicted Tm of 57°C and a corresponding selected/experimental value of 58°C and was therefore chosen for further probe-development analysis. A 15-nucleotide candidate probe was subsequently considered based on GC content, predicted hybridization characteristics, sequence composition, and potential mismatch discrimination.The study provides a preliminary framework for PKS-based molecular detection of OTA-associated Aspergillus sequences.

Keywords: Ochratoxin A; Aspergillus; polyketide synthase; PKS; DNA probe; bioinformatics; in-silico analysis; fungal detection

1. INTRODUCTION

Ochratoxin A (OTA) is a secondary fungal metabolite of considerable importance because of its occurrence in food and agricultural commodities and its potential adverse effects on human and animal health (Menka et al., 2024; Nazareth et al., 2024). OTA is primarily produced by certain species of *Aspergillus* and *Penicillium*, including *A. carbonarius, A. niger, A. westerdijkiae, A. steynii, P. verrucosum,* and *P. nordicum* (Nazareth et al., 2024; Gao et al., 2023). Consequently, early identification of fungal strains with the potential to produce OTA is important for food safety monitoring and prevention of OTA contamination (Menka et al., 2024; Guo et al., 2023).

Conventional identification of filamentous fungi generally relies on cultural, morphological, and physiological characteristics, including colony morphology, pigmentation, growth characteristics, and microscopic features of reproductive structures. However, identification based on these phenotypic characteristics can be challenging because of phenotypic variability and morphological similarities among closely related species, which may make species-level identification subjective or unreliable (Moreira et al., 2023; Visagie et al., 2024). DNA sequence-based molecular approaches have therefore become increasingly important as complementary tools for accurate fungal identification, with the internal transcribed spacer (ITS) region being one of the principal molecular targets used for fungal identification and taxonomic classification (Moreira et al., 2023). However, ITS sequencing alone may not adequately discriminate between some closely related fungal species; consequently, additional protein-coding markers such as β-tubulin (BenA), calmodulin (CaM), and RPB2 may provide greater resolution, particularly within *Aspergillus* and *Penicillium* (Visagie et al., 2024). Molecular identification can thus complement conventional characterization and improve the accuracy of identifying fungal isolates, while sequence-based approaches may also support the development of rapid and specific molecular detection assays for important fungal targets.

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Received: 25th May, 2026; Revised: 6th June, 2026; Accepted: 8th June, 2026; Available Online: 29th August, 2026

(DOI): 10.70102/AEJ.2026.18.6s.5

Ochratoxin A (OTA) biosynthesis is a complex process involving multiple enzymatic reactions and a cluster of biosynthetic and regulatory genes (Nazareth et al., 2024). The polyketide synthase (PKS), encoded by *otaA*, plays a central role in the early stage of OTA biosynthesis by catalyzing the formation of the polyketide precursor 7-methylmellein from Acetyl-CoA and malonyl-CoA (Nazareth et al., 2024; Yamina Ben Miri et al.,2024). The resulting intermediate undergoes oxidation, followed by its condensation with L-phenylalanine catalyzed by the non-ribosomal peptide synthetase (NRPS), OtaB, leading to the formation of ochratoxin B (OTB) (Nazareth et al., 2024). Subsequent chlorination of OTB by a halogenase produces the final OTA molecule, while other enzymes, including cytochrome P450 monooxygenases and flavin-dependent oxidoreductases, contribute to oxidative modifications within the pathway (Yamina Ben Miri et al.,2024; Nielsen et al., 2023). Comparative genomic studies have identified a conserved OTA biosynthetic gene cluster containing PKS (*otaA*), NRPS (*otaB*), cytochrome P450 (*otaC*), halogenase (*otaD*), and the bZIP transcription factor (*otaR1*) across several OTA-producing *Aspergillus* and *Penicillium* species (Nielsen et al., 2023). Therefore, conserved regions within OTA biosynthetic genes, particularly *pks* and other pathway-associated genes, may serve as useful molecular targets for the detection and differentiation of potentially ochratoxigenic fungal strains. Real-time PCR studies have, for example, used genes associated with the OTA biosynthetic cluster as molecular targets for detecting OTA-producing *Aspergillus* strains (Xiao Xiao et al.,2024).The development of a DNA probe requires more than the identification of a gene associated with a biosynthetic pathway. A suitable candidate region should possess appropriate sequence characteristics, permit stable probe-target hybridization, and, importantly, demonstrate sufficient sequence discrimination between intended target organisms and non-target organisms. Therefore, bioinformatic analysis provides an important preliminary stage before experimental assay development.

The present study was undertaken to investigate the possibility of selecting a candidate DNA probe targeting a PKS-associated sequence using an in-silico approach. The workflow included analysis of the OTA biosynthetic pathway, selection of candidate PKS regions, primer design and evaluation, selection of a candidate sequence, and preliminary probe-design considerations.

2. MATERIALS AND METHODS

2.1 In-Silico Analysis of the OTA Biosynthetic Pathway

The OTA biosynthetic pathway was examined using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to identify enzymes and genes associated with OTA biosynthesis. The pathway was reviewed to identify biosynthetic steps and genes that could potentially serve as molecular targets.

The pathway analysis indicated the involvement of enzymatic reactions associated with formation of the polyketide precursor and subsequent incorporation of additional components during OTA biosynthesis. On the basis of the biological relevance of the genes involved, PKS, NRPS, and chloroperoxidase-associated genes were considered as candidate targets.

2.2 Selection of candidate biosynthetic genes

Candidate genes associated with OTA biosynthesis were initially considered on the basis of their reported involvement in the biosynthetic pathway. PKS was selected as the primary molecular target for subsequent analysis.

The selection of PKS was based on its role in polyketide biosynthesis and the availability of PKS sequence information from *Aspergillus*. The objective was to identify sequence regions within PKS genes that could subsequently be evaluated for primer and probe development.

2.3 Retrieval and Comparative Analysis of PKS Sequences

PKS-related sequences were obtained from publicly available sequence databases. The study records identify several *Aspergillus niger* PKS gene identifiers, including An01g01130, An04g04340, An11g05940, An15g07920, and An18g00520.

The accession numbers corresponding to these sequences were not retained in the available study records. Therefore, accession numbers are not reported here rather than being reconstructed or assigned retrospectively.

The retrieved sequences were compared using sequence similarity analysis to identify potentially useful regions for molecular marker development. Sequence similarity analysis was used to assess relationships among candidate PKS sequences and related fungal sequences.

2.4 Selection of PKS Domains and Candidate Regions

Three PKS-associated functional regions were considered during target selection: the ketosynthase (KS), C-methyltransferase (C-MeT), and acyltransferase (AT) regions.

Candidate regions were selected from these PKS-associated regions for primer development. Six candidate targets were ultimately considered: four KS-associated regions, one C-MeT-associated region, and one AT-associated region.

The candidate genes and primer pairs were:

Gene	Region/ domain	Primer pair	Forward primer (5′–3′)	Reverse primer (5′–3′)	Ta (°C)	Amplicon (bp)
An01g01130	KS	PKS01K	CACTCCACCTTGCTTGTAA	GCCCGTTGCTCTTTGCGAA	58	536
An04g04340	KS	PKS04KS	GTGATGTAGCGTCCAAGCCT	GAGAGCCGATTAGCAAGAA	60	382
An11g05940	KS	PKS11KS	TACGAGGCTATGGAAAATG	AGAGTGGCTGACCTGGCGG	58	863
An15g07920	KS	PKS15KS	CAATGCCGTCCAACCGTATG	CCTTCGCCTCGCCCGTAG	60	776
An15g07920	C-MeT	PKS15C-MeT	GCTTTCATGGACTGGATG	CATTTCGTTGATCCCATCG	62	998
An18g00520	AT	PKS18AT	AATCCCCTATCTTCCTCCAG	GTTTACAGCAGCAATGACAGC	62	440

2.5 Primer Design and In-Silico Evaluation

Primers were designed from the selected PKS-associated candidate regions. Candidate primers were evaluated using PrimerStat for basic physicochemical characteristics, including melting temperature and GC content.

Six candidate sequences, designated SEQ1–SEQ6, were evaluated.

2.6 Selection of SEQ3

SEQ3 was selected for subsequent probe-design consideration because its predicted Tm of 57°C was close to the corresponding selected/experimental value of 58°C. Its reported GC content was approximately 50%.

Selection was therefore based on the combined consideration of melting temperature and GC content. However, these characteristics alone do not establish sequence specificity. Accordingly, the designation of SEQ3 as the preferred candidate should be interpreted as an initial in-silico selection rather than proof of diagnostic specificity.

2.7 Candidate Probe Design

A 15-nucleotide candidate probe was considered from the selected SEQ3 region. Probe design took into consideration nucleotide composition, GC content, predicted hybridization stability, and the potential for sequence discrimination.

The available study records report an approximately 50% GC content for the candidate probe. Because a 15-nucleotide sequence cannot contain exactly 50% GC when expressed as an integer number of G/C nucleotides, the GC proportion should be reported as an approximate value unless the actual sequence is available.

2.8 Probe Sequence Composition and Mismatch Considerations

The candidate probe was designed as a relatively short oligonucleotide to permit sequence discrimination. Mismatches within the probe-target duplex were considered important because their position may influence hybridization stability. Central mismatches were considered preferable for discrimination because they may have a greater effect on duplex stability than mismatches located at the terminal positions.

3. RESULTS

3.1 Analysis of the OTA Biosynthetic Pathway

The OTA biosynthetic pathway was examined using KEGG to identify genes and enzymes potentially suitable for molecular targeting. The pathway analysis indicated that OTA biosynthesis involves multiple enzymatic steps, including formation of a polyketide-derived precursor and subsequent enzymatic reactions leading to OTA formation.

Based on the pathway analysis, PKS, NRPS, and chloroperoxidase-associated genes were considered as potential molecular targets. PKS was selected for subsequent analysis because of its role in polyketide biosynthesis and the availability of sequence information.

3.2 Identification of Candidate PKS Regions

Candidate sequences from PKS-associated KS, C-MeT, and AT regions were investigated. Six candidate regions were selected for primer development.

The six candidate targets generated predicted amplicons ranging from 382 to 998 bp. Four candidate regions were associated with KS, one with C-MeT, and one with AT.

3.3 Evaluation of Candidate Sequences

The six candidate sequences showed predicted Tm values ranging from 57.00 to 64.78°C. SEQ1 had a predicted Tm of 60.15°C, SEQ2 64.78°C, SEQ3 57.00°C, SEQ4 57.18°C, SEQ5 64.50°C, and SEQ6 58.24°C.

The study records report the following predicted and selected/experimental Tm values:

Table3.3: Predicted and Selected/Experimental Tm values

Candidate	Selected/experimental value (°C)	Predicted Tm (°C)
SEQ1	58	60.15
SEQ2	60	64.78
SEQ3	58	57.00
SEQ4	60	57.18
SEQ5	62	64.50
SEQ6	62	58.24

SEQ3 showed the closest agreement between the reported selected/experimental value of 58°C and predicted value of 57°C. On this basis, together with its reported approximately 50% GC content, SEQ3 was selected for further probe-design consideration.

3.4 Selection of the Candidate Probe

A 15-nucleotide probe was subsequently considered from the selected sequence. The candidate was evaluated on the basis of nucleotide composition, GC content, hybridization considerations, and potential sequence discrimination.

The available records indicate that the candidate probe had approximately 50% GC content. Hence SEQ3 was selected as a suitable candidate.

4. DISCUSSION

The identification of molecular targets associated with OTA biosynthesis provides a promising strategy for the rapid detection of fungi with OTA-producing potential. In the present study, the OTA biosynthetic pathway was initially examined to identify genes that could serve as suitable molecular targets. **Polyketide synthase (PKS)** was selected among the candidate biosynthetic genes because of its essential role in the formation of the polyketide precursor during OTA biosynthesis and the availability of PKS sequence information from several OTA-producing fungi. Comparative genomic and functional studies have established that the PKS gene, *otaA*, is an essential component of the OTA biosynthetic pathway, with OtaA catalyzing the first committed step in the formation of the OTA precursor 7-methylmellein (Wang et al., 2018). The suitability of PKS as a molecular target is further supported by O'Callaghan et al. (2003), who identified a PKS gene in *Aspergillus ochraceus* that was expressed under OTA-producing conditions, whereas disruption of this gene resulted in complete loss of OTA production, providing direct functional evidence for its involvement in OTA biosynthesis.

Subsequent studies have demonstrated the occurrence of OTA-associated PKS genes in several ochratoxigenic species, including *A. carbonarius*, *A. niger*, *A. westerdijkiae*, *Penicillium nordicum*, and *P. verrucosum* (Gil-Serna et al., 2018). Comparative genomic analysis further demonstrated that the core OTA biosynthetic cluster is conserved across multiple OTA-producing *Aspergillus* and *Penicillium* species and contains genes encoding PKS, NRPS, cytochrome P450 monooxygenase, and halogenase. These findings provide a strong biological basis for selecting PKS as a molecular target for the detection of potentially ochratoxigenic fungi.

In the present study, three conserved PKS-associated regions—**β-ketoacyl synthase (KS), C-methyltransferase (C-MeT), and acyltransferase (AT)**—were examined, and six candidate regions were selected for primer development. The predicted amplicon sizes ranged from **382 to 998 bp**, providing a basis for comparative evaluation of the candidate sequences. The selection of conserved functional regions within fungal PKSs is supported by previous investigations in which KS and AT domains were successfully amplified and characterized in OTA-producing *Aspergillus* species. Martínez-Culebras et al. (2009), for example, used degenerate primers targeting the **AT domain** of fungal PKSs to obtain and compare PKS sequences from OTA-producing *Aspergillus* and *Penicillium* species, demonstrating the usefulness of this conserved functional region for molecular analysis of ochratoxigenic fungi. Similarly, studies investigating the diversity of PKS genes in *A. ochraceus* and *A. carbonarius* successfully amplified multiple **KS-domain sequences**, demonstrating that KS represents a conserved and experimentally accessible region of fungal PKS genes. (Atoui et al. (2006)

The relevance of KS and AT regions to OTA biosynthesis is further supported by comparative analysis of OTA-associated PKSs. Zhang et al. (2015) identified OTA-related PKS genes in *A. ochraceus* and demonstrated that the predicted proteins shared substantial similarity with PKSs from OTA-producing *A. carbonarius* and *A. niger*. Phylogenetic analysis of both the **KS and AT domains** showed relationships among OTA-associated PKSs from *A. ochraceus*, *A. carbonarius*, *A. niger*, and *A. westerdijkiae*, supporting the use of these conserved domains for comparative molecular analysis. More recently, Melki et al. (2023) also employed primer pairs targeting the **KS and AT regions** of fungal PKSs to obtain PKS fragments from *A. carbonarius*, *A. niger*, and *A. tubingensis*, further demonstrating the applicability of these domains for molecular investigation of black *Aspergillus* species.

The potential of PKS-based molecular detection has also been demonstrated experimentally. In *P. nordicum*, Geisen et al. (2004) isolated a PKS fragment using degenerate primers and subsequently developed a real-time PCR assay targeting the OTA-associated PKS gene. The target was strongly induced under OTA-producing conditions, and its expression showed a strong relationship with OTA production in wheat, demonstrating the potential of an OTA-associated PKS sequence for molecular monitoring. Importantly, the assay detected the target in *P. nordicum* but not in the closely related *P. verrucosum*, demonstrating that selection of an appropriate PKS region can provide discriminatory power even among closely related ochratoxigenic species.

Similarly, a real-time PCR assay developed for OTA-producing strains belonging to the *A. niger* aggregate targeted a PKS sequence associated with the OTA biosynthetic cluster. The assay used a short 120-bp target and was evaluated against **91 fungal strains**, including reference strains and food isolates; the assay specifically detected ochratoxigenic strains of the *A. niger* aggregate, while other food-associated fungi tested negative. This study provides important supporting evidence for the present approach because it demonstrates that an OTA-associated PKS region can be developed into a rapid molecular detection assay, provided that the selected region has sufficient sequence specificity.

PrimerStat analysis in the present study indicated differences among the six candidate sequences in their predicted melting temperatures (Tm). **SEQ3 showed a predicted Tm of 57°C and a selected/experimental value of 58°C**, indicating close agreement between the predicted and selected values. In addition, the approximately **50% GC content** of SEQ3 was considered favorable for further primer/probe development. These characteristics supported the selection of SEQ3 as a promising candidate for subsequent probe-development analysis. However, the agreement between predicted and experimental Tm and the favorable GC content should be interpreted as **initial primer/probe-design criteria rather than evidence of target specificity**. The successful PKS-based assays reported for *P. nordicum* and OTA-producing *A. niger* strains demonstrate that specificity depends on the sequence selected as well as on subsequent experimental validation against target and non-target fungi.

This consideration is particularly important when developing molecular assays for ***Aspergillus* and *Penicillium***, because PKS genes belong to large multigene families and conserved functional domains may occur in PKSs involved in the biosynthesis of different secondary metabolites. For example, analysis of KS domains in *A. ochraceus* and *A. carbonarius* identified multiple distinct KS sequences within each species, illustrating the diversity of fungal PKS genes and the potential for conserved-domain primers to amplify non-target PKS genes. Similarly, analysis of AT domains from *Aspergillus* and *Penicillium* demonstrated that these sequences can be related to PKSs involved in different mycotoxin biosynthetic pathways, emphasizing that conserved-domain detection does not automatically confer OTA specificity.

The importance of evaluating closely related species is also demonstrated by studies of the OTA biosynthetic cluster. Comparative analysis of 21 ochratoxigenic species showed that the core OTA cluster is highly conserved and contains homologous genes encoding PKS, NRPS, cytochrome P450, halogenase, and regulatory proteins. (Ferrara et al.2020) Although this conservation supports the use of biosynthetic genes as molecular targets, it also indicates that sequence similarity among related fungi must be carefully considered when designing species- or toxin-producer-specific assays. The orthologous nature of OTA biosynthetic genes across *Aspergillus* and *Penicillium* has likewise been demonstrated through comparative genomic analysis, in which OTA biosynthetic genes were consistently expressed in OTA-producing strains under permissive conditions.

Therefore, although **SEQ3 demonstrated favorable physicochemical characteristics**, including a predicted Tm of 57°C, an experimental/selected Tm of 58°C, and approximately 50% GC content, these parameters alone are insufficient to establish probe specificity. The present result should consequently be interpreted as the **preliminary selection of SEQ3 as a candidate region for further validation**. BLAST-based sequence comparison against both target and non-target fungi, multiple-sequence alignment, and in-silico specificity analysis should be performed before confirming its suitability as an OTA-producer-specific molecular target. Previous PKS-based detection studies demonstrate the importance of testing candidate targets against closely related *Aspergillus*, *Penicillium*, and other food-associated fungi before establishing assay specificity. Subsequent experimental validation using representative OTA-producing and non-OTA-producing strains, followed by determination of analytical specificity and sensitivity, would further establish the applicability of SEQ3 for rapid molecular detection of fungi with OTA-producing potential.

5. CONCLUSION

The OTA biosynthetic pathway was analysed to identify a suitable molecular target for rapid detection of potentially ochratoxigenic fungi. Among the candidate PKS, NRPS, and chloroperoxidase-associated genes, **PKS was selected** due to its central role in OTA biosynthesis and sequence conservation among OTA-producing fungi. Six candidate sequences from the KS, C-MeT, and AT regions were identified, of which **SEQ3 showed the most favourable characteristics**, with predicted and experimental Tm values of 57°C and 58°C and approximately 50% GC content. These findings support SEQ3 as a promising candidate for probe development. However, further in-silico specificity analysis and experimental validation against OTA-producing and non-producing fungi are required to confirm its diagnostic potential.

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