A PCR method to identify Ochratoxin A-producing *Aspergillus westerdijkiae* strains on dried and aged foods

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- **Highlights**
- Two OTA chemotypes were identified among *A. westerdijkiae* isolates from cheese.
- A natural deletion of *otaR* biosynthetic gene was identified in *A. westerdijkiae*
- A PCR assay targeting the *otaR* distinguishes between OTA producers and nonproducers

Abstract

Ochratoxins are a group of mycotoxins that frequently occur as contaminants in agricultural commodities and foods, including dry-cured meats and cheeses. The fungus *Aspergillus westerdijkiae* is frequently isolated from aged foods and can produce ochratoxin A (OTA). However, individual strains of the fungus can have one of two OTA production phenotypes (chemotypes): OTA production and OTA nonproduction. Monitoring and early detection of OTA-producing fungi in food are the most effective strategies to manage OTA contamination. Therefore, we examined genome sequence data from five *A. westerdijkiae* strains isolated from the surface of cheese from southern Italy to identify genetic markers indicative of the twoOTA chemotypes. This analysis revealed a naturally occurring deletion of the OTA regulatory gene, *otaR*, in an OTA-nonproducing isolate.We used this information to design a polymerase chain reaction (PCR) method that could identify *A. westerdijkiae* and distinguish between the two OTA chemotypes. In this method, the PCR primers were complementary to conserved sequences flanking *otaR* and yielded different-sized amplicons from strains with the different chemotypes. The primers did not yield *ota*-region-specific amplicons from other OTA-producing species. Because the method is specific to *A. westerdijkiae* and can distinguish between the two OTA chemotypes, it has potential to significantly improve OTA monitoring programs.

Keywords

Primers; cheese; fungi; preserved meat; surface; mycotoxin

1. Introduction

Ochratoxins are a group of mycotoxins that occur commonly as contaminants in a variety of agricultural commodities and foods, including dry-cured meats and cheese (Anelli et al., 2019; Biancardi et al.,2013; Dall'Asta et al., 2010; Pattono et al., 2013; Ramos-Pereira et al., 2019;Sakin et al., 2018). Certain species of the filamentous fungi *Aspergillus* and *Penicillium* that produce the toxins are the primary causes of these contamination issues. The ocratoxigenic species *Aspergillus ochraceus*, *A. steynii* and *A. westerdijkiae* are closely related based on phylogenetic and morphological data, and they are frequently isolated from dried and stored foods. However, the latter two species were reported to produce consistently higher levels of ochratoxin A (OTA) than *A. ochraceus* (Gil-Serna et al., 2015; Samson et al., 2014; Sartori et al., 2014).

Most OTA contamination in food was originally attributed to *A. ochraceus*, but since their descriptions as species, *A. westerdijkiae* and *A. steynii* have been acknowledged as the predominant causes of OTA contamination in dried foods. Both species have been recovered from multiple food sources, including coffee (Leitão et al., 2019; Noonim et al., 2008), grapes (Díaz et al., 2009; Gil-Serna et al., 2009), paprika (Santos et al., 2011), barley (Mateo et al., 2011), and recently, also ripened cheese (Anelli etal., 2019) and meat (Merla et al., 2018; Parussolo et al. 2019). *Aspergillus westerdijkiae* has been reported as the cause of OTA contamination of cheese, salami and dry-cured ham, where OTA can diffuse into these foods from the surface, where fungi grow during ripening (Anelli et al. 2019; Berni et al., 2017; Dall'Asta et al., 2010; Iacumin et al., 2009; Parussolo et al., 2019; Rodríguez et al., 2012; Sørensen et al., 2008, Vipotnik et al., 2017). Furthermore, the occurrence of *A. westerdijkiae* and *A. ochraceus* has been reported to occur in Argentina (Canel et al., 2013; Castellari et al., 2010; Vila et al., 2019), Brazil (Parussolo et al., 2019), Italy (Iacumin et al., 2009; 2013), as well as on surfaces of meat products from several other countries (Comi et al., 2004; Huerta et al., 1987; Nunez et al., 1996; Parussolo et al., 2019; Rojas et al., 1991;Strzelecki and

66 Badura, 1972; Sutic et al., 1972; Wang et al., 2006). Due to the worldwide distribution and previous
67 misidentification of *A. westerdijkiae* and *A. ochraceus*, the occurrence of these two species in and on
68 food is gaining attention. During some ripening processes, the surface colonization of food by
69 microorganisms from the ambient environment is allowed and even desired to impart organoleptic
70 qualities that are distinctive to particular producers and locations. In some cases, *A. westerdijkiae* and
71 *A. ochraceus* are among the colonizing microorganisms and could present a mycotoxicological risk
72 to consumers due to their ability to produce OTA.

73 Because removing OTA from food is currently not feasible, monitoring and early detection of
74 ochratoxigenic mould growth in and on food is still the most effective strategy to prevent human
75 consumption of OTA-contaminated food products (Gil-Serna et al., 2011; 2015). A polymerase chain
76 reaction (PCR)-based method would be more rapid than a morphological assay, which require
77 culturing the strains in order to identify them, and less expensive than some chemistry-based assays
78 for OTA. A rapid and specific PCR-based assay for ochratoxigenic *A. westerdijkiae* strains would
79 enhance detection and monitoring programs for this fungus. PCR assays have been developed based
80 on the *otaA* (polyketide synthase) and *otaB* (nonribosomal peptide synthase) genes in multiple OTA-
81 producing fungi (Bogs et al., 2006; Castellá and Cabañes, 2011; Geisen et al., 2004; Luque et al.,
82 2013a, 2013b). However, there are no assays specific for *A. westerdijkiae* or that can distinguish
83 between OTA-producing and nonproducing strains of the fungus.

84 PCR assays for detection of mycotoxigenic fungi rely on knowledge of genes involved in
85 biosynthesis of the target mycotoxin. Comparative sequence, gene-function and biochemical analyses
86 indicate that a cluster of five genes designated *otaA–otaD* and *otaR*. The *otaR* gene encodes a bZIP
87 transcription factor that regulates ochratoxin production by regulating expression of the other *ota*
88 genes, which encode biosynthetic enzymes (Chakrabortti et al., 2016; Han et al., 2016; Wang et al.,
89 2018).

The *ota* genes are located adjacent to one another in a gene cluster in at least seven ochratoxigenic fungal species, including *A. ochraceus*, *A. steynii* and *A. westerdijkiae* (Han et al., 2016; Susca et al. 2016; Wang et al. 2018).Wang et al. (2018) demonstrated that deletion of any of the *ota* genes in *A. ochraceus* prevents formation of OTA. The *ota* gene cluster has also been described in *A. westerdijkiae,* where the functions of the genes in OTA biosynthesis are presumed to be the same (Han et al., 2016).

Anelli et al. (2019) showed that among *A. westerdijkiae* strains isolated from cave cheese in the Apulia region of southern Italy, there were two different OTA production phenotypes (chemotypes): OTA production and OTA nonproduction. The aim of the current study was to determine whether there are nucleotide differences in the OTA biosynthetic gene cluster in strains of *A. westerdijkiae* with the different OTA chemotypes and to develop an OTA monitoring method based on any differences that are identified. We identified a naturally occurring deletion of the *otaR* gene (*Awe04185* in Han et al.,2016) in an OTA-nonproducing cheese isolate of *A. westerdijkiae* identified in the Anelli et al. (2019) study. We used the deletion to develop a PCR assay to detect *A. westerdijkiae* and distinguish between OTA-producing and nonproducing strains of the fungus.

2. Material and methods

2.1 Strains and culture conditions

In this study, we examined 34 strains of *A. westerdijkiae*, isolated from the surface of cave cheese (Anelli et al., 2019), and 17fungal strains from other ochratoxigenic species. All strains are available in the Agri-Food Toxigenic Fungi Culture Collection at the Institute of Sciences of Food Production (ISPA-CNR, Bari, Italy, http://server.ispa.cnr.it/ITEM/Collection/).

2.2 DNA Isolation

115 Each strain was grown as pure culture at 25 °C on Potato Dextrose Agar (PDA), on a cellophane film,
116 for 5-7 days, and then the resulting mycelia were scraped and frozen prior to the DNA isolation
117 procedure. DNA was isolated from 30 to 40 mg of frozen mycelium using the Wizard® Magnetic
118 Purification System for Food kit (Promega, USA) following the manufacturers protocol. Quality and
119 yield of resulting DNA were evaluated by agarose gel electrophoresis.
120
121 *2.3 Evaluation of OTA production*
122 OTA production abilities of the 34 cave-cheese isolates of *A. westerdijkiae* were previously
123 determined by Anelli et al. (2019) using the culture medium yeast extract sucrose agar (YES).The
124 OTA production ability of strains of other species were retrieved from literature (Lippolis et al.,
125 2016; Susca et al., 2016) or from ITEM collection metadata (Table 1).
126

127 **Table 1 - Fungal strains included in the study**

128

Strain ID	Strain ID	Species	OTA	Host
ITEM 18000	A101	*A. westerdijkiae*	+	C
ITEM 18001	A111	*A. westerdijkiae*	+	C
ITEM 18002	A112	*A. westerdijkiae*	+	C
ITEM 18003	A121	*A. westerdijkiae*	+	C
ITEM 18004	A122	*A. westerdijkiae*	+	C
ITEM 18005	A211	*A. westerdijkiae*	+	C
ITEM 18006	A221	*A. westerdijkiae*	+	C
ITEM 18007	A301	*A. westerdijkiae*	+	C
ITEM 18008	A302	*A. westerdijkiae*	+	C
ITEM 18009	A311	*A. westerdijkiae*	+	C
ITEM 18010	A321	*A. westerdijkiae*	+	C
ITEM 18011	B101	*A. westerdijkiae*	+	C
ITEM 18012	B201	*A. westerdijkiae*	+	C
ITEM 18013	B301	*A. westerdijkiae*	+	C
ITEM 18018	K51	*A. westerdijkiae*	+	C
ITEM 17441	K61	*A. westerdijkiae*	+	C
ITEM 18518	K7A2	*A. westerdijkiae*	+	C
ITEM 17414	L112	*A. westerdijkiae*	-	C
ITEM 18032	M22	*A. westerdijkiae*	+	C
ITEM 17418	M31	*A. westerdijkiae*	-	C

Strain ID	Strain ID	Species	OTA	Host
ITEM 18022	N81	*A. westerdijkiae*	+	C
ITEM 18023	N82	*A. westerdijkiae*	+	C
ITEM 18024	N91	*A. westerdijkiae*	+	C
ITEM 17444	N31	*A. westerdijkiae*	+	C
ITEM 18015	O11	*A. westerdijkiae*	+	C
ITEM 18016	O21	*A. westerdijkiae*	+	C
ITEM 17448	P11	*A. westerdijkiae*	+	C
ITEM 18028	T31	*A. westerdijkiae*	+	C
ITEM 4549	n.a.	*A. ochraceus*	+	G
ITEM 7043	n.a.	*A. ochraceus*	+	n.a.
ITEM 8007	n.a.	*A. ochraceus*	+	n.a.
ITEM 11738	n.a.	*A. ochraceus*	+	n.a.
ITEM 11740	n.a.	*A. ochraceus*	+	n.a.
ITEM 10997	n.a.	*A. ochraceus*	+	n.a.
ITEM 10998	n.a.	*A. ochraceus*	+	n.a.
ITEM 9634	n.a.	*P. nordicum*	+	n.a.
ITEM 13080	n.a.	*P. nordicum*	+	S
ITEM 18025	T111	*A. steynii*	+	C
ITEM 18026	T112	*A. steynii*	+	C
ITEM 9568	n.a.	*A. niger*	+	n.a.

ITEM 17419	M51	*A. westerdijkiae*	-	C
ITEM 18019	N11	*A. westerdijkiae*	+	C
ITEM 18020	N21	*A. westerdijkiae*	+	C
ITEM 18519	N62	*A. westerdijkiae*	+	C
ITEM 18021	N71	*A. westerdijkiae*	+	C
ITEM 18520	N72	*A. westerdijkiae*	+	C

ITEM 10335	n.a.	*A. niger*	+	n.a.
ITEM 9582	n.a.	*P. verrucosum*	+	R
ITEM 15065	n.a.	*A. carbonarius*	+	Co
ITEM 11937	n.a.	*A. carbonarius*	+	V
ITEM 11988	n.a.	*A. carbonarius*	+	V

C = cheese; G = grapes; S = salami; R = rye; Co = corn; V = dried vine fruit sultana;- = OTA no-producing strain; += OTA producing strain; LOD = 2 µg/kg; n.a. = not available.

Strains ITEM 17414, ITEM 17419 and ITEM 18008 were selected for additional OTA production analysis based on their OTA chemotypes, which were determined previously by Anelli et al. (2019), and the gene content of their *ota* cluster, which was determined in the current study. To assess OTA production, each *A. westerdijkiae* strain was cultured on five growth media: 1) basal medium (CIT 5) (Navaratnam et al., 1998);2) czapek's medium with autolysate yeast (CYA 6.5, pH 6.5; Gil-Serna et al., 2018);3) malt extract agar (MEA; Raper and Thom, 1949);4) YES(Frisvad and Samson, 2004); and 5) dichloran glycerol 18% (DG 18; Hocking and Pitt, 1980). To prepare the cultures, a suspension of conidia [10^6 conidia/100µL water] was first prepared from one-week-old potato dextrose agar cultures. A100-µL aliquot of the suspension was spread over one side of a cellophane film, previously laid on the surface of 20 mL of growth medium in a Petri dish. Triplicate cultures were prepared for each strain on each growth medium, and cultures were incubated for 7 days at 25 °C in the dark.

OTA were analyzed according to Susca et al. (2016) with slight modifications. One gram of culture was extract with 5 mL of acetonitrile/ methanol/ water (90: 90: 80, v/v/v) on an orbital shaker for 60 minutes. One milliliter was evaporated to dryness under a stream of nitrogen at 50°C. The residue was dissolved with 1 mL of acetonitrile/ water/ glacial acetic acid (99:99:2, v/v/v) and filtered using RC through 0.20 µm regenerated cellulose filter. Fifty microliter of extract was injected into to HPLC apparatus (Agilent 1260 Series, Agilent Technology, Santa Clara, CA, USA). The analytical

column was a Zorbax SB-C18 (4.6 x 150 mm, 5 µm) with a guard column inlet filter (0.5 µm, 3 mm diameter; Rheodyne Inc., Rohnert Park, CA) and the mobile phase consisted of a mixture of acetonitrile/ water/ glacial acetic acid (99:99:2, v/v/v) at a flow rate of 1 mL/min. The fluorometric detection was set at wavelengths of 333 nm (excitation) and 460 nm (emission).OTA was measured by comparing peak areas with calibration curve obtained with OTA standard solution. The detection limit was 2 µg/kg based on a signal to noise ratio of 3:1.

158

159 *2.4 Examination of* ota *cluster regions in genome sequences*

160 Each isolate was grown in YEDP medium (0.1 yeast extract, 0.1 peptone, 2% dextrose) for 2 days at
161 room temperature with shaking at 200 rpm. Mycelia were harvested by filtration, lyophilized, ground
162 to a powder, and genomic DNA was extracted using the method described by Raeder and Broda
163 (1985). The resulting DNA was further purified with the UltraClean DNA purification kit according
164 to the specifications of the manufacturer (MoBio Laboratories, Inc.). DNA libraries were prepared
165 using a NExtera XT DNA library Preparation Kit, and sequence data were generated with an Illumina
166 MiSeq sequencing platform as specified by the manufacturer (Illumina, San Diego, California). CLC
167 Genomics Workbench (CLC bio, Qiagen, Aarhus, Denmark) was used to process the resulting
168 sequence reads and obtain a *de novo* assembly of each genome.
169 Sequences of the *ota* cluster regions (GenBank accessions: MW526246, MW526247, MW526248,
170 MW526249, MW526250) were retrieved from genome sequences of five *A. westerdijkiae* strains that
171 were selected based on their previously tested (Anelli et al., 2019) OTA production: ITEM 18008
172 (high levels of OTA detected), ITEM 17441 and ITEM 17448 (low levels of OTA detected), and
173 ITEM 17414 and ITEM 17419 (no OTA detected). Cluster sequences were retrieved using OTA
174 biosynthetic gene sequences from *A. westerdijkiae* strain CBS 112803 and *A. steynii* strain IBT
175 23096, which are available at the Joint Genome Institute (JGI) Genome Portal website
176 (http://genome.jgi.doe.gov/).

177

178 *2.5 Species-specific primer design*

179 Oligonucleotide primers for PCR were designed to target conserved sequences in the 5' and 3'
180 flanking regions of the *otaR* gene in *A. westerdijkiae* (Han et al., 2016). The primers were designed
181 using Primer Express software v3.0 (Applied Biosystems, CA, USA) and were based on an alignment

of the *otaR* region (*otaC-otaD* intergenic region) from six strains of *A. westerdijkiae*: the species type strain CBS 112803 and the cave-cheese strains ITEM 18008, ITEM 17419, ITEM 17414, ITEM 17441 and ITEM 17448.

2.6 PCR assay for ochratoxigenic A. westerdijkiae identification

The PCR was conducted according to the following conditions in 15 µL reaction volume: template DNA 20-30 ng, sterile milliQ water (balanced with DNA template), reaction buffer 1.5 µL [10x], dNTP 2 µL [10mM], primers 0.45 µL [10µM] each, Taq DNA polymerase 0.075 µL [5U/µL]. The amplification profile was optimized as follows, carried out in T_a gradient (ranging from 56 to 60 °C, with 1 °C increment along 12 columns): 95 °C, 120 s, 40 × (95 °C, 30 s; 56 °C, 50 s; 72 °C, 180 s), 72 °C, 300 s. Beta-tubulin gene was used as internal control of PCR reactions, using BT2a/BT2b primer pair (Glass et Donaldson, 1995).

Specificity of amplified products was confirmed by DNA sequencing: The expected size according to genome data were 2890 bp in ITEM 18008, ITEM 17414, ITEM 17441, ITEM 17448 and 1169 bp in ITEM 17419. Amplicons, obtained with M51_5F/M51_6R primers, were purified with the enzymatic mixture EXO/FastAP (Exonuclease I, FastAP thermosensitive alkaline phosphatase, Thermo Scientific, Lithuania, Europe) and used as template for bidirectional DNA sequencing of amplicons. Nucleotide sequencing was performed with the BigDye v3.1 terminator kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instruction and sequences were analysed on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Alignment of the two strands was performed using the software package BioNumerics 5.1 (AppliedMaths, Sint-Martens-Latem, Belgium), with manual adjustments where necessary. *otaR* gene sequence from type strain *A. westerdijkiae* CBS 112803 (MG701896.1, CECT 2948= CBS 112803) was used as reference sequence.

*3. Results**3.1 OTA production*

In a previous study (Anelli et al. 2019), ochratoxin A production was examined in isolates of *A. westerdijkiae* recovered from cave cheese produced in the Apulia region of southern Italy. Of 34 isolates examined, 31 produced and 3 (ITEM 17414, ITEM 17418 and ITEM 17419) did not produce OTA under the culture conditions used in that study. Because ITEM 17418 and ITEM 17419 were isolated from the same cheese sample and, therefore, were potentially clonal, we excluded ITEM 17418 from OTA-production analyses. To determine whether the lack of OTA production in ITEM 17414 and ITEM 17419 was dependent on culture conditions, we grew the two strains under conditions that differed from those used by Anelli et al. (2019). That is, we grew the two strains on five culture media that differed in type and/or concentration of carbon source, nitrogen source, and mineral elements. Strain ITEM 18008 was included in the analysis as an OTA-producing reference strain.

Table 2 – OTA production for ITEM 18008, ITEM17414 and ITEM 17419 strains on different media (µg/g).

		A. westerdijkiae		
		ITEM 18008	ITEM 17414	ITEM 17419
medium	**DG18**	n.d.	0.21	n.d.
	CYA6.5	11.05	n.d.	n.d.
	CIT5	5317	n.d.	n.d.
	MEA	583.68	2.85	n.d.
	YES	2301.21	n.d.	n.d.

LOD (OTA) = 0.002 µg/g; n.d. = not detected

The level of OTA produced in ITEM 18008 cultures varied with growth medium. Production ranged from below the LOD on DG18 medium to 2301 µg/gon YES medium, confirming that growth medium can impact ochratoxin production in *A. westerdijkiae* as has been reported previously for

other on secondary metabolites in other fungi (Brakhage, 2013; Hautbergue et al., 2018; Keller, 2019; Wiemann and Keller, 2014). Strain ITEM 17419 did not produce detectable levels of OTA on any media included in this study. In contrast, strain ITEM 17414 produced low levels of OTA on MEA and DG18, but no detectable OTA on the other media (Table 2). The level of OTA produced by ITEM 17414 on MEA was approximately 200 times less than the levels produced by ITEM 18008 under the same conditions.

3.2 Examination of ota *cluster region in genome sequences.*

The presence and sequences of the OTA biosynthetic genes *otaA– otaD* and *otaR* were examined in genome sequences of five *A. westerdijkiae* strains isolated from cheese. The three strains (ITEM 18008, ITEM 17441 and ITEM 17448) that produced OTA (Anelli et al. 2019) had the five *ota* genes located adjacent to one another in the same order and orientation as previously described for the *ota* cluster orthologs in *A. ochraceus*, *A. steynii* and *A. westerdijkiae* (Figure 1) (Han et al 2016; Wang et al. 2018). The fourth strain (ITEM 17414), which produced low levels of OTA under some conditions (Table 2), also had all five *ota* genes. The fifth strain (ITEM 17419), which did not produce OTA under any conditions examined, had *otaA– otaD* but lacked *otaR* because of an 1800-bp deletion between *otaC* and *otaD* (Figure 1). Hereafter, we will refer to the intact five-gene *ota* cluster as the Type 1 cluster allele, and the four-gene *ota* cluster as the Type 2 allele.

Different start codons have been predicted for the *A. westerdijkiae otaR* gene in the JGI Mycocosm and GenBank databases. In Mycocosm, the *otaR* start codon in strain CBS 112803 (protein ID 245987) begins 75 bases upstream of the start codon in strain CECT 2948 in GenBank (accession AUS29497). Both start codons were present in the four ITEM strains examined here. We selected the start codon in GenBank, because it was also present in the *A. steynii otaR* ortholog (GenBank Accession XP_024705321), but the alternative start codon was not.

Analysis of the coding regions of the other four *ota* genes (*otaA–otaD*) in ITEM 17414 did not reveal any mutation(s) that would account for the lack or low levels of OTA production by this strain under some conditions. All four genes were predicted to yield full-length proteins that were identical or nearly identical to the predicted proteins encoded by homologs of the genes in the three OTA-producing ITEM strains and CBS 112803.

3.3 Primer design

We used the results of the comparative sequence analysis of the *ota* cluster (Figure 1) to develop a PCR assay to distinguish between the Type 1 and Type 2 *ota* cluster alleles in *A. westerdijkiae*. The assay exploited the difference in length of the *otaC–otaD* intergenic region resulting from the absence of *otaR* in the Type 2 allele and its presence in the Type 1 allele. The two primers used in the assay were designed from an alignment of the *otaC–otaD* intergenic region in the *A. westerdijkiae* strains CBS112803, ITEM 18008, ITEM 17419, ITEM 17414, ITEM 17441 and ITEM 17448. The primers were complementary to the regions upstream (M51_5F, 5'-CCAGGTACTTTTGTTTTCGT-3') and downstream (M51_6R, 5'-AGCCCTTCAGCTTGATTT-3') of the *otaR* coding region in the Type 1 allele and on either side of the 18-bp deletion in the Type 2 allele. The M51_5F/M51_6R primer pair was expected to amplify a 2890 – 2943-bp fragment from *A. westerdijkiae* strains with a Type 1 *ota* cluster and a 1169-bp fragment from strains with a Type 2 *ota* cluster.

3.4 Specificity of primer pair

Specificity of primers was tested *in silico* using BLASTn tool at NCBI, limiting to fungi and yeast, in order to detect any cross-reactivity with other species (Table 3). The M51_5F primer had the best combination of max score (40.1), total score (40.1), query cover (100%), E-value (0.026) and identity percentage (100%) values for *Aspergillus westerdijkiae* CECT 2948 in a region related to the biosynthetic cluster of OTA (Gil-Serna et al., 2018) (= CBS 112803, ATCC 22947, IBT 10738,

MUCL 39539, NRRL 3174; Mycobank search, Westerdijk Institute web site). Further results were *Zygo saccharomyces parabailii*, *Vavraiaculicis subsp. floridensis* and *Aspergillus oryzae*, which are fungal species not reported on cheese, and *Penicillium chrysogenum*, also detected on cheese (Anelli et al., 2019), but it is a non-ochratoxigenic specie (Bogs et al., 2006). The M51_6R primer had the best combination of Max score (36.2), Total score (36.2), Query cover (100%), E value (0.27) and Identity percentage (100 %) values for *A. westerdijkiae* CECT 2948 in a downstream region, matched with primer M51_5F (Gil-Serna et al., 2018) (= CBS 112803, ATCC 22947, IBT 10738, MUCL 39539, NRRL 3174; Mycobank search, Westerdijk Institute website). The following 4 results were *Ascoidea rubescens*, *Trichoderma reesei*, *Thielavia terrestris*, non-ochratoxigenic fungal species and not even reported on cheese.

Table 3– BLAST results searching M51_5F and M51_6R

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	GenBank Acc. N°
M51_5F	***Aspergillus westerdijkiae CECT:2948***	40.1	40.1	100%	0.026	100.00%	MG701896.1
	Zygosaccharomyces parabailii	34.2	62.4	85%	1.6	100.00%	CP019502.1
	Vavraiaculicis subsp. floridensis	34.2	34.2	85%	1.6	100.00%	XM_008076152.1
	Aspergillus oryzae	34.2	116	100%	1.6	100.00%	AP007151.1
	Penicillium chrysogenum	34.2	60.5	85%	1.6	100.00%	AM920437.1
M51_6R	***Aspergillus westerdijkiae CECT:2948***	36.2	36.2	100%	0.27	100.00%	MG701896.1
	Ascoidearubescens DSM 1968	34.2	34.2	94%	1.1	100.00%	XM_020192451.1
	Trichoderma reesei QM6a	34.2	34.2	94%	1.1	100.00%	XM_006968504.1
	Thielaviaterrestris NRRL 8126	34.2	167	94%	1.1	100.00%	CP003014.1
	Trichoderma reesei QM6a	34.2	60.5	94%	1.1	100.00%	CP016238.1

The results of BLAST indicated that *A. westerdijkiae* was the only species showing DNA target regions suitable for amplicon production by PCR with primerpiarM51_5F/M51_6R.

Specificity of primers was also tested by PCR using genomic DNA isolated from pure cultures of 34 *A. westerdijkiae* that were examined for OTA production in previous study (Anelli et al. 2019). In the PCR assay, genomic DNA from each *A. westerdijkiae* strain yielded an amplicon of

303 approximately 2900 bp (Figure 1). The exceptions were DNA from ITEM 17418 (data not shown)
304 and ITEM 17419 (Figure 2), which yielded a 1169 bp amplicon.
305 Sanger sequence analysis of selected amplicons generated with M51_5F/M51_6R primers confirmed
306 that the amplicons from OTA-producing strains were 100% identical to the *otaC-otaD* intergenic
307 region in *A. westerdijkiae* CBS 112803 and included *otaR* (GenBank Accession number:
308 MG701896.1).
309 We also assessed the specificity of primers in PCR with other OTA-producing species of fungi: *A.*
310 *ochraceus* (7), A. *niger* (2), *A. steynii* (2), *A. carbonarius* (3), *Penicillium nordicum* (2) and *P.*
311 *verrucosum* (1) (Figure 3).
312
313 PCR with genomic DNA from these six fungal species did not yield the same sized bands that were
314 amplified from genomic DNA of *A. westerdijkiae* strains. But, genomic DNA from some of the other
315 species yielded a faint band or multiple bands. When these bands were subjected to Sanger
316 sequencing, and the resulting sequences were used as queries in BLAST analysis against GenBank's
317 nonredundant fungal database, there were no significant matches.
318
319 *4. Discussion*
320 OTA is produced by some species of *Penicillium* and *Aspergillus* and is among the mycotoxins of
321 most concern to food and feed safety because of its toxicity and its occurrence in a wide range of
322 food and feed. For decades, researchers have reported that grapes, cereals, spices and coffee are
323 frequently contaminated with OTA. The European Commission has set OTA limits of 0.5–10 µg/kg
324 (EC, 1881/2006) and 10–250 µg/kg (EC, 2016/1319) for food and feed, respectively. Recently, the
325 Commission asked EFSA (EFSA, 2020) to update the 2006 opinion on OTA in food, based on recent
326 studies showing OTA contamination in a broader range of food and feed, including eggs, dry-cured

meat, and dairy products (Bozzo et al 2011; Dall'Asta et al 2008, 2010; Denli et al 2010, Pattono et al 2013; Becker-Algeri et al 2016, Persi et al 2014, Anelli et al 2019).

Previous studies have demonstrated that *A. westerdijkiae* is an ochratoxigenic species that frequently occurs on the surface of cheese (Anelli et al., 2019;Berni et al., 2017; Dall'Asta et al., 2010; Iacumin et al., 2009; Rodríguez et al., 2012; Parussolo et al., 2019; Sørensen et al., 2008). Therefore, a rapid method for detection of OTA-producing strains of *A. westerdijkiae* would be useful to assess risk of OTA contamination in cheese and to aid OTA monitoring programs.

Traditional morphology-based fungal identification methods are time-consuming, involve multiple steps, and require mycological expertise (Dao et al., 2005). By contrast, DNA-based methods, such as PCR, are usually rapid, sensitive, and allow for accurate species identification with less time and expertise than morphology-based methods (Geisen et al., 2004; Borman et al., 2008). However, DNA-based methods require preliminary analyses of DNA sequences of the mycotoxin-producing fungi.

In the current study, therefore, we generated and compared DNA sequences for the *ota* cluster in five strains of *A. westerdijkiae* isolated from cheese. The comparison revealed that one of the cluster genes, *otaR*, was present in OTA-producing strains but absent in a nonproducing strains. A previous gene-function analysis indicates that *otaR* is essential for OTA production (Han et al., 2016). Presumably, it is essential because the bZIP transcription factor, that it encodes, regulates expression of the enzyme-encoding genes in the *ota* cluster. Thus, the absence of *otaR* is consistent with and predictive of the lack of OTA production in the nonproducing strains. The variation in the *ota* cluster sequence identified in the current study provided a basis for a diagnostic PCR method to distinguish between OTA-producing and nonproducing strains of *A. westerdijkiae*.

The primers used in the PCR method (M51_5F and M51_6R) were complementary to conserved sequences in the upstream and downstream regions of *otaR* and, as a result, yielded different sized amplification products for OTA-producing strains, which have an intact *otaR*, versus

OTA-nonproducing strains, which lack *otaR*. In addition, these primers were specific to *A. westerdijkiae* in that they did not amplify *ota* cluster-specific bands from the ocratoxigenic species *A. ochraceus, A. steynii, A. niger, A. carbonarius, P. nordicum,* and *P. verrucosum*. Given the results reported in the current study, the PCR method has multiple potential applications. For example, it could be used to determine whether a fungal isolate is *A. westerdijkiae*; determination of whether an isolate is OTA-producing or nonproducing strain; and whether food samples are contaminated with OTA-producing strains, an OTA-nonproducing strains, or both types of strains of the fungus. With respect to the latter application, the ability to determine the chemotype of *A. westerdijkiae* present in food would allow implementation of appropriate control measures to prevent humans from eating OTA-contaminated products.

Previous studies have demonstrated that OTA production is dependent on culture conditions (Brakhage, 2013; Hautbergue et al., 2018; Keller, 2019; Wiemann and Keller, 2014). The OTA-production assays done in the current study, using multiple media and conditions for the fungal culture growth, provide further evidence that *otaR* is essential for OTA production. That is, while variation of some culture conditions caused fluctuations in OTA production in ITEM 18008 and ITEM 17414, ITEM 17419 did not produce detectable levels of OTA under any of the culture conditions examined. The ability to induce low level OTA production in ITEM 17414 by manipulating the composition of the growth medium, highlights the important role that the composition likely has on production of secondary metabolites in substrates in which fungi occur naturally (e.g., crops and food) (Brakhage, 2013; Keller, 2019).

The PCR diagnostic method developed in the current study predicted that strain ITEM 17414 produces OTA even though this strain did not produce or produced low levels of OTA under laboratory conditions. The PCR result with ITEM 17414 is informative, because the low levels of OTA that ITEM 17414 produces constitute a potential risk. It remains to be determined whether strains with an ITEM 17414-like OTA chemotype occur at a substantial frequency on food and

whether they contribute significantly to OTA contamination despite their limited OTA production ability. Therefore, future studies should address the frequency of occurrence of such strains and whether they contribute significantly to OTA contamination in cheese and other food. If the strains contribute significantly to the contamination, then the PCR method developed in the current study would be an accurate predictor of their OTA contamination potential. If their contribution is not significant, then additional analyses would be required to identify genetic markers that can be used to distinguish them from strains that cause significant contamination. Such markers could then be used to refine the PCR method. Comparative genomic analyses of multiple strains with each of the three known OTA chemotypes (no production, no or low-level production, and high-level production) would facilitate such an effort. To the best of our knowledge, the current study is the first report of a naturally occurring deletion in the *ota*cluster in *A. westerdijkiae* that blocks OTA biosynthesis. All the strains examined in the study were isolated from the same region of southern Italy and from the same substrate, cheese. Our results do not exclude the possibility for the existence of other nucleotide variation that blocks OTA production in *A. westerdijkiae* strains from other regions and/or substrates. If such strains exist, the PCR method developed in the current study could yield inaccurate predictions of their OTA chemotype. However, the PCR method could be used in combination with chemical analysis to screen OTA-nonproducing strains of *A. westerdijkiae* for novel mutation in the *ota* cluster or other regions of the genome. If novel mutations are identified, the PCR method could be used in combination with chemical analysis to identify OTA-nonproducing strains of *A. westerdijkiae* with novel mutation in the *ota* cluster or other regions of the genome.

The surface of cheese is nutritionally rich and as a result can have complex microbial communities (Dugat-Bony et al. 2015; Montel et al. 2014). Although it was among the 24 fungal species isolated from the surface of Apulian cave cheese, it remains to be determined whether *A. westerdijkiae* contributes to the organoleptic properties of the cheese (Anelli et al. 2019). Therefore, future studies should address the effect of *A. westerdijkiae* on the aroma, flavour, and texture of

cheese. Because OTA contamination constitutes a significant food safety concern, future studies should also address whether *A. westerdijkiae* strains that have lost the genetic potential to produce OTA can be applied to the surface of cheese or other aged food in order to prevent OTA contamination by excluding colonization by OTA-producing strains during ripening. The OTA-nonproducing strains characterized in the current study (ITEM 17418 and ITEM 17419) are appealing candidates for such applications because not only have they lost the genetic potential to produce OTA, but they occur naturally in the same environment as OTA-producing strains. Thus if *A. westerdijkiae* contributes to the organoleptic qualities of cheese, strains ITEM 17418 and ITEM 17419 might impart similar properties as other *A. westerdijkiae* strains from the same environment. Biological control efforts to reduce OTA contamination using OTA-nonproducing strains should also address whether OTA-nonproducing strains can regain the ability to produce OTA by reacquisition of functional *ota* genes. Such knowledge would aid in the evaluation of the potential effectiveness of OTA-nonproducing strains as biological control agents and as microorganisms that are safe for use in food production.

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Captations

Figure 1.

Two types of the OTA biosynthetic gene (*ota*)cluster alleles in *A. westerdijkiae* strains. Type 1, intact cluster with five previously described *ota*genes (*otaA*– *otaD* and *otaR*); Type 2, cluster that lacks *otaR*due to 1800-bp deletion between *otaC*and *otaD*. The previously described *ota*cluster in strain CBS 112803 served a reference (Han et al 2016). All other strains shown are cave-cheese isolates with ITEM strain designations (e.g., 18008 = ITEM 18008). Strains 18008, 17441, 17448 produced OTA (Table 1), whereas strains strain 17414 produced low levels of OTA under some conditions, and 17419 did not produce OTA under any conditions examined (Table 2).

Figure 2

PCR amplicons obtained with primer pairs M51_5F/M51_6R from genomic DNA from *A. westerdijkiae* strains.

Lane 1: OTA producing strain of *A. westerdijkiae* (2890-2943 bp). Lane 2: OTA non-producing strain of *A. westerdijkiae* (1169 bp). Lane 3: DNA ladder "GeneRuler 1 kb DNA Ladder" (Thermo Fisher). Lane 4: not template control (NTC). Lanes 5-19: *A. westerdijkiae*OTA-producing strains. The figures shows results for 17 of 34 *A. westerdijkiae* strains that were examined by PCR. Numbers above each lane refer to ITEM accessions. The low molecular weight band in most lanes corresponds to primer dimers.

Figure3

PCR amplicons obtained with M51_5F/M51_6R primers against 17 ochratoxigenic isolates of 6 fungal species other than *A. westerdijkiae*.

Lane 1 = "GeneRuler 1 kb DNA Ladder" (Thermo Fisher); lane 2 = positive control *A. westerdijkiae* ITEM 18008; lanes 3-8 = *A. ochraceus*; lanes 9-11 = *A. niger*; lanes 12-13 = *A. steynii*; lanes 14-15 = *P. nordicum*; lane 16 = *P. verrucosum*; lanes 17-19 = *A. carbonarius*. Numbers above each lane refers to ITEM accessions. The low molecular weight band in most lanes corresponds to primer dimers.