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Study of non-conventional Aspergilli and Penicillia Ochratoxin A producers

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Abstract

The continuous growth of the population is pushing the available natural resources to the limit, posing a threat to the balance of the planet. This increase in population means that more food is needed. Consequently, food safety is, nowadays, a matter of interest to the scientific community and world leaders. However, problems related to food/feed contamination have been frequently reported, including those related to fungi. Fungi are diverse, ubiquitous and their role and impact in food security is now an urgent concern. One thing that makes fungi so dangerous is their ability to resist the treatments during the food making process and their ability to produce harmful secondary metabolites like mycotoxins. Mycotoxins are fungal secondary metabolites that can cause severe health issues in either humans or domesticated animals when ingested, inhaled and/or absorbed. From all the mycotoxins reported, some are more studied due to the higher health risks. Ochratoxin A (OTA) is among those and is present in several food/feed products. *Aspergillus* and *Penicillium* species are commonly associated with food spoilage and both genera contain species that can produce this toxin. The major OTA producers are *P. verrucosum*, *P. nordicum* and *A. carbonarius*, but more than 20 species of *Aspergillus* can produce this toxin. However, recent studies reported the presence of OTA in food matrices where known OTA producers are not present. This triggered the scientists involved to try to find the origin of OTA. Based on previous evidence other species like *P. crustosum* and *A. fumigatus* are now being considered. Therefore, the main goal of this work was to search for potential OTA producers among *P. crustosum* and *A. fumigatus* strains, with different geographic origins, and try to find potential genetic differences at the sub-species level.

A set of 28 *Penicillium crustosum* strains and 7 *Aspergillus fumigatus* strains, kindly supplied by Micoteca da Universidade do Minho (MUM), and 16 *Penicillium crustosum* strains, by Colección Chilena de Cultivos Tipo (CCCT), were studied. The *Penicillium* isolates are from four different countries: 16 from Italy, 16 from Chile, 6 from Portugal and 4 from Tunisia. The *Aspergillus* strains are all from Portugal, Spain or with unknown origin. Mycotoxin production was analysed by HPLC-FLD and results were compared with a standard sample. In addition, genes associated with OTA production [two ochratoxin polyketide synthase (*Penicillium* and *Aspergillus* related), ochratoxin non-ribosomal peptide synthetase and an ochratoxin transport protein] were tested. RAPD-PCR fingerprinting [M13 and $(GACA)_4$] and beta-tubulin gene (*BenA*) sequencing were used to perform a wide molecular characterisation.

Under the studied conditions, and with a HPLC-FLD detection limit of 7.6 ng/ml, preliminary results showed that OTA was not detected for all studied strains. However, regarding the genes associated with OTA production, there were 4 positive strains of *P. crustosum* for the 3 genes. Genetic differences, based on RAPD fingerprints, between *P. crustosum* isolates were found allowing the clustering of strains from the same geographic region, except for isolates from Europe. The low number of *A. fumigatus* strains did not allowed to draw conclusions, although they also presented genetic differences. Sequencing with *BenA* did not revealed any SNPs. Nevertheless, further studies with a broader array of conditions needs to be considered.

Keywords: Food Safety; Ochratoxin A; *P. crustosum*; *A. fumigatus*; RAPD; Biosynthetic genes.

Resumo

O crescimento exponencial da população mundial está a levar ao limite os recursos naturais disponíveis representando um risco para o equilíbrio do planeta. O aumento da população tem como consequência o aumento da necessidade de mais alimentos. Consequentemente, a segurança alimentar é, hoje em dia, uma preocupação para a comunidade científica e para os líderes mundiais. Contudo, problemas relacionados com comida e rações animais contaminadas têm sido frequentemente reportados, incluindo aqueles com fungos. Os fungos são um grupo de organismos eucarióticos, diversos, ubíquos e têm um grande impacto na segurança alimentar.

A capacidade de os fungos resistirem aos processos da indústria alimentar, como tratamentos térmicos, e a sua capacidade de produzirem metabolitos secundários prejudiciais, torna-os perigosos contaminantes. Um exemplo desses metabolitos produzidos são as micotoxinas. As micotoxinas quando ingeridas, inaladas e/ou absorvidas podem causar problemas severos de saúde a pessoas e animais. A preocupação com este assunto iniciou-se aquando da morte de cerca de 10000 perus no reino unido, que se deveu a uma contaminação com aflatoxinas. *Aspergillus* e *Penicillium* são dois grupos de fungos filamentosos que têm um grande impacto na contaminação alimentar particularmente pela capacidade de produzirem micotoxinas. As micotoxinas mais associadas a estes géneros são aflatoxinas, ocratoxina A, patulina e citrinina. A maior preocupação com estas toxinas é a sua toxicidade aguda e/ou crónica, que pode levar à morte ou a outras patologias. A maioria das micotoxinas são estáveis, resistentes ao calor e podem permanecer mesmo em produtos tratados (por exemplo, produtos pasteurizados). De todas as micotoxinas, a ocratoxina A (OTA) é uma das mais estudadas, devido à sua presença em vários produtos alimentares e também pelos problemas de saúde que pode causar.

A OTA foi descoberta em 1965 no *Aspergillus ochraceus*. É considerada neurotóxica, nefrotóxica, carcinogénica, hepatotóxica e teratogénica, para diversas espécies. Está classificada com o grupo 2B - possivelmente carcinogénica para humanos. Está presente em diversos produtos alimentares como cereais, vinho, queijo e café. De entre os cereais, o centeio, o trigo e a cevada são os que apresentam os maiores níveis de contaminação. Isto representa um problema pois estes cereais são muito usados no fabrico de farinhas e rações destinadas à alimentação de animais. Consequentemente, os animais domésticos são os mais suscetíveis a ter reações adversas devido à presença da toxina, uma vez que estão expostas a ela por mais tempo e com maior frequência. Os maiores produtores de OTA são *P. verrucosum*, *P. nordicum* e *A. carbonarius*, no entanto cerca de mais 20 espécies de *Aspergillus* são capazes de a sintetizar. A identificação e a quantificação da OTA são comummente feitas por cromatografia líquida de alta eficiência com um detetor de fluorescência (HPLC-FLD). Além disso há genes que estão associados com a ocratoxina A e que podem ser usados como possíveis marcadores genéticos da capacidade de um fungo a produzir. Recentemente, um estudo identificou a presença de OTA em diversas amostras de queijos Italianos. Contudo, não foi possível identificar nenhum dos conhecidos produtores da toxina. Assim, surgiu a dúvida de qual seria a origem da OTA. Baseados em estudos anteriores, *P. crustosum* e *A. fumigatus* estão a ser considerados como possíveis novos produtores. Estes fungos são ubíquos e são diversas vezes encontrados em produtos alimentares. São produtores de toxinas, mas apenas foram descritos uma (*A. fumigatus*) ou duas (*P. crustosum*) vezes como produtores de OTA.

O objetivo principal deste trabalho é procurar potenciais produtores de OTA entre um conjunto de estirpes de *P. crustosum* e de *A. fumigatus* (com diferentes origens geográficas) e tentar encontrar potenciais diferenças ao nível da subespécie.

Um conjunto de 28 estirpes de *P. crustosum e 7 estirpes de A. fumigatus* fornecidas pela Micoteca da Universidade do Minho (MUM), e 16 estirpes de *P. crustosum*, fornecidas pela Colección Chilena de

Cultivos Tipo (CCCT), foram estudadas. Os isolados de *Penicillium* provieram de quatro países e matrizes diferentes: 16 foram isolados de queijos italianos, 16 de merkén do Chile, 6 de diferentes origens em Portugal e 4 de maçãs da Tunísia. As estirpes de *Aspergillus* são, na sua maioria, de Portugal ou Espanha, no entanto algumas têm origem desconhecida.

A produção de ocratoxina A foi avaliada por HPLC-FLD e os resultados foram comparados com um padrão de concentração conhecida. As estirpes de *P. crustosum* foram cultivadas em meio enriquecido com sal de forma a terem um *stress* externo que conduza à produção de micotoxinas. Genes relacionados com a produção de OTA (PKS, NRPS e um transportador) foram também procurados. Adicionalmente foi feito um estudo genético complementar. Apesar de todas as estirpes terem origem em coleções de culturas, o que à partida valida a sua identificação, o gene da beta tubulina foi amplificado. Uma vez que o estudo envolve uma panóplia de estirpes da mesma espécie, principalmente em *P. crustosum*, com origens geográficas diferentes, há uma possibilidade de nessas estirpes terem ocorrido processos de especiação e haver espécies crípticas por revelar. Espécies crípticas são espécies que são morfologicamente idênticas, no entanto apresentam diferenças genéticas. Para complementar este estudo foram feitas análises de perfis genómicos (*fingerprinting*) usando pequenos primers: M13 e $(GACA)_4$.

Nas condições testadas, tendo a HPLC um limite de deteção de 7,6 ng/ml, os resultados preliminares mostraram que não se detetou produção de OTA para nenhuma das 51 estirpes. Os cromatogramas obtidos de cada estirpe foram comparados com o cromatograma padrão, onde foi possível observar um pico aos 13 min. No entanto, em relação aos genes relacionados com a biossíntese de OTA os resultados foram mais promissores, exceto para o gene relacionado com a produção de OTA em *Aspergillus* (*Acpks*). Para esse gene apenas o controlo positivo foi amplificado e não em nenhuma das estirpes em estudo. Os restantes genes estavam relacionados com *Penicillium* [*otapks* (≈500 bp); *otanps* (≈700bp) e *otatra* (≈420 bp)]. Para estes genes, 8% estirpes amplificaram todos os genes (sendo todas do Chile), 16% amplificaram somente *otapks*; 10% *otapks* e *otatra*, 8% *otanps* e *otatra*; 45% apenas *otatra;* e 14% não amplificaram nenhum gene. O facto de haver espécies a amplificarem os três genes testados é um indicador que estas podem ter a capacidade de produzir OTA. Além disso a grande diversidade de padrões verificados mostra que as estirpes são distintas entre si. O estudo com os primers de *fingerprinting* de onde se obtiveram perfis genéticos e com os quais foram construídos dendrogramas mostraram a mesma distinção. Diferentes padrões foram obtidos podendo individualizar cada indivíduo. Além disso, fazendo uma análise aos dendrogramas foi possível verificar que as estirpes chilenas formam um *cluster* assim como as estirpes da Tunísia. Isto pode demonstrar que a distância geográfica poderá criar processos de especiação. Por outro lado, as estirpes Italianas e Portuguesas não formam nenhum *cluster*. Estes dois países encontram-se na Europa onde não há muitas restrições de fronteiras. Em relação a *A. fumigatus*, o baixo número de estirpes, e a falta de informação de algumas delas, não permitiu a formação de *clusters*, apenas se verificando que também apresentam perfis diferentes.

Este foi um estudo preliminar em que não foi possível a quantificação de OTA nem em *P. crustosum* nem em *A. fumigatus*. No entanto, os resultados dos genes biossintéticos mostram que algumas das estirpes podem ter a capacidade de a produzir. Também foi possível verificar que RAPD é uma boa técnica de tipificação e que espécies afastadas geograficamente apresentam perfis genéticos diferentes. Não obstante, estudos com métodos adicionais e com diferentes condições precisam ser considerados.

Palavras chave: Segurança Alimentar; Ocratoxina A; *P. crustosum*; *A. fumigatus*; RAPD; Genes biossintéticos.

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List of Abbreviations

AFLP – Amplified Fragment Length Polymorphism
BenA – β-tubulin
BLAST – Basic Local Alignment Search Tool
CaM – Calmodulin
CCCT – Colección Chilena de Cultivos Tipo
CTAB – Cetyltrimethylammonium bromide
DNA – Deoxyribonucleic acid
dsDNA – Double stranded DNA
EFSA – European Food Safety Authority
ELISA – Enzyme-Linked Immunosorbent Assay
FAO – Food and Agriculture Organization
GC – Gas Chromatography
GRAS – Generally Regarded As Safe
HPLC-FLD – High Performance Liquid Chromatography with Fluorescence Detection
HPTLC – High Performance Thin-Layer Chromatography
IARC – International Agency for Research on Cancer
ITS – Internal Transcribed Spacer
IUPAC – International Union of Pure and Applied Chemistry
LOD – Limit of Detection
LOQ – Limit of Quantification
MALDI-TOF MS – Matrix-Assisted Laser Desorption Ionization-Time-Of-Flight Mass Spectrometry
MEA – Malt Extract Agar
MGYP – Malt Extract Glucose Yeast Extract Peptone Medium
MLST – Multilocus Sequence Typing
MS – Mass Spectrometry
MUM – Micoteca da Universidade do Minho
NGMLST – Next Generation Multilocus Sequence Typing
NGS - Next Generation Sequencing
NRPS – Non-Ribosomal Peptide Synthases
OTA – Ochratoxin A
OTB – Ochratoxin B
OTα – Ochratoxin α
OTβ – Ochratoxin β
PCR – Polymerase Chain Reaction
PFGE – Pulse Field Gel Electrophoresis
PKS – Polyketide Synthases
RAPD-PCR – Random Amplification of Polymorphic DNA
RNA – Ribonucleic acid
RPB2 – RNA polymerase II second largest subunit
rRNA – Ribosomal ribonucleic acid
SNPs – Single Nucleotide Polymorphisms
TAE – Tris acetate EDTA
UPGMA – Unweighted Pair Group Method Using Arithmetic Average
WGST – Whole Genome Sequence Typing

1. Introduction

The world population is continuously growing posing a threat to the balance of the planet. Recent projections show that in 2050 the world population is going to reach 9.8 billions (United Nations, 2019). Ensuring the health and safety of all of them will be a priority to world leaders. One of the main concerns is food availability. To meet this demand, substantial increases in food production will be required as well as the need for the safety of the food produced. It is also important to ensure that the food is nutritious, affordable and uses the least resources possible.

In the food chain, the consumers (humans or animals) are the priority. Problems related to food/feed contamination harmful to their health have been increasing (Steinwider *et al*., 2019). Food spoilage can occur due to microbiological, physical or chemical changes. Microbial spoilage is a major concern since it can cause severe health issues.

The kingdom Fungi encompasses a variety of eukaryotic organisms either unicellular (yeasts) or multicellular (filamentous fungi) that are absorptive chemoorganoheterotrophs. Estimates propose that there are 2.2 to 3.8 million species of fungal species in the world with only 120000 being validly described (Hawksworth and Lücking, 2017). Fungi are diverse, ubiquitous and their role and impact in food security is now an urgent concern. This impact is due to the capacity to resist under the conditions, like extreme heat, used in food production (Biango-Daniels *et al*., 2019).

Another concern related to fungi is their capacity to produce mycotoxins. Mycotoxins are fungal secondary metabolites that can cause severe health issues in either humans or domesticated animals when ingested, inhaled and/or absorbed. The awareness for this issue started in 1960 when an unknown disease killed more than 100000 turkeys in the United Kingdom. This disease was called “Turkey X disease” (Blount, 1961) and only later it was found to be caused by aflatoxins contamination.

Aspergillus and *Penicillium* are filamentous fungi of high importance in food contamination, particularly from mycotoxin production. Major mycotoxins associated with aspergilli and penicillia are: aflatoxins [*A. flavus* and *A. parasiticus* (Klich, 2007)], ochratoxin A (OTA) [*A. carbonarius*, *P. verrucosum* and *P. nordicum* (Cabañes *et al*., 2010; Varga *et al*., 2015)], patulin [*P. expansum* (Frisvad, 2018)] and citrinin [*P. expansum* and *P. citrinun* (Ostry *et al*., 2013)]. The main concern of these naturally occurring toxins is their acute and/or chronic toxicity, which can cause death and/or deleterious effects. Most of the known mycotoxins are relatively stable, heat resistant and are expected to remain in the heat-treated product.

1.1. Ochratoxin A (OTA)

Ochratoxin A (OTA) is one of the most important toxins. It was first discovered in *Aspergillus ochraceus* by Van der Merve *et al*. (1965) who also established its structure. It is considered, among other possible effects, neurotoxic (Paradells *et al*., 2014), nephrotoxic (Zhao *et al*., 2016), carcinogenic (Pfohl-Leszkowicz and Manderville, 2007), hepatotoxic and teratogenic (Wu *et al*., 2018) in various species. The International Agency for Research on Cancer (IARC) classified OTA as a possible human carcinogen (group 2B) (IARC, 1993). This mycotoxin is present in a wide diversity of food and feed products, such as cereals, wine, coffee, dried fruits, beer and in feeds for animals (Joshi *et al*., 2017). Among cereals, rye, wheat and barley are the ones with the highest levels of contamination (Marin *et al*., 2013). This represents a serious problem because these cereals are extensively used to make flours, and other derivatives that are the basis for animal feeds. Due to this, livestock are the animals more susceptible to have adverse reactions due to the presence of OTA, as they may be exposed to the toxin for a long period of time.

As aforementioned, the major producers of OTA are *P. verrucosum, P. nordicum* and *Aspergillus carbonarius*. Many other species within *Aspergillus* genus can be OTA producers (Varga *et al*., 2015), such as *A. niger, A. steynii, A. westerdijkiae and A. alliaceus. P. verrucosum* is the main source of OTA contamination in cereals and their products in cold and temperate climates. In contrast, *P. nordicum* is usually recovered from dry-cured meat products and cheese and it may be the cause of OTA contamination in these foods (Cabañes *et al*., 2010). Recently, another *Penicillium* joined the group of the known OTA producers – *Penicillium thymicola*, isolated from Canadian cheddar cheese (Nguyen *et al*., 2016).

OTA was considered, for many years, the cause of the Balkan endemic nephropathy. This disease is characterised by chronic renal disease frequently associated with upper urothelial cancer. This data is plausible because the disease only affects residents from farming villages in the Balkans, that are exposed to relatively high concentrations of OTA. More recent studies demonstrate that OTA is not the cause for the disease but can influence the metabolism of other carcinogens (Stiborová *et al*., 2015).

To prevent outbreaks of the ochratoxigenic fungi, the Codex Allimentarius (FAO), created Codes of Practice for the prevention and reduction of OTA contamination in cereals (2000), wine (2007), coffee (2009) and cocoa (2013). Mycotoxin production in crops can occur at various points in the food chain: at pre-harvest, harvest, drying, and storage so these codes contemplate all phases. Based on many studies and after assembling of a panel, the European Food Safety Authority (EFSA), established a tolerable weekly intake for OTA of 120 ng/kg body weight (EFSA, 2010).

OTA quantification is commonly performed by high-performance liquid chromatography with fluorescence detection (HPLC-FLD) (Abrunhosa *et al*., 2014; Mishra *et al.*, 2016; Bonerba *et al*., 2017). Immunoaffinity columns can be used to obtain more pure and concentrated extracts (Zhang *et al*., 2018). Other techniques may be used like enzyme-linked immunosorbent assay (ELISA), high-performance thin-layer chromatography (HPTLC), gas chromatography (GC) and mass spectrometry (MS) (Flajs *et al*., 2009; Zhang *et al*., 2018; Zhang *et al*., 2019). Due to the high cost of the majority of these methods HPLC is still the most commonly used technique.

1.2. OTA biosynthetic genes

Knowing and understanding the genetic pathway involved in the biosynthesis of mycotoxins is of the utmost importance, as it could provide tools for diagnostic methods and prevention strategies, for example. In recent years, several works have been conducted to understand the metabolic pathway and the biosynthetic genes involved in OTA production. However, unlike other mycotoxins, this process is still not entirely known.

OTA is a pentaketide composed of a dihydroisocoumarin linked via amide bond to the amino acid phenylalanine (IUPAC, 1992). Polyketide synthases (PKSs) and non-ribosomal peptide synthases (NRPSs) are multimodular enzymes that have roles in the production of fungal secondary metabolites (Nielsen *et al*., 2017) and OTA is not an exception. The PKS and NRPS were identified in the greatest number of species such as *Aspergillus ochraceus* (O'Callaghan *et al*., 2003), *Aspergillus carbonarius* (Gallo *et al*., 2012; Gallo *et al*., 2014), *Penicillium verrucosum* (O'Callaghan *et al*., 2013), among others.

Besides these two synthases, a cytochrome P450, a halogenase and a bZIP transcription factor (except in *Penicillium verrucosum*) were also found in a great number of species: *Aspergillus westerdijkiae* (Chakrabortti *et al*., 2016, Gil-Serna *et al*., 2018), *Aspergillus steynii* (Gil-Serna *et al*., 2015, Gil-Serna *et al*., 2018), *Aspergillus niger* (Gil-Serna *et al*., 2018), *Aspergillus carbonarious* (Gil-Serna *et al*., 2018), *Penicillium nordicum* (Geisen *et al*., 2018, Gil-Serna *et al*., 2018) and *Penicillium verrucosum* (Geisen *et al*., 2018). Thus, the most recent studies propose that OTA biosynthesis begins with the

biotransformation of malonyl-CoA and acetyl-CoA by a PKS enzyme. Then it is catalysed to ochratoxin β (OTβ) by a cytochrome P450 monooxygenase, that when linked to l-phenylalanine, by NRPS, forms ochratoxin B (OTB). Finally, this compound is chlorinated, by a halogenase, to produce OTA (Wang et al., 2018) (Fig. 1.1). The belief that ochratoxin α, initially proposed by Huff and Hamilton (1979) and others (Harris and Mantle, 2001), was an intermediate in OTA biosynthesis was not corroborated, which indicates that this compound is only a product of OTA hydrolysis.

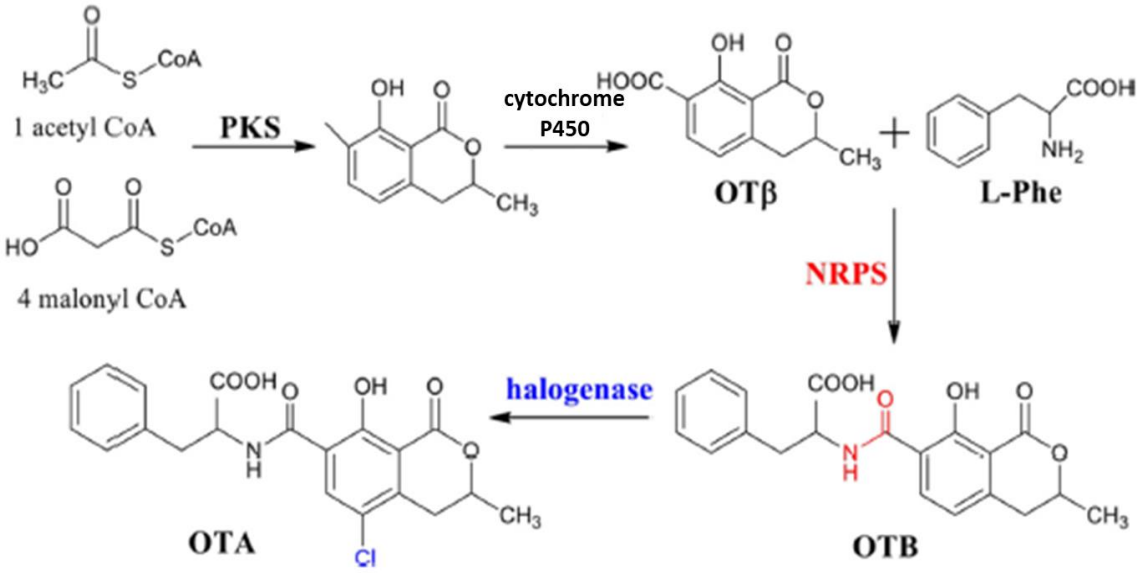

Figure 1.1. - Representation of OTA biosynthetic pathway. OTA biosynthesis begins with biotransformation of malonyl-CoA and acetyl-CoA by a PKS enzyme. Then it is catalysed to ochratoxin β (OTβ) by a cytochrome P450 monooxygenase, that when linked to l-phenylalanin by NRPS, forms ochratoxin B (OTB). Finally, this compound is chlorinated, by a halogenase, to produce OTA (adapted from Wang *et al*., 2018).

1.3. *Penicillium crustosum*

P. crustosum is a ubiquitous fungus, found all over the globe. The fungus presents, typically, low colonies with the surface appearing powdery due to the heavy conidial production (Fig. 1.2). Colonies tend to have a dull green colour on the obverse and on the reverse side yellow to orange-brown (Pitt and Hocking, 2009).

This fungus is usually found associated with food/feed (Wigmann *et al*., 2016; Decontardi *et al*., 2017; Greeff-Laubscher *et al*., 2018). It is, for example, a normal contaminant in cheese (Hymery *et al*., 2014).

P. crustosum is a consistent producer of several mycotoxins such as roquefortine C, penitrem A–F, thomitrem A and E (Rundberget *et al*., 2004). However, in 2006, Vega and collaborators identified a *P. crustosum* strain, isolated from a berry in Mexico, that was able to produce OTA (Vega *et al.*, 2006). Though, the amount of OTA produced was below the allowed by law. Furthermore, recent preliminary studies (personal communication) discovered that several different strains of *P. crustosum* in specific conditions can produce OTA. These results are very concerning because this fungus may be contaminating several different foods, which represents a safety hazard.

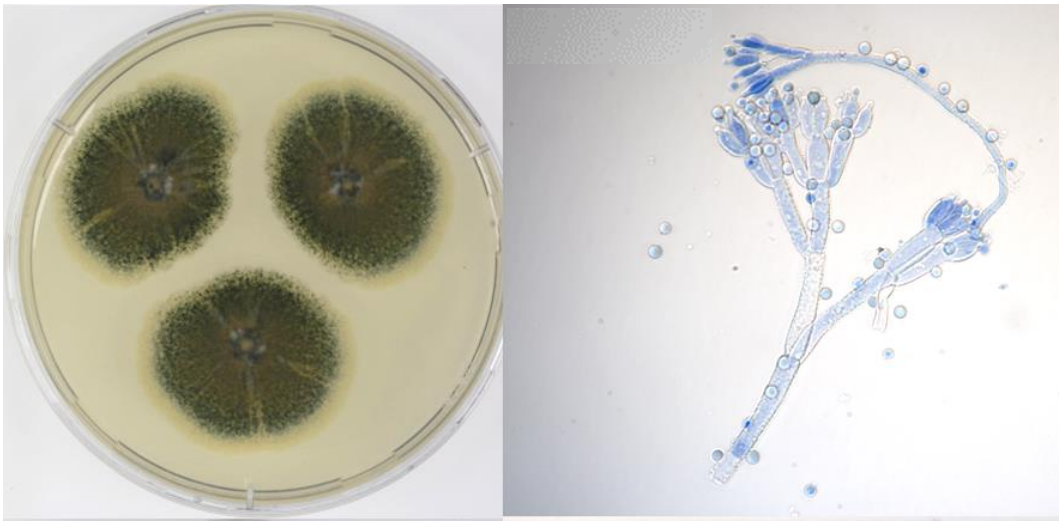

Figure 1.2 – Macro- and micromorphology of *P. crustosum* (MUM 16.11), after 7 days growth in MEA at 25 ºC. (Images kindly provided by Célia Soares, curator of Micoteca da Universidade do Minho)

1.4. *Aspergillus fumigatus*

A. fumigatus is also a ubiquitous fungus, highly associated with human health due to the capacity to invade human tissue causing aspergillosis, especially in immunocompromised patients (Lee *et al*., 2019). This fungus presents low, blue-green colonies, with a velvety texture (Fig. 1.3) (Pitt and Hocking, 2009).

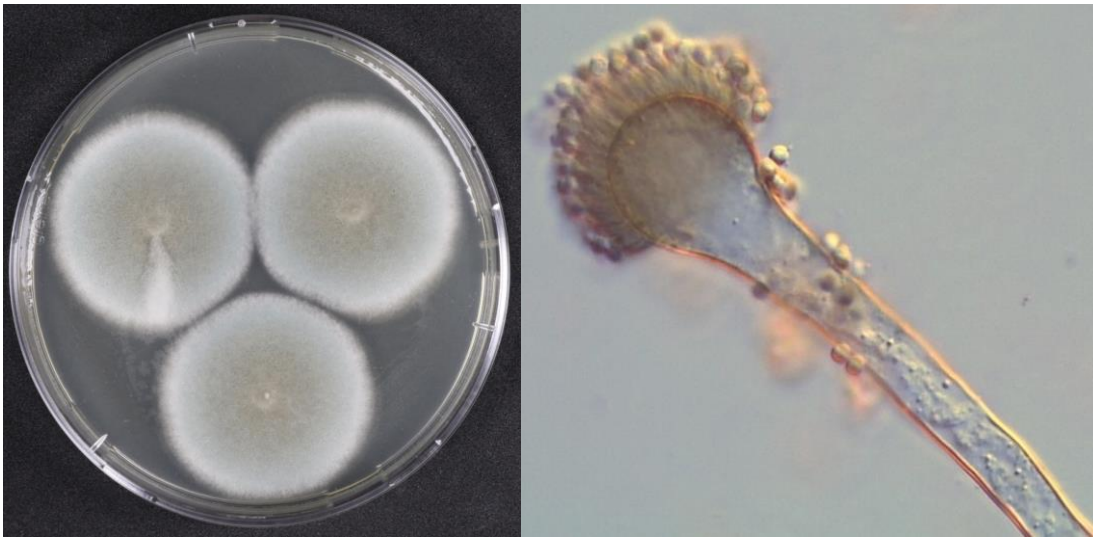

Figure 1.3 - Macro- and micromorphology of *A. fumigatus* (MUM 14.30), after 3 days growth in MEA at 37 ºC. (Images kindly provided by Célia Soares, curator of Micoteca da Universidade do Minho)

The prime habitat for *A. fumigatus* is decaying vegetation, however it can be found in food or feed samples (Shapaval *et al*., 2013; Greco *et al*., 2014). On this matrix it represents a danger to the consumers' safety since it can resist high temperatures. This fungus can produce several toxins like gliotoxin, verruculogen, fumagillin and helvolic acid (Boudra and Morgavi, 2005). In 1997, Albarca and collaborators reported an *A. fumigatus* strain (isolated from food-related materials) that was able to produce OTA (Albarca *et al*., 1997). In the same study, *A. versicolor* was also identified as an OTA producer, yet none of the strains was described as an OTA producer after that.

1.5. Identification of filamentous fungi

The identification of foodborne fungi is very important because with a name comes a lot of knowledge (*i.e*. resistance under extreme heat, low/higher water activity, ability to produce mycotoxins). An effective and rapid identification can prevent food outbreaks which are a crucial feature in the food industry.

The identification of fungi is traditionally made by classical microbiological techniques. These methods use morphologic criteria and microscopic features, that are grounded on phenotypic characters. Though, fungi have a variety of different forms (anamorphic and teleomorphic) which leads to giving different names to the same species (Hawksworth, 2011). For that reason, the identification based on conventional techniques is rather subjective, time-consuming and prone to errors.

The development of molecular biology allowed to do rapid and sensitive identification of filamentous fungi on food/feed samples (Rico-Munoz *et al*., 2019). Still using traditional techniques should not be excluded. So, an integration of a phenotypic approach together with a genotypic approach, in a multiple step method is the most effective way to identify foodborne fungi (Simões *et al*., 2013; Decontardi *et al*., 2018).

When identifying bacteria, in most of the cases, a well know gene, 16S rRNA, is used as the universal marker since a long time (Fox *et al.*, 1977). Meanwhile, regarding fungi, choosing a universal barcode DNA marker for identification has not been so easy. Only recently, the nuclear ribosomal internal transcribed spacer (*ITS*) region was chosen for this purpose (Schoch *et al.*, 2012). Ribosomes are responsible for synthetize proteins, and because of this they are highly conserved (Rorbach *et al*., 2017), thus an excellent target for identification. Albeit *ITS* is a good DNA marker, is not always the best marker to discriminate to the species level some groups, like *Aspergillus* or *Penicillium*. This might be due to the genetic differences that the ITS region presents (Kiss, 2012). For this, secondary markers were chosen for the identification of these groups. For these groups 3 options were chosen: calmodulin (*CaM*), β-tubulin (*BenA*) or the RNA polymerase II second largest subunit (*RPB2*), being *CaM* more suitable for *Aspergillus* and *BenA* for *Penicillium* (Samson *et al.*, 2014; Visagie *et al.*, 2014).

In the Eumycota kingdom there is a high prevalence of morphologically defined species, with similar phenotypes, *i.e.* cryptic species. These species, as has been said, are morphologically indistinguishable but have different nucleic acid characters (Perrone and Susca, 2017). The study of a broad array of strains from different species, resorting to PCR based methods, allows the discovery of these "sister species". To identify and define species boundaries is important to understand processes related to adaptation and speciation. For this, it is crucial to have a strong database that gathers all the information, in this case, gene sequences, that will allow the comparison and recognition of inconsistencies if present (Federhen, 2014). Over the years, several cryptic species have been acknowledged (Cruse *et al*., 2002; Perrone *et al*., 2011; Lackner *et al*., 2019). The ability to classify these species is important in foodborne fungi once similar species can produce different secondary metabolites such as mycotoxins. This causes concern since species with the status of generally regarded as safe (GRAS) may have "sister species" that produce harmful components that can cause health issues to the consumers (Schuster *et al*., 2002). Notwithstanding this, resolving cryptic species may not be easy. Due to all the evolutionary processes that all livings beings are exposed to, one DNA marker may not be enough to resolve the relationship between closely related fungi. Balasundaram and collaborators (2015), using species from the genus *Serpula*, demonstrate that to reveal cryptic species with high confidence it may be needed 5 independent loci (different genetic markers). They also showed that *ITS* was not the best marker to disclose so close related species.

1.6. Typing techniques

Typing techniques allow to identify strains and discriminate them to subspecies level. When food security is at stake using these techniques are important to find different individuals within the same species, that might pose different risks, or to try to detect the origin of the contamination.

The Matrix-Assisted Laser Desorption Ionization-Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) technique can be used to type. A soft ionisation results in minimum fragmentation of different biomolecules, such as peptides, proteins, sugars or polymers (Lima and Santos, 2017). This technique has been used for detection of different food-borne fungi (Santos *et al*., 2016; Elbehiry *et al*., 2017; Lauterbach *et al*., 2017).

Amplified fragment length polymorphism (AFLP) is a genotyping technique that uses restriction endonucleases digestion to obtain fragments from a particular genome (Umesha and Manukumar, 2018). Kure *et al.* (2003) could precise which *Penicillium* strains and in which stage of the production chain the contamination of cheese was occurring. Pawlik *et al.* (2012) used AFLP to identify *Pleurotus* strains to take the most advantage of their potential for the food industry.

RAPD-PCR fingerprinting (random amplification of polymorphic DNA) has also been used to study fungi. It uses short arbitrary primers that produce distinct banding patterns (Umesha and Manukumar, 2018). The congruence of a group of *P. nordicum* strains, isolated from cured meats, was studied (Bogs *et al.*, 2006). Decontardi *et al*. (2018) also used this technique to study the clustering of Penicillia and Aspergilli isolated from Italian grana cheese.

A plethora of other techniques can be used, such as microsatellite analysis, multilocus sequence typing (MLST), pulse field gel electrophoresis (PFGE), whole genome sequence typing (WGST), Next Generation Multilocus Sequence Typing (NGMLST), among others (Umesha and Manukumar, 2018; Rico-Munoz *et al*., 2019). It is the responsibility of the investigator, based on previous knowledge and the resources available, to choose the most adequate techniques that allow a rapid and effective identification.

1.7. Objectives

So, if the major Aspergilli and Penicillia producers of OTA are well recognized, other species have been currently assigned as potential OTA producers. Taking this into consideration, the present work will try to answer a fundamental question: Are other Aspergilli and Penicillia species able to produce OTA? For the reasons mentioned above, the focus will be *Penicillium crustosum* and *Aspergillus fumigatus*. Several isolates from different backgrounds will be analysed for the potential of producing this mycotoxin, either by quantification or search of OTA related genes. Apart from that, a typing study of all the isolates will try to address potential genetic differences at the sub-species level.

2. Materials and Methods

2.1. Fungal strains

A set of 28 *Penicillium crustosum* strains and 7 *Aspergillus fumigatus* strains, kindly supplied by Micoteca da Universidade do Minho (MUM), and 16 *Penicillium crustosum* strains, by Colección Chilena de Cultivos Tipo (CCCT), were studied. The *Penicillium* isolates have different backgrounds (Fig. 2.1): 16 were isolated from Italian cheeses (Decontardi *et al*., 2018), 16 from Chilean *Capsicum* pepper and in its derivatives such as Merkén (traditional Chilean chili pepper powder), 4 from Tunisian apples, and 6 from different substrates from Portugal. The *Aspergillus* strains were all from different substrates from Portugal, Spain or unknown origin (Attachment I – Table 7.1).

Figure 2.1 - Map of the origin of the *P. crustosum* strains in the world. Chile is painted in blue, Italy in purple, Portugal in green and Tunisia in pink.

2.2. DNA extraction

All strains were grown for seven days on Malt Extract Agar [(MEA), agar 20 g/L, glucose 20 g/L, 20 g/L malt extract, 1 g/L peptone)] at 25 ºC in the dark. Spores were collected and preserved in soft agar (4%) pending on further inoculations. With the help of a sterile loop, these suspensions were used to inoculate 50 mL Falcon tubes containing 25 mL of Malt Extract Glucose Yeast Extract Peptone Medium [(MGYP, malt extract 3 g/L, glucose 10 g/L, yeast extract 3 g/L, peptone 5g/L)]. Samples were incubated at room temperature for 4-6 days in an orbital shaker. Fungal biomasses were filtrated and, subsequently, stored at -20 ºC.

For DNA extraction, 200 mg of biomass were placed in a 1.5 mL tube filled with 670 mg of glass beads (425-600 µm). Mechanical lysis was performed using a FastPrep-24^{TM} 5G Instrument (MP Biomedicals, Santa Ana, California, USA) with 1 mL of CTAB (2%) for 30 s at a velocity of 6.0 m/s. Samples were centrifuged at 14000 × g for 8 min at room temperature and 800 µL of the supernatant were transferred to a new 2 mL tube. Polysaccharides and proteins were precipitated by adding 1 mL of cold sodium acetate (3 M, pH 5.5). Samples were gently mixed by inversion, placed at -20 °C for 10 min and centrifuged at 14000 x g for 10 min at room temperature. Then, 1 mL of the supernatant was transferred to a new tube along with 1 mL of isopropanol at room temperature. Samples were again gently mixed by inversion and incubated 1 h at room temperature. To precipitate the nucleic acid pellet, samples were centrifuged ate 14000 x g for 10 min. The pellet was washed twice with cold 70% ethanol, centrifuged at 6000 x g for 7 min and dried at 40 ºC for 5 min using a SpeedVac Concentrator (Thermo

Scientific, Waltham, Massachusetts, USA). Pellets were suspended on 100 µL of sterile ultra-pure water, and incubated in a water bath, at 56 ºC for at least 3 h. DNA samples were stored at -20 ºC. The DNA quality was assessed by quantification of total DNA using NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) and by electrophoresis agarose gel 1% (w/v) for 30 min at 80 V. SYBR® Safe DNA Gel Stain (Invitrogen, Waltham, Massachusetts, USA) was used as a staining element and NZYDNA ladder III (NZYTech, Lisbon, Portugal) was used as a DNA molecular weight marker.

2.3. Molecular identification

Sequence analysis of the β-tubulin gene (*BenA*) was used to reconfirm and enlarge the knowledge of the previous identification of the fungal strains based on the *ITS* analysis. The extracted DNA was amplified using the primers Bt2a (5´-GGTAACCAAATCGGTGCTGCTTTC-3´) and Bt2b (5´-ACCCTCAGTGTAGTGACCCTTGGC-3´) design by Glass and Donaldson (1995). PCR was carried in a 25 µL mixture containing 12.5 µL of NZYTaq 2× Green Master Mix (NZYTech, Lisbon, Portugal), 0.25 µM of each primer, 25 ng of DNA and ultra-pure water until the final volume. The PCR program was performed in C1000™ Thermo Cycler (Bio-Rad, Hercules, California, USA) and consisted in an initial denaturing step of 5 min at 95 ºC; 35 cycles of 60 s at 95 ºC, 45 s at 56 ºC, and 90 s at 72 ºC; and a final extension of 10 min at 72 ºC.

PCR products were visualized in ChemiDoc after electrophoresis of 30 min at 80 V, in a 1% agarose gel in 0.5 × Tris-acetate-EDTA (TAE) buffer. SYBR® Safe DNA Gel Stain (Invitrogen, Waltham, Massachusetts, USA) was used as a staining element and NZYDNA ladder III (NZYTech, Lisbon, Portugal) was used as a DNA molecular weight marker.

Amplicons were purified using E.Z.N.A Cycle Pure Kit (Omega Bio-tek, Norcross, Georgia, USA) according to the manufacturer's instructions. The identity of amplicons was confirmed by sequencing at Stab Vida (Madan Parque, Caparica, Portugal).

Gene sequences were provided in AB1 format file. Sequence analysis was carried out using BioEdit Sequence Alignment Editor. The processed sequences were compared with the Gene Bank database sequences using the Basic Local Alignment Search Tool (BLAST https://blast.ncbi.nlm.nih.gov/). The phylogenetic trees (one for *Penicillium* samples and one for *Aspergillus* samples) were constructed using Mega X software [Molecular Evolutionary Genetics Analysis across computing platforms (Kumar *et al.*, 2018)]. Sequences were aligned using Clustal W alignment (Thompson *et al.*, 1994). Dendrograms were then deduced using MEGAX tool to find the most suitable model for the data. Kimura 2-parameter model was chosen and Maximum Likelihood statistical method was used with a bootstrap resampling method considering 1000 replicates. *Penicillium nordicum* ATCC 44219 (KJ834476) and *Penicillium discolor* IMI 285513 (AY674348) were used as outgroups in the *Penicillium* dendrogram and *P. crustosum* IMI 91917 (AY674353) as type strain. *Aspergillus felis* CBS 130245 (JX021700) and *Aspergillus lentulus* BPI 863540 (EF669825) were used as outgroups in the *Aspergillus* dendrogram, and Aspergillus fumigatus IMI 16152 (EF669791) as type strain.

2.4. Ochratoxin A (OTA) extraction and quantitative analysis

All *P. crustosum* strains were three-point inoculated, starting from the spore soft agar suspension, in MEA plates supplemented with 10% NaCl, for 14 days at 25 ºC. The *Aspergillus* strains were also three-point inoculated in MEA, without any supplementation, for 7 days at 25 ºC.

Procedures to extract OTA from all plates were conducted. The agar medium was cut in small pieces and covered with 20 mL of an extraction solution (2% acetic acid; 20% methanol; 78% acetonitrile) on 50 mL tubes. The solution was left on constant stirring for at least one hour. The tubes were centrifuged for 10 min to precipitate the agar medium. Then the solution was filtered twice: first with a 0.45 µm nylon filter followed by a second step using a 0.2 µm nylon filter. The tubes were kept at 4 ºC.

Quantification of OTA was performed using High Performance Liquid Chromatography instrument comprised by a C18 column (YMC-Pack ODS-A/ 5µm, 12 nm, 250x4.6mm equipped with a Varian 9002 pump (Agilent, Palo Alto, CA, USA), a Varian Prostar 410 autosampler and Jasco FP-920 fluorescence detector (Jasco Europe, Cremella, Italy). Excitation and emission wavelengths were set at 333 and 460 nm, respectively. The mobile phase was a mixture of acetonitrile (49.5%), distillate water (49.5%) and acetic acid (1%) that was filtered using a 0.2 µm filter and degassed using a sonicator.

The presence of OTA in samples was identified by comparing the retention time of the peak from the standard and peaks that might be formed in each sample. The limit of detection (LOD) and the limit of quantification (LOQ) were, 7.6 ng/ml and 23.2 ng/ml, respectively.

2.5. Ochratoxin A (OTA) biosynthetic genes

Aiming to explore the bioactive potential, *i.e.* ability to produce OTA, the molecular screening of OTA related genes was carried out in all the studied isolates.

The primers designed by Geisen *et al*. (2006) were used to test this ability in *Penicillium* strains: *otapks*_for (5´-TACGGCCATCTTGAG CAACGGCACTGCC-3´); *otapks*_rev (5´-ATGCCTTTCTGGGTCCGATA-3´); *otanps*_for (5´-AGTCTTCGCTGGGTGCTTCC-3`); *otanps*_rev (5´-CAGCACTTTTCCCTCCATCTATCC-3`); *otatra*_for (5´-GGTCGGGCCGATGTTTGATCG-3`); *otatra*_rev (5´-CCTCGCATCTTGTAAGG AACGC-3`). *Penicillium nordicum* (MUM 16.08) and *Fusarium oxysporum* (MUM 18.58) were used as a positive and negative control, respectively.

For the *Aspergillus* samples, only one set of primers was used: *Acpks*_for (5´-GCAGCGGGAGTCAATGTAAT-3´); *Acpks*_rev2 (5´-GCGTCGTACAAAGC CTCTT-3´) (Rossi *et al*., 2010). *Aspergillus carbonarius* (MUM 01.08) and *Fusarium oxysporum* (MUM 18.58) were used as positive and negative control, respectively. In a second phase, *Penicillium* primers were tested in *Aspergillus* strains and vice-versa.

PCR reactions for *Penicillium* primers were performed in a 12.5 µL mixture of 6.25 µL of NZYTaq 2× Green Master Mix (NZYTech, Lisbon, Portugal), 0.20 µM of each primer, 12 ng of DNA and ultra-pure water until the final volume. The PCR program was performed in C1000™ Thermo Cycler (Bio-Rad, Hercules, California, USA) and consisted in an initial denaturing step of 3 min at 95 ºC; 33 cycles of 30 s at 95 ºC, 40 s at 60 ºC and 60 s at 72 ºC; and a final extension of 10 min at 72 ºC.

PCR reactions for *Aspergillus* primers were also performed in a 12.5 µL mixture of 6.25 µL of NZYTaq 2× Green Master Mix (NZYTech, Lisbon, Portugal), 0.20 µM of each primer, 12 ng of DNA and ultra-pure water until the final volume. The PCR program was performed in C1000™ Thermo Cycler (Bio-Rad, Hercules, California, USA) and consisted in an initial denaturing step of 2 min at 94 ºC; 30 cycles of 45 s at 94 ºC, 60 s at 67.1ºC and 60 s at 72 ºC; and a final extension of 7 min at 72 ºC.

PCR products were visualized in ChemiDoc after electrophoresis of 30 min at 80 V, in a 1% agarose gel in 0.5 × Tris acetate EDTA (TAE) buffer. SYBR® Safe DNA Gel Stain (Invitrogen, Waltham, Massachusetts, USA) was used as a staining element and NZYDNA ladder III (NZYTech, Lisbon, Portugal) was used as a DNA molecular weight marker.

2.6. RAPD-PCR fingerprinting

To differentiate all the strains from the same species, RAPD-PCR technique was used. The primers M13, 5´-GAGGGTGGCGGTTCT-3´ (Vassart *et al*., 1987), and $(GACA)_4$ (Ali *et al*., 1986) were used. Using Qubit® 2.0 Fluorometer and Qubit dsDNA Broad Range Assay Kit (Invitrogen, Waltham, Massachusetts, USA), DNA quantification was made, according to manufacturer's instructions. Qubit device allows to distinguish DNA from RNA which means that the measurements are more precise and accurate. DNA dilutions were conducted so that all samples had the same amount of DNA for the RAPD-PCR technique.

For M13 primer reactions were carried out in a 25 µL mixture of 12.5 µL of NZYTaq 2× Green Master Mix (NZYTech, Lisbon, Portugal), 0.80 µM of primer, 50 ng of DNA and ultra-pure water until the final volume. The PCR program was performed in C1000™ Thermo Cycler (Bio-Rad, Hercules, California, USA) and consisted in an initial denaturing step of 2 min at 94 ºC; 40 cycles of 20 s at 94 ºC, 60 s at 50 ºC and 20 s at 72 ºC; and a final extension of 6 min at 72 ºC.

For $(GACA)_4$ primer reactions were carried out in a 25 µL mixture of 12.5 µL of NZYTaq 2× Green Master Mix (NZYTech, Lisbon, Portugal), 0.80 µM of primer, 50 ng of DNA and ultra-pure water until the final volume. The PCR program was performed in C1000™ Thermo Cycler (Bio-Rad, Hercules, California, USA) and consisted in an initial denaturing step of 2 min at 94 ºC; 40 cycles of 20 s at 94 ºC, 60 s at 48 ºC and 20 s at 72 ºC; and a final extension of 6 min at 72 ºC.

PCR products were visualized in ChemiDoc after electrophoresis of 100 min at 60 V, in a 1.5% agarose gel in 0.5 × Tris acetate EDTA (TAE) buffer. SYBR® Safe DNA Gel Stain (Invitrogen, Waltham, Massachusetts, USA) was used as a staining element and NZYDNA ladder III (NZYTech, Lisbon, Portugal) was used as a DNA molecular weight marker. Images were saved as TIFF files and processed in BioNumerics software (version 7.6, created by Applied Maths NV. Available from http://www.applied-maths.com). Calculation of similarity between fingerprinting profiles was based on Dice band matching coefficient with 1% band position tolerance. The dendrograms for each primer were deduced from the matrix of similarities by the unweighted pair group method using arithmetic average (UPGMA) algorithm. The co-phenetic correlation was calculated. A dendrogram merging both analyses, *i.e.* of each primer, was created. Cluster analysis was done using the average of all experiments method.

3. Results

3.1. Phylogenetic Analysis

For phylogenetic analysis, the β-tubulin gene of 7 *Aspergillus fumigatus* and 44 *Penicillium crustosum* strains were amplified. A search using BLAST (Basic Local Alignment Search Tool) performed on the *BenA* sequences confirmed the respective identification of each strain, either for *A. fumigatus* or *P. crustosum*. Dendrograms were constructed, using the type strains *A. fumigatus* IMI 16152 (EF669791) and *P. crustosum* IMI 91917 (AY674353) as references for each. The dendrograms show that all *A. fumigatus* strains aligned perfectly with the type strain without any nucleotide differences (Fig. 3.1). The bootstrap value of 100% indicates a perfect clustering. This was also verified for the *P. crustosum* strains (Fig 3.2), also with a high bootstrap value of 99%, and again no nucleotide differences were observed.

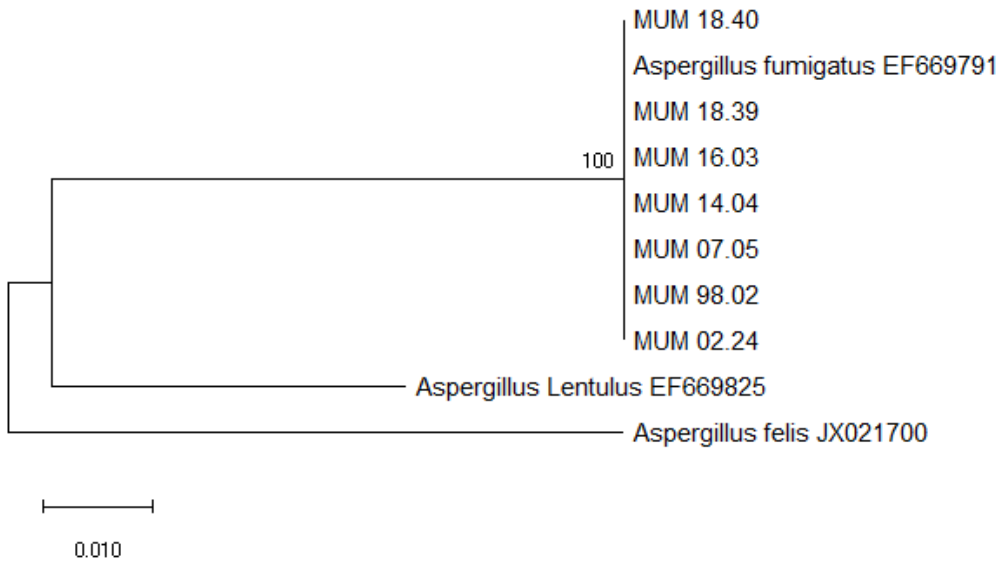

Figure 3.1 - Evolutionary relationships of *A. fumigatus* strains (*BenA*). The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-763.88) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 378 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

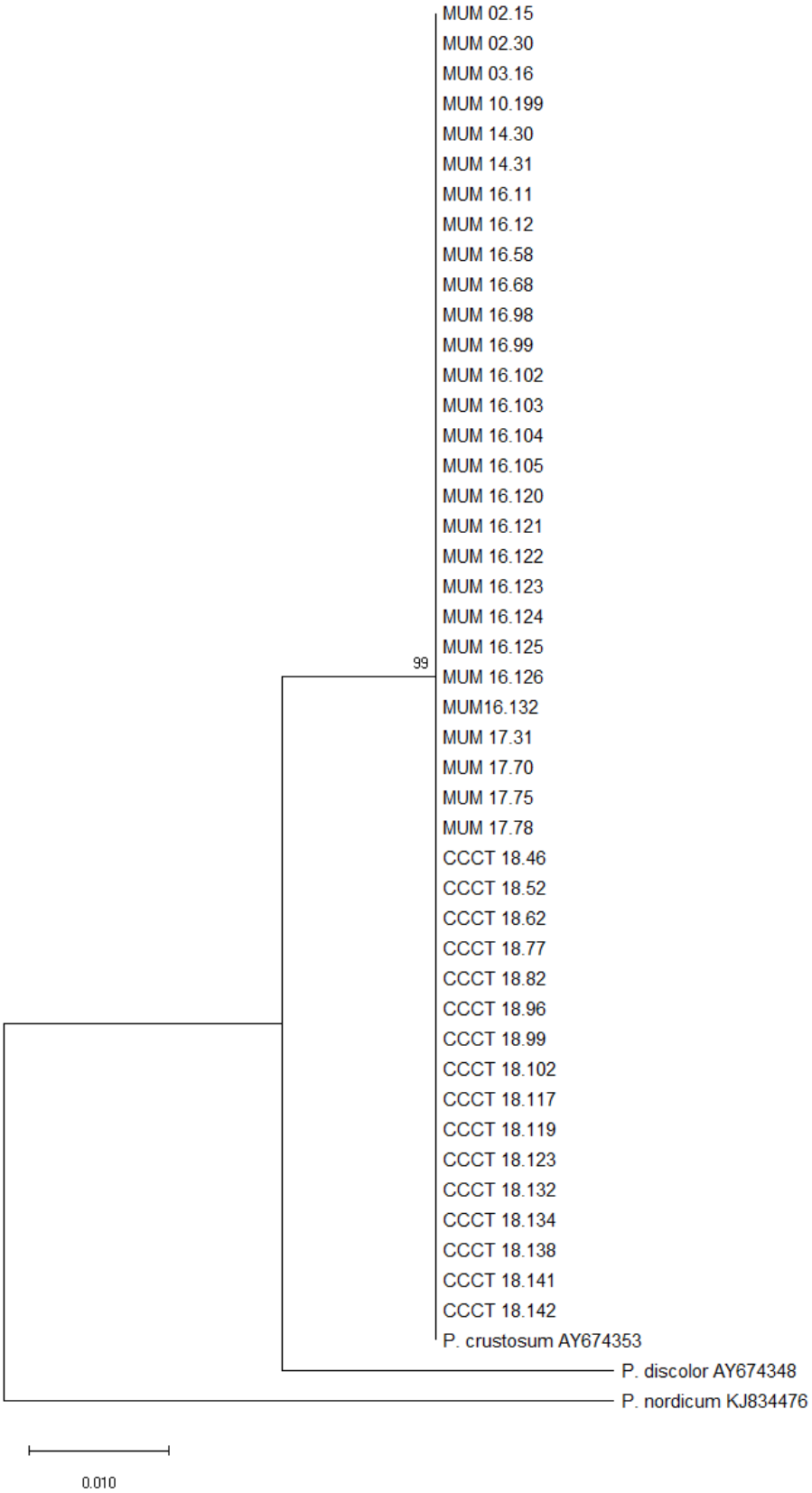

Figure 3.2 - Evolutionary relationships of *P. crustosum* strains (*BenA*). The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-555.71) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 47 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 298 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

3.2. Ochratoxin-A (OTA) quantitative analysis

To detect the presence of OTA 44 *P. crustosum strains* and 7 *A. fumigatus* strains were analysed by HPLC with a fluorescent detector. Alongside a standard sample was included to obtain the retention time of the toxin and for comparison purposes. The retention time was around 13 minutes (Fig. 3.3). However, under the studied conditions, there were not detected OTA producers above the HPLC-FLD detection limit, neither for the *Penicillium* or the *Aspergillus* strains.

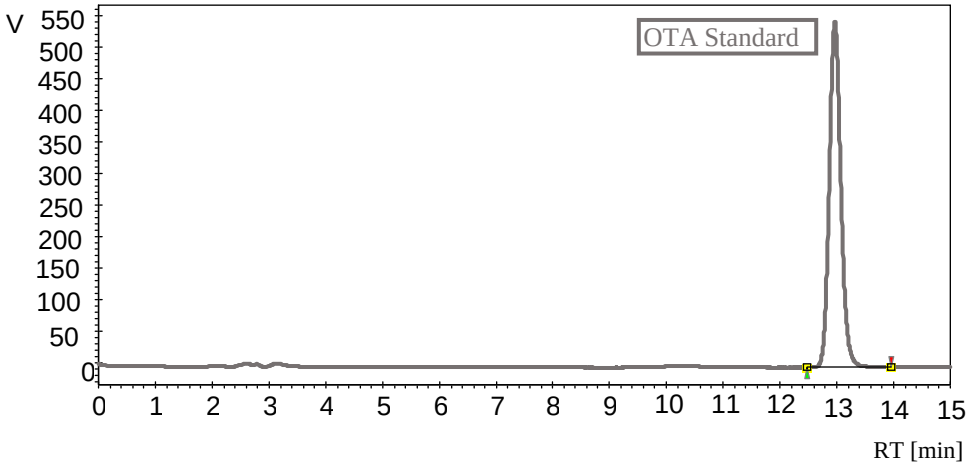

Figure 3.3 - Chromatogram of an OTA standard (118 ng/mL) with retention time at 13 minutes.

3.3. OTA biosynthetic genes

The molecular screening of OTA related genes was conducted. This search was a preliminary attempt to find an OTA cassette that each species harbour. Forty-four strains of *P. crustosum* and seven of *A. fumigatus* were screened against 3 known OTA genes of *P. nordicum* (*otapks*, *otanps* and *otatra*) and 1 from *A. carbonarius* (*Acpks)*. *P. nordicum* (MUM 16.08) and *A. carbonarius* (MUM 01.08) were used as positive controls and *F. oxysporum* (MUM 18.58) as the negative control.

None of the strains was positive for all genes. Regarding *Acpks* only on the positive control, *A. carbonarius* (MUM 01.08), was possible to see amplification (Table 3.1).

Considering the other 3 genes studied the results were more complex (Table 3.1). Of the 51 strains tested: around 14% of the strains did not amplify any gene; 16% amplified *otapks* (≈ 500 bp); 10% *otapks* and *otatra* (≈ 420 bp); 8% *otanps* (≈ 700bp) and *otatra*; 45% only *otatra*; and 8% amplified the three genes (see Attachment II, Fig. 7.1, for examples).

The Portuguese strains (MUM 02.15 - MUM 14.31) and the Tunisian strains (MUM 17.31 – MUM 17.78) of *P. crustosum* were consistent only amplifying *otatra*. Some of the strains, from Portugal, had nonspecific amplification in *otapks* however for the study they were considered negative.

Almost all the Italian strains (MUM 16.11 – MUM 16.132) amplified *otatra*, except for MUM 16.12, 16.58, 16.68, 16.98 and 16.99. On the other side, two of these strains, MUM 16.68 and MUM 16.98, were positive for *otapks*. Three other strains of Italy, MUM 16.102, 16.104 and 16.105, also have dissimilar results, amplifying *otanps* besides *otatra*. In this group 4 strains, MUM 16.12, 16.58, 16.99 and 16.123, were negative for all the genes studied.

Regarding the Chilean strains (CCCT 18.46 - CCCT 18.142) all of them amplified *otapks*, except for CCCT 18.142, that did not amplify any of the genes. Besides these, this gene was only amplified by the positive control (MUM 16.08) and the two Italian strains aforementioned. The majority of the strains

amplified *otatra*, still 7 did not, which is a large percentage compared to other groups, *i.e.* Portuguese, Italian and Tunisian strains. Four strains amplifying all the genes, CCCT 18.82, 18.96, 18.102 and 18.117, as the positive control.

Five of the *A. fumigatus* strains amplified *otatra* except for MUM 98.02 and 18.39. Only another strain, MUM 18.40, amplified a different gene: *otanps*. It was registered a nonspecific amplification in MUM 16.03 of *otanps*, but, once more, not considered pertinent.

Table 3.1 - Results of the PCR amplification of OTA related genes (*otapks*, *otanps*, *otatra* and *Acpks*).

Strain	*otapks*	*otanps*	*otatra*	*Acpks*
MUM 02.15	ns	-	+	-
MUM 02.30	ns	-	+	-
MUM 03.16	ns	-	+	-
MUM 10.199	-	-	+	-
MUM 14.30	ns	-	+	-
MUM 14.31	-	-	+	-
MUM 16.11	ns	-	+	-
MUM 16.12	-	-	-	-
MUM 16.58	-	-	-	-
MUM 16.68	+	-	-	-
MUM 16.98	+	-	-	-
MUM 16.99	-	-	-	-
MUM 16.102	-	+	+	-
MUM 16.103	-	-	+	-
MUM 16.104	-	+	+	-
MUM 16.105	-	+	+	-
MUM 16.120	ns	-	+	-
MUM 16.121	ns	-	+	-
MUM 16.122	-	-	+	-
MUM 16.123	-	-	-	-
MUM 16.124	-	-	+	-
MUM 16.125	-	-	+	-
MUM 16.126	-	-	+	-
MUM 16.132	-	-	+	-
MUM 17.31	-	-	+	-
MUM 17.70	-	-	+	-
MUM 17.75	-	-	+	-

Table 3.1 – Cont.

Strain	*otapks*	*otanps*	*otatra*	*Acpks*
MUM 17.78	-	-	+	-
CCCT 18.46	+	-	-	-
CCCT 18.52	+	-	-	-
CCCT 18.62	+	-	-	-
CCCT 18.77	+	-	+	-
CCCT 18.82	+	+	+	-
CCCT 18.96	+	+	+	-
CCCT 18.99	+	-	+	-
CCCT 18.102	+	+	+	-
CCCT 18.117	+	+	+	-
CCCT 18.119	+	-	+	-
CCCT 18.123	+	-	+	-
CCCT 18.132	+	-	+	-
CCCT 18.134	+	-	-	-
CCCT 18.138	+	-	-	-
CCCT 18.141	+	-	-	-
CCCT 18.142	-	-	-	-
MUM 98.02	-	-	-	-
MUM 02.24	-	-	+	-
MUM 07.05	-	-	+	-
MUM 14.04	-	-	+	-
MUM 16.03	-	ns	+	-
MUM 18.39	-	-	-	-
MUM 18.40	-	+	+	-
MUM 16.08	+	+	+	-
MUM 01.08	-	-	-	+
MUM 18.58	-	-	-	-

[+] positive (amplifies the expected band size, but can also have non specific amplification); [-] negative (does not amplify ; no bands observed); [ns] non specific amplification (only amplifies unspecific bands)

3.4. RAPD-PCR fingerprinting

Once there were a lot of strains from just two different species, fingerprinting analyses were conducted to discriminate them. All the strains amplified two primers: M13 and $(GACA)_4$. Fingerprinting profiles were visualized in agarose gel and analysed using BioNumerics software to construct dendrograms.

3.4.1 *Aspergillus fumigatus*

Seven *A. fumigatus* fingerprint profiles of a combined analyse of $(GACA)_4$ and M13 primers were studied to build a dendrogram (Fig. 3.5). It was not possible to highlight any specific clusters between the different strains. Nor for their matrix of origin (for example air or food) nor their geographic origin (Portugal or Spain). However, differences in fingerprint profiles are noticeable (Fig. 3.4) The strain MUM 98.02 has the most different band pattern in both genes, as MUM 16.03 and MUM 18.39.

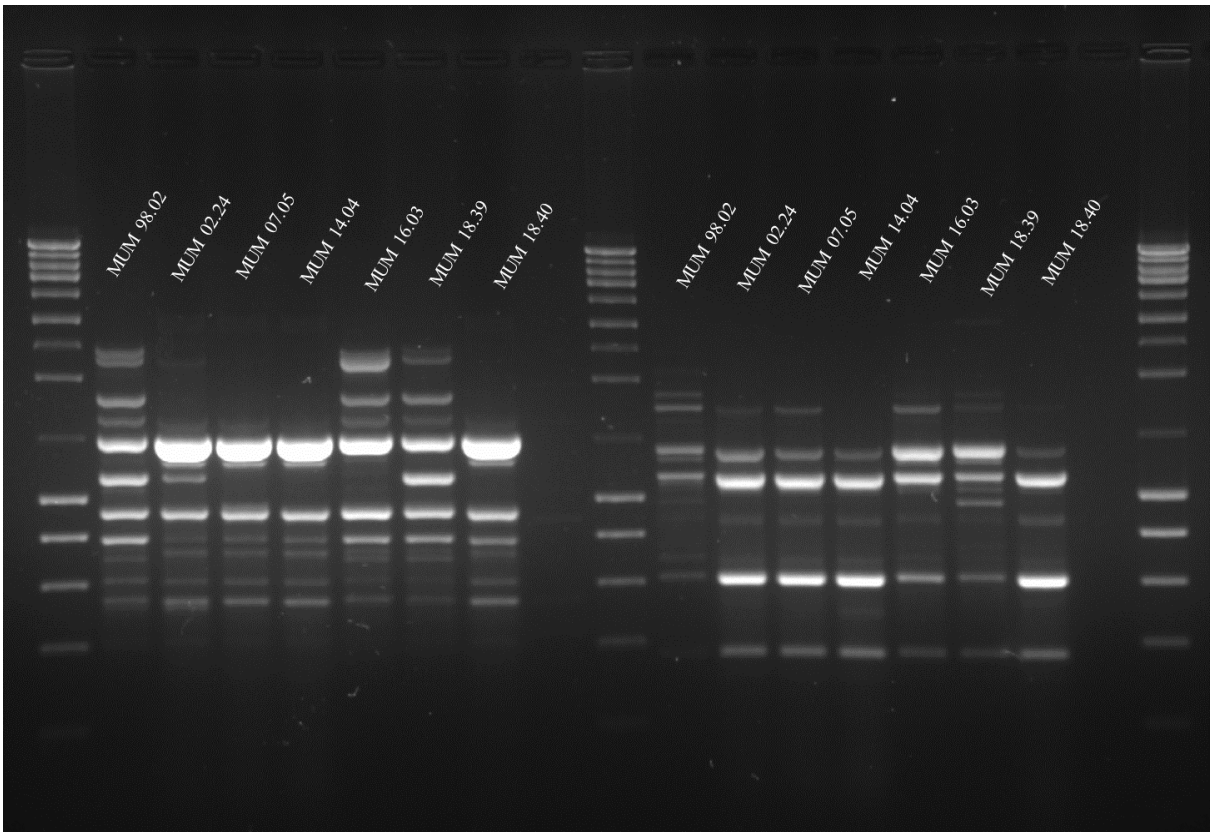

Figure 3.4 - Fingerprint profiles of the *A. fumigatus* strains. Amplification with M13 (left) and $(GACA)_4$ (right).

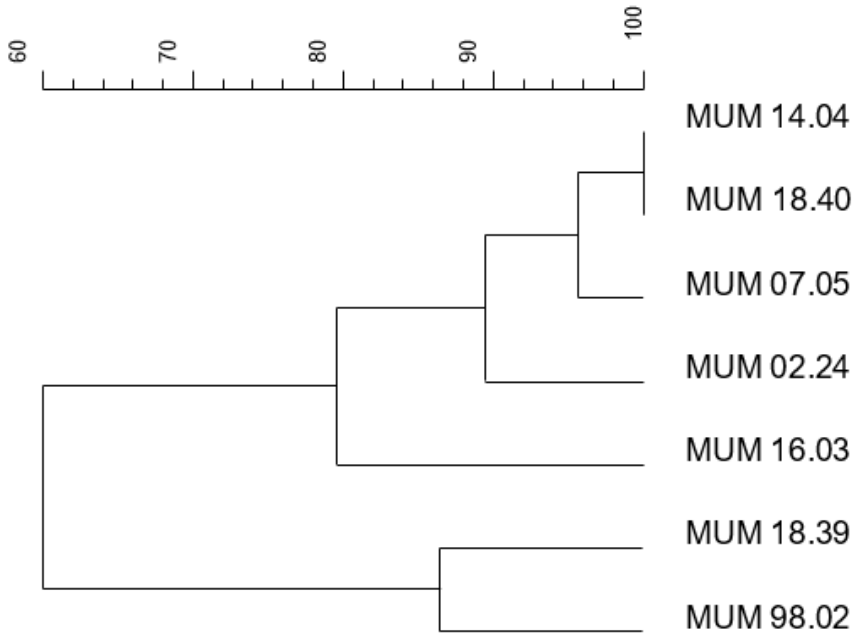

Figure 3.5 - Joined dendrogram of M13 and $(GACA)_4$ fingerprinting of *A. fumigatus* strains, generated in BioNumerics using the average of all experiments method.

3.4.2 *Penicillium crustosum*

Forty-four *P. crustosum* fingerprint profiles were analysed to build a dendrogram (Fig. 3.8). Genetic differences were found allowing clustering. The Chilean strains (blue) clustered all together as the Tunisian strains (pink). Even though they cluster together, there are some differences in the fingerprint profiles (Figs. 3.6 and 3.7).

The Portuguese strains (green) are scattered between the Italian and the Tunisian strains. They do not show particular similarities between them. All of them are closer together with the Italian strains, apart from MUM 14.30 that is closer with the Tunisian strains.

Finally, the Italian strains (purple) are spread all over the dendrogram. Most of them cluster together with the Portuguese and the Tunisian strains, except for 3 strains (MUM 16.121, 16.122 and 16.124) that cluster at the end of the dendrogram close to the Chilean strains.

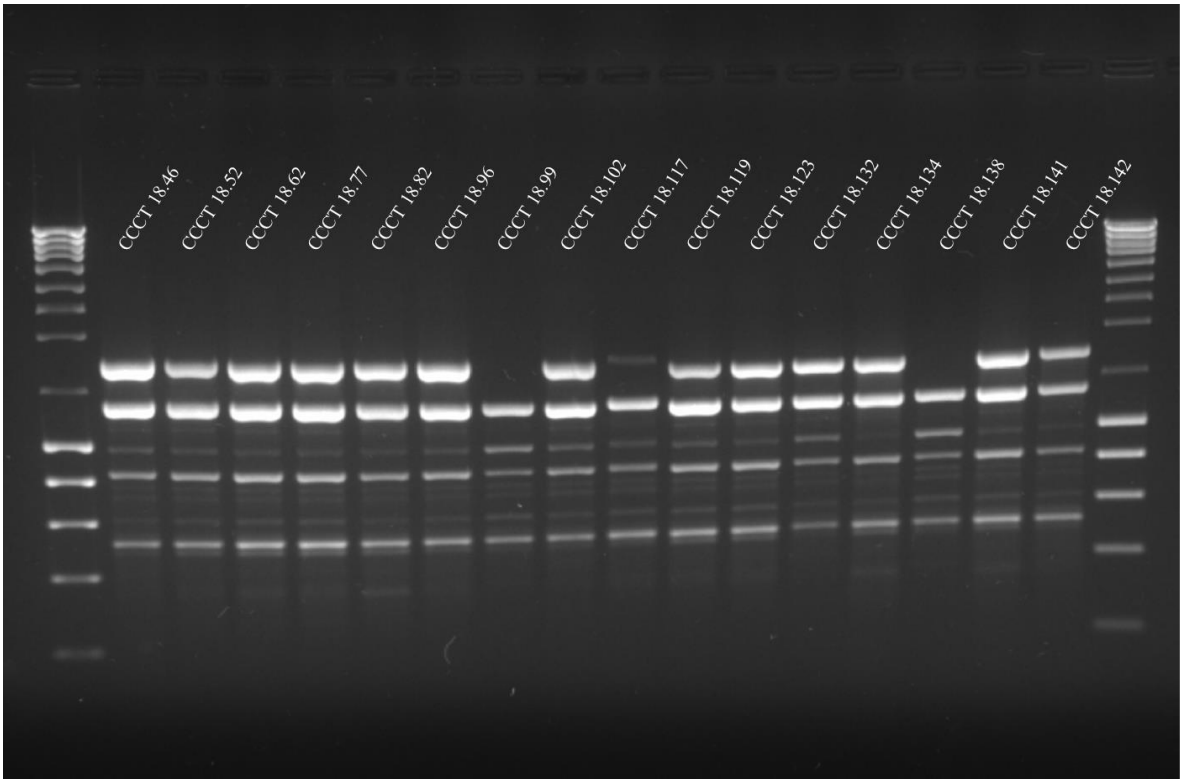

Figure 3.6 - Fingerprint profiles of the Chilean strains $(GACA)_4$.

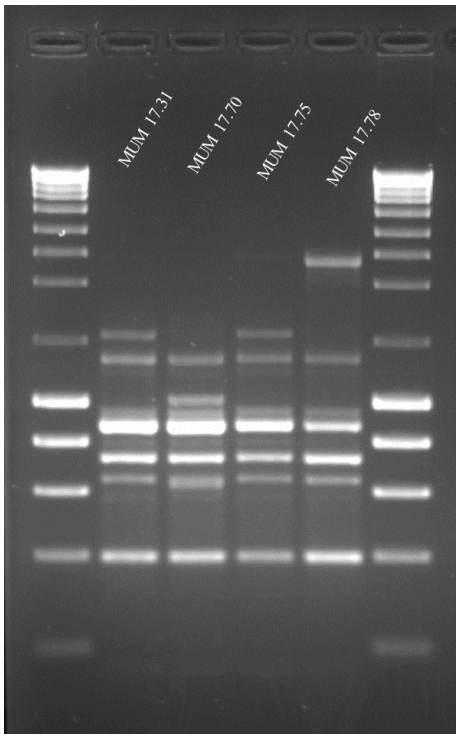

Figure 3.7 - Fingerprint profiles of the Tunisian strains (M13).

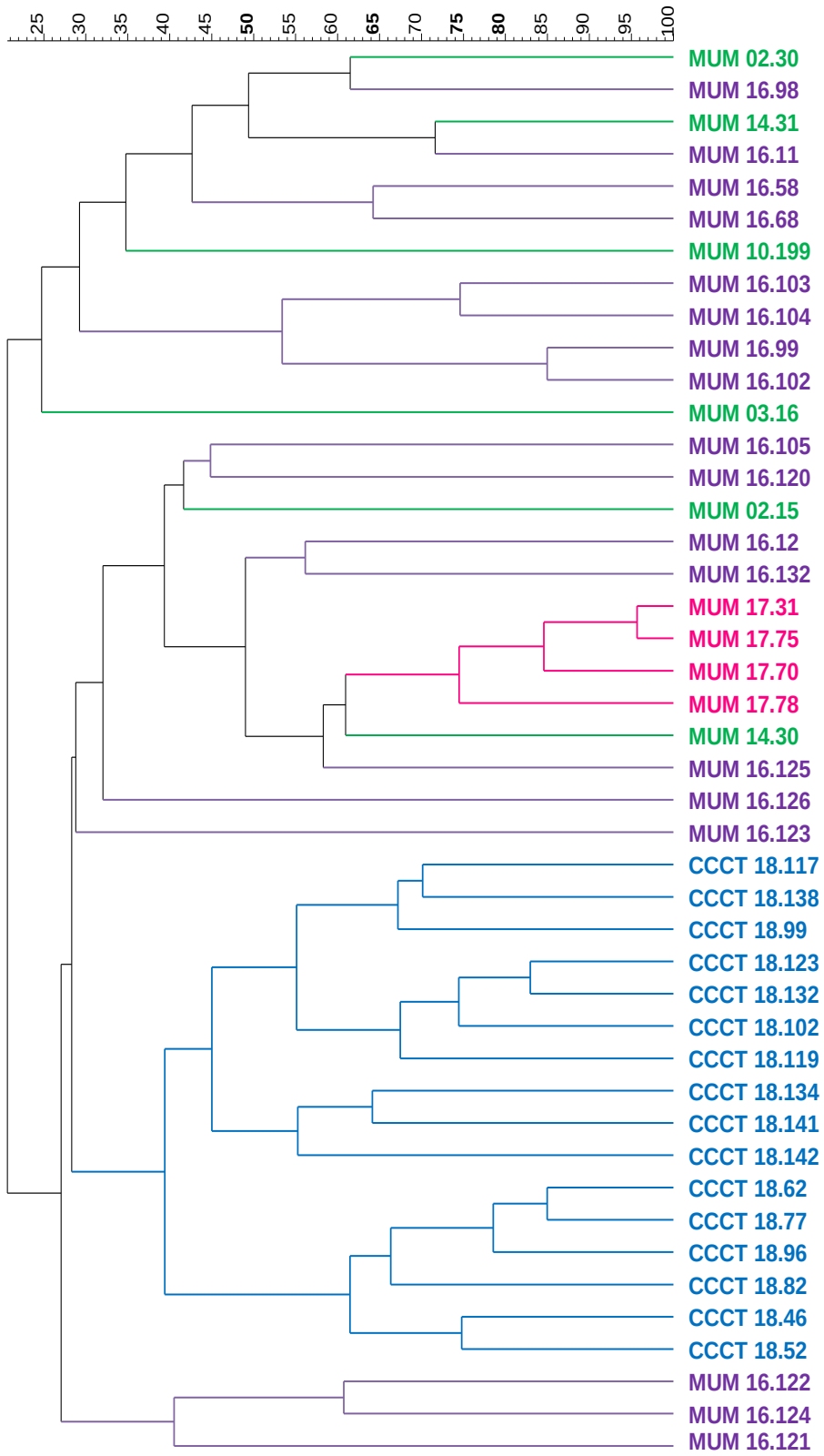

Figure 3.8 - Joined dendrogram of M13 and $(GACA)_4$ fingerprinting of *P. crustosum* strains, generated in BioNumerics using the average of all experiments method. Portuguese strains are represented in green, Italian's in purple, Tunisian's in pink and Chilean's in blue

4. Discussion

Due to significant economic losses, and negative impact on human and animal health, mycotoxins are a matter of concern to the scientific and political communities. Ochratoxin A has been a highly studied mycotoxin since it can cause various deleterious effects, on humans or animals, when consumed in contaminated products (Paterson and Lima, 2010b). Besides being present in raw materials, OTA is also able to resist some food processes, like making bread, as reported by Milani and Heidari (2016). For this, it is essential to keep control in all phases of food production.

The OTA producing fungi have been established, being *Aspergillus* or *Penicillium* species. There are around 27 species of *Aspergillus* that can produce OTA like: *A. steynii, A. ochraceus, A. westerdijkiae* from section *Circundati, A. carbonarius* and *A. niger*, from section *Nigri* and *A. alliaceus* from section *Flavi* (Perrone and Gallo, 2017). On the other side, there are only three OTA producing *Penicillium*: P. nordicum, *P. verrucosum* and *P. thymicola* (Nguyen *et al*., 2016; Perrone and Susca, 2017). Regardless these fungi are the recognize OTA producers by the scientific community additional reports have been published with other species being proposed as having OTA producing ability. *P. brevicompactum, P. crustosum, P. olsonii* and *P.oxalicum* were reported by Vega and collaborators (2006). *P. chrysogenum, P. glycyrrhizacola, P. polonicum,* isolated from liquorice, were also acknowledged as OTA producers (Chen *et al*., 2013). *A. fumigatus* and *A. versicolor* are also in the group of potential OTA producers (Abarca *et al*., 1997).

Recently, Decontardi and colaborators (2017) studied the presence of OTA in Italian cheeses. They found that all the samples of cheese were contaminated with OTA ranging from 1 to 1432 μg/kg. All the cheeses were colonized by fungi with *Penicillium* being generally dominant, but with the presence of some *Aspergillus*. However, none of the isolates was identified as any of the major producers of OTA, that is, they were not identified as any Aspergilli OTA producers or any *P. verrucosum, P. nordicum* or *P. thymicola*. These were intriguing results because the presence of OTA was indubitable. The authors propose that the isolation of the OTA producers Penicillia may have been hampered by a large number of other *Penicillium* species non-OTA producers. *P. crustosum* was one of those isolated fungi. Moreover, it's possible to hypothesise that maybe one of the other isolated species was the source of the OTA contamination. As *P. crustosum* has already been described in another independent study as a producer, this study focused on this species to try to understand if this fungus could be a threat to food safety as an OTA producer. Concurrently, *A. fumigatus* also was scrutinized.

A thorough study was conducted on 44 *P. crustosum* and 7 *A. fumigatus* strains. Once that all the strains came from culture collections it is expected that the identifications are accurate. For this, no classical methods of identification, to study the morphology of each strain, took place. Withal some species are morphologically indistinguishable, referred to as cryptical species, but are genetically different (Bickford *et al*., 2007). Therefore, the use of molecular methods is crucial since it does not depend on culture conditions, interpretation from a specialist and could reveal the hidden identity of new species (fixing the names based on DNA barcodes) (Hawksworth, 2015). Once that so many strains of the same species are being studied from different matrices and different parts of the globe, specially *P. crustosum* strains, there is a possibility that some of them have gone through speciation events.

The authenticity of the identification of each strain was verified by the amplification of the housekeeping *BenA* gene. This gene was recognized as the best gene, instead of the universal barcode (*ITS*), to differentiate most of the *Penicillium* to the species level (Visagie *et al*., 2014). For *Aspergillus*, the corresponding gene would be *CaM*, but *BenA* is suitable as well (Samson *et al*., 2014). So, to standardize the present study the same gene was used in both genus. No genetic differences were found

in either of the groups. They form one single cluster with bootstrap levels of 99% for *P. crustosum* strains and 100% for *A. fumigatus*. At first, these results might indicate that all the strains are equal and that there are not any cryptical species among them. However, the power of discrimination of *BenA* can be not enough to differentiate to the subspecies level. To reveal cryptic species, it might be needed more than one DNA marker. Using the genus *Serpula* as a model, Balasundaram and collaborators (2015), proved that to find cryptic species with high confidence there is a need to include five loci. They suggested the use of *tub*, *rpb2*, *hsp* and *tef* together with *ITS*, although they tested others. Gathering multi-locus sequencing analysis and MALDI-TOF MS is also a good strategy to unveil cryptic species (Gautier *et al*., 2016). A new *Aspergillus* species, *A. suttoniae*, and the identification of cryptical species with clinical origin were possible with the use of four DNA markers, *CaM*, *BenA*, *RPB2* and *ITS* (Siqueira *et al*., 2018). The identification of cryptical species in the clinical context is critical to reveal new potential pathogens and to choose the appropriate course of treatment. In the food industry the same principle applies: it is important to identify possible spoilage organisms and determinate reliable controls, proper processing parameters and prevention guidelines (Snyder and Worobo, 2018). All of this is an ongoing work of the scientific community that aims to the establishment of food quality programs to reduce the growth of fungi (yeasts or filamentous fungi).

To differentiate strains from the same species it is possible to resort to typing techniques. As already discussed, in food industry identify isolates to the subspecies level is of the utmost importance. RAPD-PCR fingerprinting is a popular technique because of its simplicity and reduced cost. It is considered a fragment-based method. The main advantage is that no previous knowledge of the DNA sequence is needed (Ruppitsch, 2016). The technique depends on the amplification of short arbitrary primers under low stringency conditions and migration on agarose (or acrylamide) gel. The products are complex band patterns, similar to barcodes, which can be considered a genetic fingerprint for each strain. Despite that, RAPD's simplicity has some drawbacks: the low stringency conditions and the use of random primers lead to low reproducibility (Araújo and Sampaio-Maia, 2018). RAPD is also dependent on DNA quality and does not detect accidental mixtures. In the genomic era, whole genome sequence is a tool that can give us a lot of information. On the other side, it is costly and time-consuming. RAPD, once that only analyses a small fraction of the genome can compare hundreds to thousands of strains in a faster and cheaper way. It is an excellent first assessment of the degree of polymorphism and can be complemented with other methods. RAPD has been broadly used to study fungi (Hadrich and Ranque, 2015; Kermani *et al*., 2016; Decontardi *et al*., 2018; Verhasselt *et al*., 2019).

Once that no genetic single nucleotide polymorphisms (SNPs) were verified with *BenA*, and before sequencing other primers (since is time-consuming and expensive), RAPD was used to understand if sequencing other genes would be worth it. Since this method is dependent on DNA quality and quantity, and alterations in these parameters can cause polymorphism (Rodriguez *et al*., 2019), all the DNA's were extracted with the same method and quantified by Qubit. Fluorometer. This method offers more precise quantification of the DNA present in the sample, and unlike NanoDrop, can distinguish the DNA from RNA (Invitrogen). Qubit is also used for next generation sequencing (NGS) where DNA quantification is also critical (Robin *et al*., 2016).

All the strains were amplified with two primers: M13 and $(GACA)_4$. Amplification products were run in a thick agarose gel at low voltage to obtain the best separation of the bands. Fingerprint profiles of each strain were analysed in BioNumerics software by the similarity of the band patterns. Comparative studies on the two primers were performed and two dendrograms (one for *P. crustosum* and another for *A. fumigatus* strains) were constructed.

The resulting dendrogram of *A. fumigatus* did not allowed conclusions, besides that RAPD can, distinguish species to subspecies level. From the fingerprint profiles (Fig. 3.4) it is possible to see that there are some genetic differences, so it is possible to differentiate individuals. There were only seven strains all from the Iberian Peninsula and not all the strains have information available (Attachment I). With more strains from different parts of the globe, a more comprehensive and understandable study would be achieved. This fungus is not only a problem for food safety but also a threat to humans and animals' health. That being said this kind of studies should be pursued. Ashu and collaborators (2017) studied 2026 *A. fumigatus* isolates from 13 countries in four continents. They were able to group them in eight differentiated genetic clusters among geographic and ecological niches (some of the genetic difference was due to the geographic separation).

On the other hand, resulting from a large number of strains tested, the *P. crustosum* dendrogram allowed withdraw several conclusions. As it happened for *A. fumigatus*, RAPD technique could differentiate the strains in the study, proving that this can be used to detect the presence of polymorphisms between strains from the same species even if no nucleotide differences existed when sequencing. These results agree with the ones obtained for *Trichophyton mentagrophytes* where amplification of ITS1/ITS4 primers did not distinguish between strains, but the band patterns obtained by the amplification of $(GACA)_4$ could do it. It is still proved that this primer is a good and rapid method for the differentiation of different dermatophytes species (Shehata *et al*., 2008). To differentiate *Botryosphaeriaceae* species M13 and $(GACA)_4$ primers (together with other primers and other techniques) were also used. For these species, these primers are a good solution to differentiate them, but it was also concluded that, although the fingerprint profiles from the same species were similar, it was possible to detect intraspecies variability (Alves *et al*., 2007). Furthermore, these two primers, together with $(GTG)_5$, could segregate several serotypes of *Cryptococcus neoformans* (Meyer *et al*., 1993). A study made on different *Aspergillus* species determined that RAPD is able to determine inter- and intra-species variability and that the procedure should be used for strain characterization, particularly for preliminary evaluation over extensive collections (Kermani *et al*., 2016). Another thing to take into account is the choice of the primers regarding the species in the study. For example, $(GACA)_4$ and $(ACA)_5$ have the same fingerprint pattern for *Trichophyton rubrum* and *Trichophyton mentagrophytes*, reason that these two primers do not have a high differentiation power for these species (Dobrowolska *et al*., 2011). In contrast, the same author showed that using $(GACA)_4$ it is possible to get four different profiles of *Trichophyton ajelloi* strains.

RAPD is also a good technique to detect the fungi and source of contamination in fungi outbreaks (Hadrich and Ranque, 2015). The same way, RAPD is a tool to study fungi that contaminate food which can translate in economic losses or health risks for the consumers (Akram *et al*., 2017). Geisen and collaborators (2001) demonstrated that patterns created by RAPD primers can distinguish between strains on *Penicillium roqueforti* that produce different secondary metabolites. They proved, as well, that strains of this species can be differentiated by the production of larger or smaller numbers of metabolites. This is evidence that RAPD should be used in mycotoxigenic fungi, as *P. crustosum* and *A. fumigatus*, to identify possible harmful strains.

In the dendrogram constructed based on the similarities of fingerprint profiles, it is possible to verify that the Chilean and the Tunisian strains clustered together. The geographic distance between the strains likely made them adapt to the environment that they inhabit. Therefore, it is possible to infer that there is some genetic variation based on their geographic origin. This issue has been poorly addressed since there is a common belief that fungi are ubiquitous and do not have distinct distribution patterns (Lumbsch *et al*., 2008). Notwithstanding, some studies have addressed this issue. Sonjak and collaborators (2007) also studied *P. crustosum* strains. There were different fingerprint profiles

(obtained by AFLP and M13 primer) between Artic and non-Artic isolates, demonstrating that the ecological niches they inhabit has influence and can create genetic polymorphisms. Besides the genetic polymorphisms, phenotypic differences were verified in the Artic strains, showing a speciation event to this cold environment. The Chilean strains are also very isolated, once that Chile has a very particular location: from one side is isolated by the Andes and from the other by the Pacific Ocean. This makes a perfect habitat for unique adaptations to occur (almost like the ones that happened in islands or extreme environments, like the Artic). A study made on *Malassezia furfur* M13 fingerprints, showed that different strains tend to associate with the host's geographic region. Then this fungus, that causes dermatoses, can be used for phylogeographic studies (Gaitanis *et al*., 2009). Economic important truffles, *Tuber magnatum*, were studied too. Population located more at north or south proved to be significantly differentiated from the rest of the populations (Rubini *et al*., 2005).

On the other side, the European strains, *i.e.* Portuguese and Italian, did not form specific clusters. This might be due to lack of physical barriers that isolates either of the countries and the ongoing trade that happens all over Europe. Additionally, both countries have similar climates. This agrees with the results obtained from Sonjak *et al*. (2007) that did not find high polymorphisms for the non-Artic strains. However, it might depend on the species to be studied. *Cryptococcus neoformans* strains isolated only in Italy showed that two strains with different pathogenicity are differently distributed in the country: one this more present in the north and another in the south. The results were obtained based on the amplification of $(GACA)_4$ (Pini *et al*., 2017). This demonstrates that although for *P. crustosum* only distant isolated strains have genetic differences (similar to the other isolates from the same geographic region), in other species that might not be the case.

Concerning the production of OTA, it was not possible, under the studied conditions, to detect the presence of OTA (nor for *P. crustosum* strains neither for *A. fumigatus* strains). All the *P. crustosum* strains grew in NaCl enriched medium. This fungus is a common contaminant in cheese (Hymery *et al*., 2014; Decontardi *et al*., 2017) and for that is adapted to salt-rich environments. One adaptation reported to these niches is the production of Ochratoxin A (Geisen *et al*., 2017). In *P. nordicum* the production of OTA gives the advantage to grow in NaCl rich foods. A mutant strain, unable to produce this secondary metabolite, has a drastic growth in this kind of environment (Schmidt-Heydt *et al*., 2012). This represents a safety hazard for this kind of food products (Geisen *et al*., 2018). *P. verrucosum* is also producer of OTA as well as citrinin. However, the production of the two toxins is dependent on the environment that the fungus inhabits. To cope with high concentrations of NaCl the fungus produces OTA at the expense of the citrinin production. Citrinin on the other side is an adaptation to oxidative stress, as the excess of light. OTA can also be present without the presence of NaCl – for example, wheat contaminated with *P. verrucosum* in the field produces citrinin (due to light exposure), however if the wheat is contaminated during storage, under low light conditions, the fungus produces OTA (Schmidt-Heydt *et al*., 2015). In contrast, high NaCl environments are normally not suitable for *Aspergillus* species and may even inhibit OTA production. An example is *A. carbonarius*, that in the presence of salt has a decrease in the growth (Stoll *et al*., 2013). Hence, *A. fumigatus* strains grew in MEA without any enrichment. The presence of glucose and galactose can also influence the produce of OTA (O'Callaghan *et al*., 2013). Since no OTA production was detected, in the future, trying to replace glucose for galactose in an option to induce OTA production.

The identification of OTA was done with HPLC with fluorescence detection. This method has high sensitivity and the strong native fluorescence activity of OTA makes this technique a good option to detect it (Jalili and Jinap, 2015). It has been used in several occasions to detect OTA in food samples like cheese (Dall'Asta *et al*., 2008), in foodstuffs of animal and plant origin like pork meat and blood, coffee, cocoa, peppers (Skarkova *et al*., 2013) or in wines (Abrunhosa *et al*., 2014). According to

Leggieri and collaborators (2017) the temperature used (25 ºC) is appropriate for OTA production. The use of HPLC-FLD, as well as the use of an extraction solution with methanol, is proper for this toxin once that is soluble in polar organic solvents (Zhang *et al*., 2018).

Even though, the appropriate methods for extraction, detection and quantification of OTA were used, no OTA producers' strains were identified, in this study. This might be due to the low concentrations of the toxin. Other studies propose to use concentration methods like immunoaffinity columns. These columns clean up the sample and have a membrane specific for the toxin in the study which allows a good purification, enhancing OTA fluorescent signal and lowering the detection limit (Dall'Asta *et al*., 2008). Yet, these columns are expensive and, for that, not always an option. One disadvantage of these columns is that if they are specific for OTA, they cannot retain other OTA similar molecules like OTα (Muñoz, 2010). Sometimes OTA is degraded and it's only possible to identify the product of that process, that is OTα. Taking this into account, a few strains, from this study, were chosen to try to identify the presence of this metabolite in their extracts (data not presented), but as it happened before for OTA, no OTα was detected, under the conditions tested. To increase the chances to find a toxin HPLC connected to mass spectrometry detector can be used. This method is more sensitive than HPLC-FLD, but not all laboratories have access to it, because it is more expensive. Even though, it is now becoming more popular (Zhang *et al*., 2016; Castellá *et al*., 2018). Using state of the art appliances and the most appropriate methods does not guarantee OTA detection. The genetic differences that can occur between strains from the same species, that were already demonstrated in this study, may have repercussion on the production of secondary metabolites. The discovery of *A. carbonarius* strains that do not produce OTA is an example of this. In these strains OTA was not detected despite that several conditions were tested, and HPLC-MS was used (Castellá *et al*., 2018).

Although OTA was not detected in any of the strains, molecular screening of potential OTA biosynthetic genes was conducted. The biosynthetic pathway of OTA is not entirely clarified and can have differences between species. Nevertheless, the role of a PKS is indubitable. It has been deeply studied in many ochratoxigenic species like *A. carbonarius* (Gallo *et al*., 2014), *P. verrucosum* (O'Callaghan *et al*., 2013), *A. ochraceus* (O'Callaghan *et al*., 2003; Wang *et al*., 2015), *P. nordicum* (Geisen *et al*., 2006), *A. niger* (Zhang *et al*., 2016a), among others. Another enzyme that has an important role in all OTA producers is NRPS (Geisen *et al*., 2006; Gallo *et al*., 2012; Gil-Serna *et al*., 2015; Chakrabortti *et al*., 2016). It is expected that genes are similar between *Penicillium* species and the same for *Aspergillus*. Nevertheless, the recent description of *P. thymicola* as a new ochratoxigenic fungus proved otherwise. A gene cluster containing a PKS and a NRPS hybrid was found, and it has a high similarity with OTA biosynthetic enzymes of *A. carbonarius* and *A. ochraceus*. No OTA biosynthetic genes were similar neither to *P. nordicum* nor *P. verrucosum* (Nguyen *et al*., 2016). Therefore, in this study *Penicillium* related primers were tested in *Aspergillus* strains and vice-versa.

One PKS related to *P. nordicum* (*otapks*) and one related to *A. carbonarius* (*Acpks*) were tested. Apart from the positive control, none of the strains was positive for *Acpks*. This set of primers was conceived for *A. carbonarius*, still, is also apt for *A. niger* (De Rossi *et al*., 2009). For this, it was chosen to test in *A. fumigatus* strains. In light of the results, other primers related to other *Aspergillus* species should be experienced. Regarding *otapks* results, 17 strains could amplify it. Almost all of these strains were from Chile (except one strain from this region that did not amplified this gene) demonstrating, once again, that this group of strains has some genetic differences compared to the others (from Portugal, Italy and Tunisia). These results were incongruous with the ones obtained in other studies. This specific primer was tested in food-related fungi, including *P. crustosum*, and it was only positive for *P. nordicum* (used here as positive control) (Bogs *et al*., 2006; Geisen *et al*., 2006). On these studies only one strain *of P. crustosum* was tested, and even though the majority of the strains tested here were not positive a

considerable amount (≈ 39%) were. Furthermore, the use of this primer was established to detect *P. nordicum*, among *Penicillium* isolated from cured meat (Bogs *et al*., 2006). This means that the amplification of *otapks* is not exclusive of *P. nordicum*, and the use of this primer as a molecular marker should be revised.

The importance of NRPS was already established here, and for that, a primer targeting this enzyme was tested - *otanps*. Only 8 of the 51 strains tested had positive results for this primer. Three *P. crustosum* strains from Italy strains, four from Chile and surprisingly one *A. fumigatus* strain. Again, these results do not agree with other studies (Bogs *et al*., 2006; Geisen *et al*., 2006). Only *P. verrucosum* and *P. nalgiovense* were able to amplify it, besides *P. nordicum*, even though that *P. nalgiovense* only presented a faint band. Although *A. fumigatus* was not used in those studies, *P. crustosum* and other *Aspergillus* species were, and none was positive. The absence of this gene does not mean that these strains are not able to synthetize OTA. The linkage between OTβ and l-phenylalanine can be done by a different gene, as it happens in *P. thymicola*.

On the other hand, the results for *otatra* were more consistent with the ones reported before (Bogs *et al*., 2006; Geisen *et al*., 2006). Almost all the strains were positive for this primer, even the *A. fumigatus* strains. This gene encodes for an ochratoxin transport protein. This protein may have other functions besides transporting ochratoxin, and for that is transversal to various species. Surprisingly, the group that least amplified this primer was Chile, demonstrating, again, the genetic differences present in these strains. Tunisian and Portuguese strains only amplified this primer and are probably similar to the one strain of *P. crustosum* tested in the former studies.

Four Chilean strains (CCCT 18.82, 18.96, 18.102 and 18.117) were positive for the three *P. nordicum* primers (*otapks*, *otanps* and *otatra*). The amplification indicates that these strains have very similar or equal genes to the ones needed to produce OTA. This implies that, from all of the isolates they are prime candidates to produce OTA. Yet, having these three genes is not enough. An halogenase and a cytochrome P450 monooxygenase are also needed to complete OTA biosynthesis (Gil-Serna *et al*., 2015; Chakrabortti *et al*., 2016; Ferrara et al., 2016; Geisen *et al*., 2018, Gil-Serna *et al*., 2018; Wang *et al*., 2018). Even if all the described genes for OTA production are present, it does not mean that the species/strains that have it will be able to produce OTA. Three *A. carbonarius* strains, with no deletion on those genes were not able to produce OTA (Castellá *et al*., 2018). Besides the OTA known genes, other regulatory genes may influence the biosynthesis (Wang *et al*., 2016). Furthermore, the external stimulus can also influence the regulation of biosynthetic genes of secondary metabolites. The regulation of OTA genes in *P. nordicum* and *P. verrucosum*, for example, is influenced by the presence of NaCl (Schmidt-Heydt *et al*., 2011; Schmidt-Heydt *et al*., 2015). In high NaCl conditions, the regulation of OTA is mediated by HOG MAP kinase signal transduction pathway (when present).

In terms of the number of genes, the secondary metabolism is the most variable subsystem, within Penicillia. Consequently, these fungi might acquire or change biosynthetic genes in order adapt to new environments or improve their fitness within the environment they already inhabit (Nielsen *et al*., 2017). The geographic isolation of the Chilean strains may have made them adapt and differentiate from others. Moreover, in the same study, the authors hypothesize that in *Penicillium* genomes there are some conserved domains like PKS and NRPS, that can serve to evaluate the similarity between other possible biosynthetic gene clusters. Taking this into account, an alignment of PKS primers was conducted in MEGAX software, in an attempt to create a degenerate primer to simplify the search for potential OTA producers (data not shown). Mainly due to the difference is the size of the different sequences, this attempt was unsuccessful.

Nowadays, a lot more is known about OTA biosynthetic pathway, however it is not completely understood. Continuing with these studies is of the utmost importance, especially in the food industry. The transcription of the genes always precedes the phenotypic production of the mycotoxins (Schmidt-Heydt *et al*., 2011). For this, if a good molecular method is defined to detect OTA prematurely (before its synthesis), outbreaks can be prevented. Moreover, the understanding between mycotoxins production and external signals, and the regulatory genes that might possibly control the biosynthesis of OTA are also subjects of interest.

Reports have been made on the influence of climate change on the production of mycotoxins (Paterson and Lima, 2010a; 2011; Medina *et al*., 2017). These reports hypothesise that some fungi might disappear, but that mostly the production of mycotoxins might change, and even new mycotoxins can appear. Cold regions are going to be more susceptible to mycotoxin contaminations. Studies on these matters should be one of the priorities of the food industry to predict and prevent outbreaks of mycotoxigenic fungi that can mean tremendous economic losses and put in danger peoples and animals' health.

5. Conclusions and Future Perspectives

The present work conducted a deep study on non-conventional Aspergilli and Penicillia Ochratoxin A producers. *P. crustosum* and *A. fumigatus* were the chosen to represent each group based on previous evidence. Under the studied conditions, it was not possible to detect OTA above the HPLC-FLD detection limit. However, this does not mean that OTA cannot be produced by these two species. Further conditions should be tested, like the use of different media with inducive OTA conditions such as different temperatures and water activities. Other extraction methods and the use of immunoaffinity columns can increase the chance of detecting OTA. Moreover, instead of using HPLC-FLD, HPLC-MS can be an option once that is a more sensitive apparatus.

A comprehensive study of each strain was conducted as well. Amplification of *BenA* gene and the use of RAPD technique were used to differentiate each individual, and even, if present, identify cryptic species. Due to the low number of A. fumigatus strains, it was only possible to see that was diversity between strains, but a higher number of isolates will give better and elucidating results on the intraspecies diversity. On the contrary, with the high number of *P. crustosum* strains from different backgrounds, it was possible to conclude that there is a lot of intraspecies variability, and that a process of speciation might have occurred in Chile. Trying to identify cryptic species is more challenging. For this, at least four more housekeeping genes should be amplified. Besides that, other typing techniques can be performed like MALDI-TOF MS or RFLP. Still, the goal should be sequencing the complete genome to secure the most information possible.

Studying the biosynthetic pathway of OTA is also a matter to take into account. Here three genes were investigated. The results also demonstrated the intraspecies diversity, and once again the isolates from Chile stood out. Four isolates of this country amplified the three genes which might indicate the ability to produce OTA. Though, these results do not invalidate that other strains can also produce OTA. In the end, with the current data, this theory cannot be supported. To better study this possibility other primers can be tested on these strains. However, sequencing the complete genome and further annotation is probably the best course of action.

OTA contamination is an urgent concern in the field of food safety. Overpopulation and climate changes can cause the emergence of new risks. Scientists and world leaders need to be prepared for these. Therefore, the knowledge of possible new producing species and matrices should increase. In this way developing predictions, preventive measures and diagnostic methods can avoid big economic losses and food outbreaks potentially harmful for humans and animals.

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7. Attachments

7.1. Attachment I

Table 7.1 - List of studied strains, their origin and the isolation substrate.

Strain	Substrate	Origin	Strain	Substrate	Origin
MUM 02.15	Water	Portugal	**MUM 17.75**	Apple	Tunisia
MUM 02.30	Bread	Portugal	**MUM 17.78**	Apple	Tunisia
MUM 03.16	Clothes	Portugal	**CCCT 18.46**	Merkén	Chile
MUM 10.199	Grapes	Portugal	**CCCT 18.52**	Merkén	Chile
MUM 14.30	Grape must	Portugal	**CCCT 18.62**	Merkén	Chile
MUM 14.31	Grapes	Portugal	**CCCT 18.77**	Merkén	Chile
MUM 16.11	Cheese rind	Italy	**CCCT 18.82**	Merkén	Chile
MUM 16.12	Cheese rind	Italy	**CCCT 18.96**	Merkén	Chile
MUM 16.58	Cheese rind	Italy	**CCCT 18.99**	Merkén	Chile
MUM 16.68	Cheese rind	Italy	**CCCT 18.102**	Merkén	Chile
MUM 16.98	Cheese rind	Italy	**CCCT 18.117**	Merkén	Chile
MUM 16.99	Cheese rind	Italy	**CCCT 18.119**	Merkén	Chile
MUM 16.102	Cheese rind	Italy	**CCCT 18.123**	Merkén	Chile
MUM 16.103	Cheese rind*	Italy	**CCCT 18.132**	Merkén	Chile
MUM 16.104	Cheese rind*	Italy	**CCCT 18.134**	Merkén	Chile
MUM 16.105	Cheese rind*	Italy	**CCCT 18.138**	Merkén	Chile
MUM 16.120	Cheese rind	Italy	**CCCT 18.141**	Merkén	Chile
MUM 16.121	Cheese rind	Italy	**CCCT 18.142**	Merkén	Chile
MUM 16.122	Cheese rind	Italy	**MUM 98.02**	Tree bark	Unknown
MUM 16.123	Cheese rind	Italy	**MUM 02.24**	Air	Portugal
MUM 16.124	Cheese rind	Italy	**MUM 07.05**	Air	Portugal
MUM 16.125	Cheese rind	Italy	**MUM 14.04**	Unknown	Unknown
MUM 16.126	Cheese rind	Italy	**MUM 16.03**	Orange skin	Portugal
MUM 16.132	Cheese rind	Italy	**MUM 18.39**	Unknown	Spain
MUM 17.31	Apple	Tunisia	**MUM 18.40**	Unknown	Unknown
MUM 17.70	Apple	Tunisia			

All the cheeses were isolated from cheese rind from Grana Padano, except for the ones marked with "*" that were isolated from cheese rind of Parmigiano Reggiano.

7.2. Attachment II

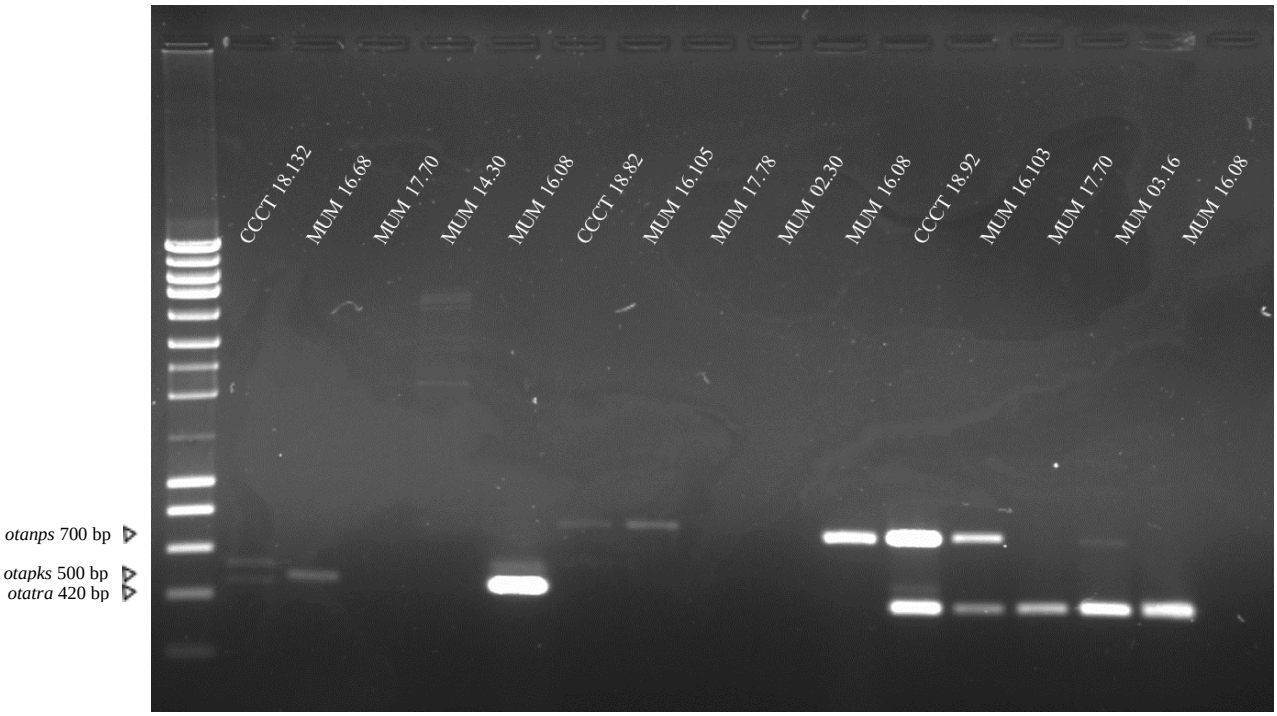

Figure 7.1 - Representation of PCR amplification of OTA byossinthetic genes: *otapks* (line 1-5), *otanps* (line 6-10) and *otatra* (line 11-15). Amplicons were visualized on 1% agarose gels. MUM 16.08 was used as positive control for all genes. The expected band size, for each gene, are in evidence. For *otapks* CCCT 18.132 and MUM 16.68, are positive even though that the Chilean strain evidence a non specific band. For this gene is also possible to see non specific amplification in MUM 14.30. For *otanps* there are also 2 positive strains (CCCT 18.82 and MUM 16.105). Lastly, for *otatra* all the strains are positive, but only MUM 17.70 does not evidence a non specific band.