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Short communication

Survey of Penicillia associated with Italian grana cheese

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ABSTRACT

The present work aimed to contribute information on the mycobiota associated with ripening grana cheese, with focus on the genus *Penicillium* as potential mycotoxin producers. Eighteen wheels of grana cheese, aged in different storehouses situated in Northern Italy, were sampled to isolate associated fungi. *Penicillium* spp. were commonly dominant; morphological observation and gene sequencing were applied to identify *Penicillium* at species level. *P. crustosum* and *P. solitum* were the dominant species. Citrinin and ochratoxin A mycotoxins were analysed and the latter was found in all grana cheese samples. These results confirmed that a polyphasic approach is mandatory for *Penicillium* identification at species level.

1. Introduction

Grana is an Italian hard cheese, made from unpasteurised, partially skimmed cow's milk, aged for a period ranging from nine months up to four years. The word "grana" comes from "grainy" to describe its fine granular texture. This cheese is of utmost importance in Italian food culture and economy. The Protected Designation of Origin (PDO) is attributed to "Grana Padano" and "Parmigiano Reggiano" brands. Nevertheless, information on the mycobiota associated with this type of cheese is poor, with only one paper published on this topic in recent decades (Dragoni et al., 1983). The development of uncontrolled moulds during ripening and ageing has been reported for other cheese types, causing spoilage and possibly leading to mycotoxin production (Ropars et al., 2012). The genera *Aspergillus*, *Cladosporium*, *Geotrichum*, *Mucor*, *Penicillium*, *Trichoderma* (Sengun et al., 2008) and *Fusarium* (Lund et al., 1995; Montagna et al., 2004) have been reported as cheese contaminants. In addition, some related mycotoxins such as citrinin (CIT) (Bailly et al., 2002; Cooper et al., 1982; Ostry et al., 2013; Pugazhenthi et al., 2000) and ochratoxin A (OTA) (Dall'Asta et al., 2008; Jarvis, 1983) have been detected in cheese. In particular, OTA contamination was recently reported in packed grated cheese, commonly produced with grana cheese (Biancardi et al., 2013). Mycotoxins are a main concern in food and feed: CIT showed nephrotoxic, immunosuppresive, teratogenic and mutagenic effects in past studies, and seems able to cause hemolysis of human erythrocytes (Ambrose and Deeds, 1946; Houbraken et al., 2010; IARC, 1986; Lurá et al., 2004; Ostry et al., 2013) while OTA is mainly toxic for the kidneys and liver (Richard, 2007) and classified in group 2B by the International Agency for Research on Cancer (IARC, 1987). Therefore, the present work aimed to contribute by filling in these gaps in knowledge with a first description of the mycobiota associated with ripening grana cheese, with focus on the genus *Penicillium*, as potential mycotoxin producers. A polyphasic approach, based on morphological and molecular observations and on mycotoxin analysis, was used.

2. Materials and methods

2.1. Chemicals

Mycological peptone, microbiological grade agar, malt extract and yeast extract were purchased from Himedia Laboratories (Mumbai, India); Dichloran Rose Bengal Chloramphenicol Agar (DRBC) and Czapek Dox Agar (CZ) ready prepared culture media were purchased from Oxoid (Basingstoke, U.K.); D(+) glucose was purchased from Sigma-Aldrich (Merck, Darmstadt, Germany); salts ($CuSO_4 \cdot 5H_2O$; $ZnSO_4 \cdot 7H_2O$; $K_2HPO_4 \cdot 3H_2O$; $MgSO_4 \cdot 7H_2O$; KCl; $FeSO_4 \cdot 7H_2O$) were purchased form Carlo Erba Reagents S. r. l. (Milano, Italy); a DNA "Plant II" extraction kit was purchased from Macherey-Nagel (Düren, Germany); PCR master mix was purchased from Promega Corporation (Madison, Wisconsin, USA); an Exosap cleaning kit was purchased from Euroclone (Pero (MI), Italy).

2.2. Sampling

Eighteen ripening storehouses for grana cheese were sampled in 2013, in five sites in northern Italy. Seven storehouses (1–7) were

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located in Brescia (BS), four in Cremona (8–11, CR), four in Mantova (12–15, MN), two in Piacenza (16 and 17, PC) and one in Verona (18, VR).

One ripening wheel was randomly chosen for each storehouse and sampled collecting 10 g of cheese rind, scratched from 5 different areas, to 0.5 mm depth.

2.3. Mycological analysis

One gram of scratched cheese rind was sub-sampled and diluted in 9 mL of physiological solution (1% peptone), accurately mixed and serially diluted up to 10^{-7}. Dilutions were plated in triplicate using DRBC agar medium and incubated for 7 days in the dark at 25 °C. Growing colonies were counted and reported as colony forming units per g of cheese rind (CFU/g) as a rough estimation of fungal bioload. Fungi were also identified to genus level using optical microscopy.

2.3.1. Strain selection

Three to five representative colonies of *Penicillium* were selected from each wheel, repeatedly transferred onto CZ to obtain pure cultures and subsequently managed to obtain monosporic strains destined for identification at species level.

Monosporic cultures were grown on Blakeslee Malt Extract Autolysate Agar (MEA, Samson and Frisvad, 2004; malt extract 30 g; mycological peptone 1 g; glucose 20 g; $CuSO_4 \cdot 5H_2O$ 0.005 g; $ZnSO_4 \cdot 7H_2O$ 0.01 g; agar 20 g; bidistilled water 1 L, adjusted to pH 5.3 ± 0.3), at 25 °C in the dark for one week. Plugs were then removed from each colony, put into bidistilled sterile water and stored at 4 °C until their use.

2.3.2. Morphological identification

All monosporic strains were 3-point inoculated on Czapek Yeast Agar (CYA, Samson and Frisvad, 2004; yeast extract 5 g; sucrose 30 g; $K_2HPO_4 \cdot 3H_2O$ 1.3 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; KCl 0.5 g; $FeSO_4 \cdot 7H_2O$ 0.01 g; $CuSO_4 \cdot 5H_2O$ 0.005 g; $ZnSO_4 \cdot 7H_2O$ 0.01 g; agar 15 g; bidistilled water 1 L, adjusted to pH 6.3 ± 0.2) and MEA and incubated at 25 °C in the dark for 7 days. At the end of incubation, they were observed for macroscopic (colony diameter, obverse and reverse colour, margins, presence/absence of exudate droplets) and microscopic characters (shape and size of penicillus and spores, roughness of the stipe) and tentatively grouped at species level.

Reference strains of the potential species of interest were obtained from culture collections (Table 1) and used as reference in the following steps of the study for comparisons with the strains selected according to Section 2.3.1.

2.3.3. Molecular identification

Monosporic strains were identified by *BenA* (encoding for β-Tubulin) and *COI* (encoding for Cytochrome Oxidase subunit I) gene sequencing. Samples were inoculated in 8 mL of Malt Extract Broth (MEB, Pitt, 1979; malt extract 30 g; mycological peptone 1 g; glucose 20 g; bidistilled water 1 L) and incubated at room temperature for 7 days in 15 mL Falcon® tubes; the tubes were kept on moving using an orbital shaker (100 rpm) for the whole incubation period.

DNA was extracted using a "Plant II" kit according to the handbook supplied by the producer. Diluted genomic DNA (1:10, about 2 ng/μL) was employed for PCR amplification using the primer pairs *Bt2a-Bt2b* (Glass and Donaldson, 1995) for *BenA* and *PenF1-AspR1* for *COI* (Seifert et al., 2007).

PCR reactions for both genes were performed in 7 μL reaction mixtures containing 3.5 μL Promega master mix (Promega Corporation, Wisconsin, USA), 1.5 μL genomic DNA, 0.25 μL of each primer (0.5 μM) and 1.5 μL bidistilled sterile water. Amplification conditions were the following: 95 °C for 2 min, 35 cycles at 95 °C for 1 min, 55 °C for 45 s, and 72 °C for 1 min, 72 °C for 10 min and rest at 4 °C.

Cleaning of the PCR product was performed using an Exosap kit according to the protocol provided by the producer. Sequencing was performed using a 48-capillary 3730 DNA Analyser (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Sequences (forward and reverse) provided in AB1 format file were opened using the pattern analysis software package Bioedit Sequence Alignment Editor v.7.2.5.0 (Alzohairy, 2011) and processed; identification at the species level was performed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Dendrograms were then deduced opening the final sequence using the pattern analysis software package Mega7 (Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets; Kumar et al., 2016) and using the Neighbor-Joining (NJ) method for dendrogram design (Saitou and Nei, 1987). The robustness of tree topology for each analysis was evaluated by 1000 bootstrap replicates in both cases.

Sequences of reference strains coming from official culture collections were obtained from the website of the respective collection, from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/), or by sequencing according to Section 2.3.3 if not available, and included in the dendrograms for clustering. More sequences were obtained from the GenBank database in case unexpected species were detected (type or neotype strains were chosen when possible).

2.4. Mycotoxin analysis

CIT and OTA were analysed in the 18 cheese samples, according to Somorin et al. (2016), and reported in μg/kg. Briefly, 9 g of scratched cheese rind were used; CIT was extracted using 50 mL of ortho-phosphoric acid and 10 mM methanol solution (3:7 v/v) using a rotary-shaking stirrer; then, after filtration through a folded filter paper, 2 mL of the filtrate were diluted with 20 mL of PBS and purified through immunoaffinity columns (R-Biopharm, Darmstadt, Germany). The column was washed with 2 mL of PBS and CIT slowly eluted (0.5 mL/min) with 4 mL of methanol into a graduated glass vial; the eluate was then concentrated under a gentle stream of nitrogen, brought to 1 mL with methanol and water solution (3:7 v/v) and vortexed for a few seconds. The extract was filtered before LC-MS/MS analysis. The instrumental analysis was carried out using a LC-MS/MS system in positive mode consisting of a LC 1.4 Surveyor pump, a Quantum Discovery Max triple-quadrupole mass spectrometer (Thermo-Fisher Scientific, San Jose, California, USA) and a PAL 1.3.1 sampling system (CTC Analitycs AG, Zwingen, Switzerland). Excalibur 1.4 software (Thermo-Fisher Scientific, San Jose, CA, USA) controlled the whole system. Limits of detection (LOD) and of quantification (LOQ) were 0.5 and 1.5 μg/kg, respectively.

OTA was quantified using a HPLC-FLD (fluorescence detector) system Perkin Elmer 200 (Perkin Elmer, Norwalk, Connecticut, USA) after extraction with 100 mL of sodium bicarbonate 0.13 M methanol (1:1 v/v) using a stirrer. The suspension was then filtered and 5 mL of the filtrate were diluted with PBS (50 mL) and purified through immunoaffinity columns (Ochratest WB, Vicam, Watertown, MA, USA), which was washed with 2 mL PBS so that OTA was slowly eluted (0.5 mL/min) with 3 mL of acetonitrile into a glass vial. The eluate was then concentrated under a gentle stream of nitrogen, brought to 1 mL with

Table 1
Reference strains from fungal collections used in this work.

Fungal collection	Strain code	Origin	Species
CBS	115992	The Netherlands	*P. citrinum*
CBS	122396	USA	*P. citrinum*
CBS	101025	Portugal	*P. crustosum*
CBS	115503	Scotland	*P. crustosum*
BFE	856	Italy	*P. nordicum*
CBS	110769	Spain	*P. nordicum*
CBS	112573	Italy	*P. nordicum*
CBS	323.92	Denmark	*P. verrucosum*
CBS	325.92	Denmark	*P. verrucosum*

CBS: CBS-KNAW Fungal Biodiversity Centre, The Netherlands.
BFE: Federal Research Centre for Nutrition and Food, Germany.

acetonitrile and 2% acetic acid solution (41:59 v/v) and vortexed for some seconds. The extract was filtered before analysis. The HPLC Perkin Elmer 200 system (Perkin Elmer, Norwalk, CT, USA) was equipped with a Jasco AS 1555 sampling system and a FP 1520 fluorescence detector (Jasco Corporation, Tokyo, Japan). A Borwin 1.5 software (Jasco Corporation, Tokyo, Japan) allowed the whole system to work. LOD and LOQ were 0.1 and 0.3 μg/kg, respectively.

3. Results

3.1. Fungal bioload

The eighteen cheese wheels sampled were all colonized by fungi varying between 10^3 and 10^7 CFU/g, with *Penicillium* ranging from 10^2 to 10^6 CFU/g. *Penicillium* was generally dominant with the exception

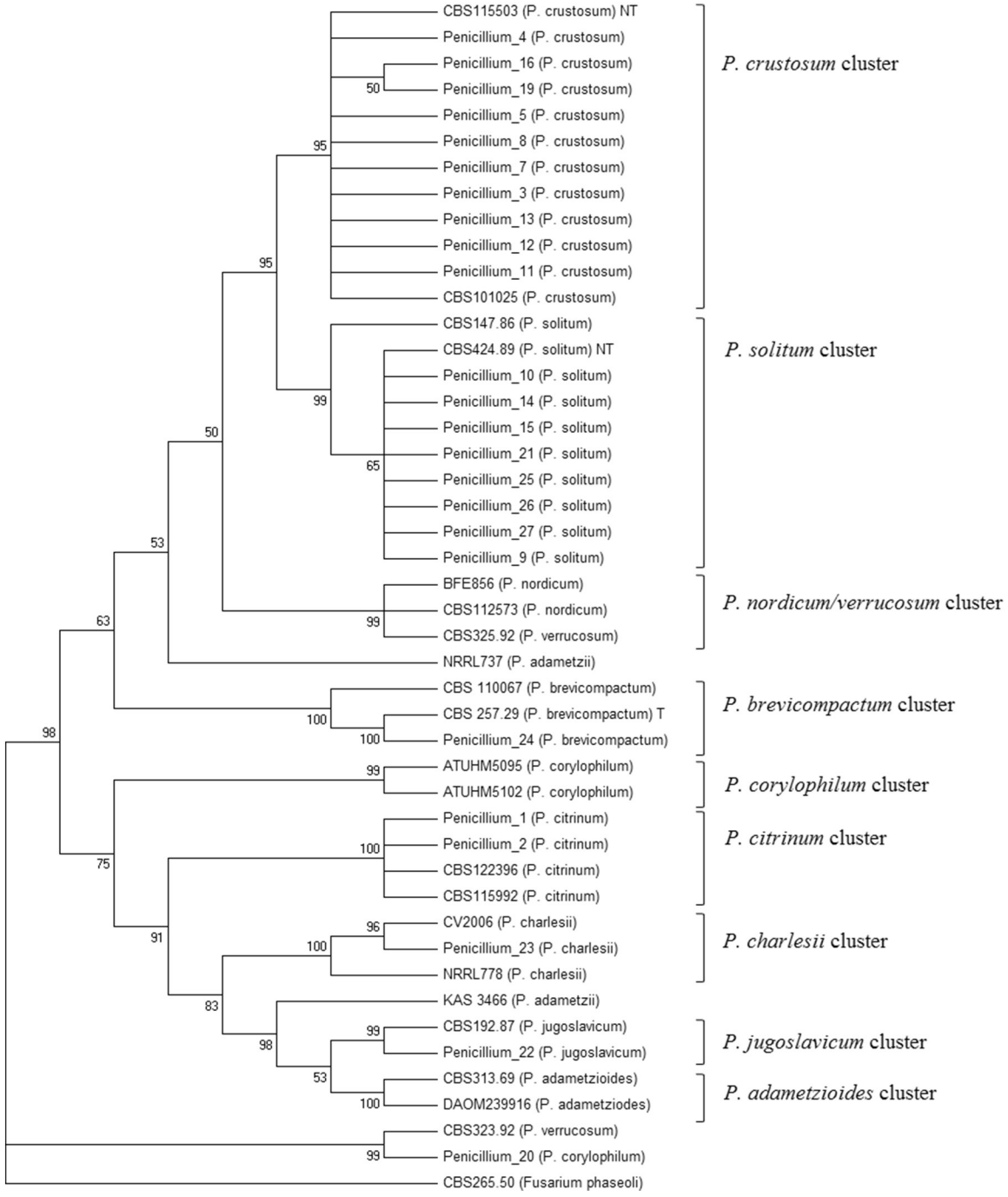

Fig. 1. Evolutionary relationships of taxa for the strains obtained from cheese rind monitoring (*BenA*). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 3.02079598 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The analysis involved 46 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. There were a total of 260 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). "T": type strain. "NT": neotype strain.

of samples 15, 17 and 18 where it was not isolated. Aspergilli were occasionally detected: *Aspergillus* section *Flavi* was isolated in samples coming from storehouses 10, 11 and 12. *Cladosporium*, *Rhizopus* and *Trichoderma* were also isolated.

3.2. Morphological identification

Twenty seven *Penicillium* spp. strains were selected in the survey; morphological observations of monosporic colonies grown on CYA

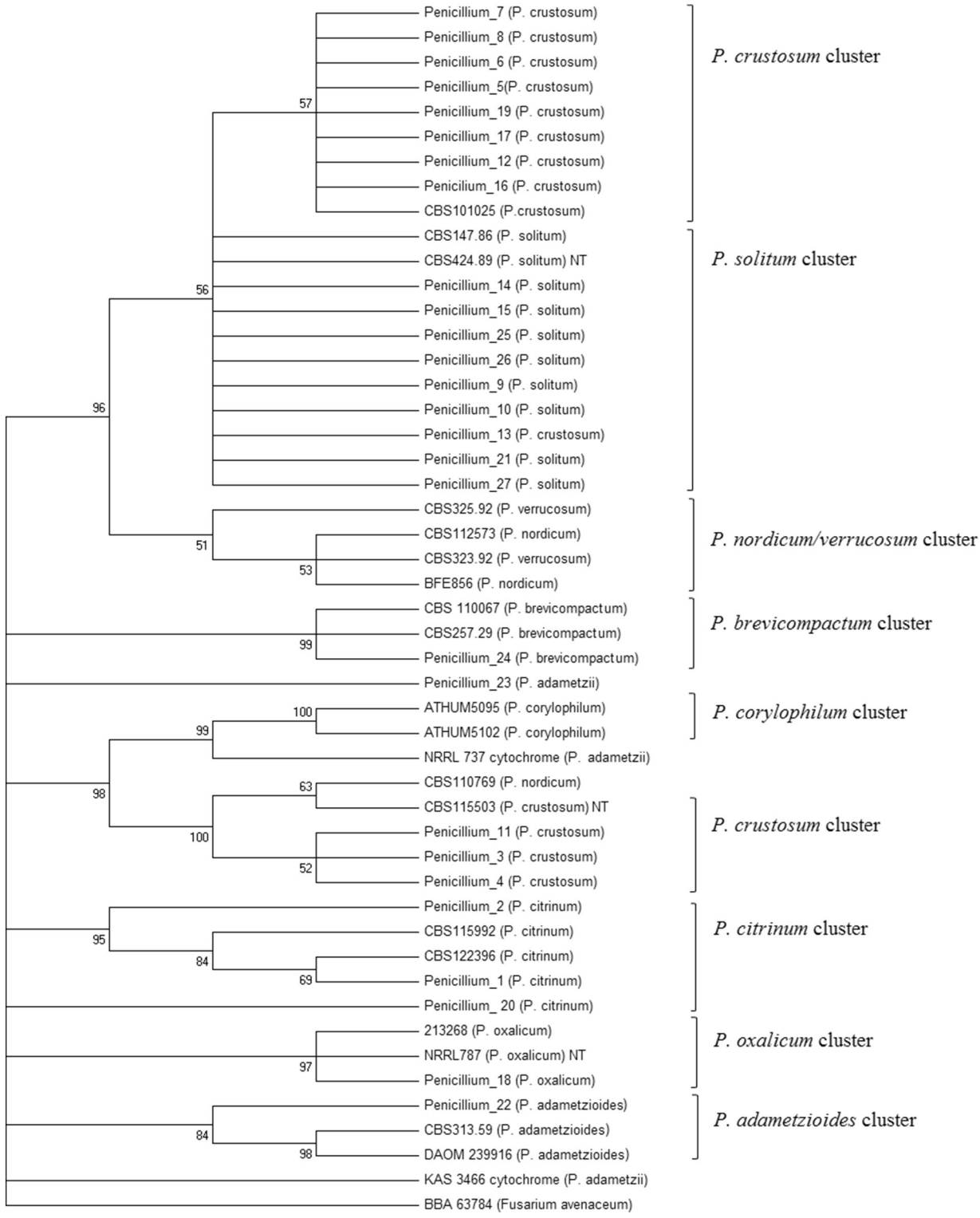

Fig. 2. Evolutionary relationships of taxa for the strains obtained from cheese rind monitoring (*COI*). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 2.29578242 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The analysis involved 49 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. There were a total of 324 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). "T": type strain. "NT": neotype strain.

and MEA and visual comparison with reference strains led to the identification of the majority of them as *P. crustosum*, *P. nalgiovense* and *P. citrinum*, one as *P. brevicompactum* while 3 were not identified at the species level using this approach.

3.3. Molecular identification

Gene sequencing results (Table 3) were generally well supported by their respective dendrograms. In particular, for *BenA* (Fig. 1), all *P. crustosum* sequences available (10) clustered very well with their references, and the same occurred also for *P. solitum* (8 sequences), *P. citrinum* (2 sequences), *P. brevicompactum*, *P. corylophilum*, *P. charlesii* and *P. jugoslavicum* (1 sequence each). Those clusters were generally supported by high (>70%) bootstrap values. Two outliers were found: the reference strain *P. verrucosum* CBS 323.92, not matching with the other *P. nordicum/verrucosum* reference strains and strain n° 20 identified as *P. corylophilum* in *BenA* sequencing. Considering *COI*, sequencing results coming from BLAST analysis differed from *BenA* only in a few cases (samples 20, 22 and 23), with a poorer clustering (Fig. 2). *Penicillium crustosum* sequences were shared in two clusters: one at the top of the dendrogram, with 9 sequences of strains collected in the study and 1 reference, and the other in the middle, with 3 sequences from this study; one reference and sample n° 13 were outlier. *Penicillium solitum* sequences were shared in one cluster, with 8 sequences of strains collected in this study and 2 references, and 2 outliers. Considering the other species, the clustering was comparably good as for *BenA*, except for strain n° 20, which was identified as a *P. citrinum*, but not clustered with the other *P. citrinum* sequences, n° 23 which was identified as *P. adametzii*, and CBS 110769, a *P. nordicum* reference strain. In *COI* dendrogram bootstrap values which support the clustering were far lower, but above 50%.

Therefore, identification based on morphology was only partially confirmed: all the strains belonging to *P. crustosum* and *P. citrinum* were correctly identified. On the contrary, using the molecular approach, putative *P. nalgiovense* strains were identified as *P. solitum*.

The final molecular identification of the strains was obtained merging results from both gene sequencing and sequence clustering: 12 (44.4%) *P. crustosum* (10 of which univocally identified by both of the 2 genes considered), 8 (29.6%) *P. solitum* and 2 (7.4%) *P. citrinum* strains were identified. Moreover, 3 other species (*P. brevicompactum*, *P. charlesii* and *P. oxalicum*) were identified as single strains. After the final identification, all the strains were deposited in the MUM (Micoteca da Universidade do Minho, Braga, Portugal) culture collection and a code was assigned (Table 3).

3.4. Mycotoxins

Regarding mycotoxin contamination, OTA was detected in all the grana cheese samples, ranging from 1 to 1432 μg/kg whereas CIT was detected only in 4 cheese wheels (from storehouses 7, 13, 15, 17) ranging from 42 to 100 μg/kg (Table 2).

4. Discussion

Morphological observation of the colonies on CZ, CYA and MEA, was useful to group strains and get a first presumptive identification: *P. crustosum* strains looked very similar to the reference strains CBS 115503 and CBS 101025 and their assignment to the correct species was quite straightforward. On the contrary, *P. solitum* strains were more difficult to assign, being quite similar in shape, size and general appearance to colonies of other species, including *P. nalgiovense*.

Thus, to get an accurate and reliable identification at species level the use of molecular techniques was crucial. Results obtained from the sequencing of both genes were generally in agreement, but *BenA* region seems more suitable, having shown a very good clustering, both for the strains obtained from cheese and for the reference strains.

Only a few outliers were found. Strain n° 20 was identified as *P. corylophilum*, but since it was far from the *P. corylophilum* sequences deposited in the GenBank and since its identity was not confirmed in *COI* sequencing, identification remained uncertain. Strain n° 22 was identified as *P. jugoslavicum* in *BenA* dendrogram, but no reference sequence was available in the GenBank for *COI* region; therefore, in this case, it was identified as the closely related species *P. adametzioides* (Visagie et al., 2013). Strain n° 23, based on *BenA*, was identified as *P. charlesii*, *P. adametzii* according to *COI* sequencing; no *P. charlesii* sequence was available for *COI* gene.

Strains n° 6 and 18 showed no clear evidence of being *P. crustosum* from a molecular point of view, since it was not possible to amplify their *BenA* regions and they clustered separately in the *COI* dendrogram; therefore, molecular and morphological identifications are in disagreement.

Penicillium brevicompactum, *P. crustosum*, *P. citrinum* and *P. solitum* were reported in past studies in cheese and/or cheese manufacturing and ripening environments (Houbraken et al., 2010; Kure and Skaar, 2000; Kure et al., 2004; Samson and Frisvad, 2004; Serra et al., 2003), as well as *P. citrinum* and *P. crustosum,* but none of them is known as OTA producer.

OTA and CIT were the only mycotoxins considered in this study, because OTA has been reported in grated cheese (Biancardi et al., 2013) and the main possible producers were *P. nordicum* (OTA producer) and *P. verrucosum* (CIT and OTA producer). OTA was detected in all the samples, ranging from 1 to 1431.9 μg/kg, but none of the producing species was detected using the classic, media-dependent isolation approach and the molecular characterisation of selected strains. Probably, presence of OTA producing species was limited compared with *P. crustosum* and *P. solitum* and this contributed to the difficulty of their isolation/selection. On the other hand, CIT presence, previously reported in cheese by Taniwaki and van Dender (2001) is supported by the isolation of *P. citrinum*. In the present study, the average OTA concentration was 183 μg/kg. Contamination detected was commonly higher than that reported by Biancardi et al. (2013), between 1.62 and 54.07 μg/kg. This is not surprising because the data are not exactly comparable. Biancardi et al. (2013) analysed commercially packed grated cheese where 18% v/v of cheese rind is allowed, whereas rind constituted 100% in our samples. OTA presence was also reported in semi-hard cheese by Pattono et al. (2013) in concentrations ranging from 1.0 to 262.2 μg/kg, in 6 of 32 cheese rind samples. Rind surface contamination during ripening is supposed to be responsible for OTA contamination;

Table 2
Fungal bioload, including *Penicillium* spp., and CIT and OTA concentrations obtained in the eighteen grana cheese samples of ripening storehouses studied.

Storehouse code	Storehouse place	Fungi CFU/g	*Penicillium* CFU/g	CIT μg/kg	OTA μg/kg
1	BS	$1 * 10^7$	$4 * 10^6$	<LOD	145
2	BS	$4 * 10^5$	$8 * 10^4$	<LOD	5
3	BS	$6 * 10^3$	$3 * 10^2$	<LOD	2
4	BS	$1 * 10^5$	$1 * 10^4$	<LOD	1
5	BS	$6 * 10^4$	$4 * 10^2$	<LOD	1432
6	BS	$7 * 10^3$	$1 * 10^2$	<LOD	126
7	BS	$7 * 10^6$	$3 * 10^5$	97	96
8	CR	$1 * 10^4$	$1 * 10^2$	<LOD	17
9	CR	$8 * 10^3$	$2 * 10^3$	<LOD	3
10	CR	$4 * 10^6$	$5 * 10^5$	<LOD	221
11	CR	$3 * 10^6$	$2 * 10^5$	<LOD	6
12	MN	$6 * 10^6$	$3 * 10^4$	<LOD	543
13	MN	$2 * 10^7$	$1 * 10^5$	100	77
14	MN	$5 * 10^3$	$3 * 10^2$	<LOD	153
15	MN	$7 * 10^7$	No	42	8
16	PC	$7 * 10^7$	$1 * 10^6$	<LOD	8
17	PC	$3 * 10^6$	No	68	401
18	VR	$3 * 10^6$	No	<LOD	51

LOD: limit of detection = 0.5 μg/kg (CIT) and 0.1 μg/kg (OTA).

Table 3
Results of morphological characterisation, gene sequencing (*COI* and *BenA*) and final identification for the 27 *Penicillium* strains isolated from cheese crust in 2013 and fungal code assigned in the MUM culture collection.

Strain	Store house	Morph. ID	*COI*	*BenA*	Final ID	MUM code
1	10	*P. citrinum*	*P. citrinum*	*P. citrinum*	*P. citrinum*	16.09
2	12	*P. citrinum*	*P. citrinum*	*P. citrinum*	*P. citrinum*	16.10
3	9	*P. crustosum*	*P. crustosum*	*P. crustosum*	*P. crustosum*	16.11
4	16	*P. crustosum*	*P. crustosum*	*P. crustosum*	*P. crustosum*	16.120
5	17	*P. crustosum*	*P. crustosum*	*P. crustosum*	*P. crustosum*	16.12
6	17	*P. crustosum*	*P. crustosum*	–	*P. crustosum*	16.121
7	7	*P. crustosum*	*P. crustosum*	*P. crustosum*	*P. crustosum*	16.122
8	7	*P. crustosum*	*P. crustosum*	*P. crustosum*	*P. crustosum*	16.123
9	7	*P. nalgiovense*	*P. solitum*	*P. solitum*	*P. solitum*	16.13
10	15	*P. nalgiovense*	*P. solitum*	*P. solitum*	*P. solitum*	16.14
11	16	*P. crustosum*	*P. crustosum*	*P. crustosum*	*P. crustosum*	16.124
12	12	*P. crustosum*	*P. crustosum*	*P. crustosum*	*P. crustosum*	16.125
13	17	*P. crustosum*	*P. crustosum*	*P. crustosum*	*P. crustosum*	16.126
14	4	*P. nalgiovense*	*P. solitum*	*P. solitum*	*P. solitum*	16.127
15	10	*P. nalgiovense*	*P. solitum*	*P. solitum*	*P. solitum*	16.128
16	18	*P. crustosum*	*P. crustosum*	*P. crustosum*	*P. crustosum*	16.129
17	9	*P. crustosum*	*P. crustosum*	–	*P. crustosum*	16.130
18	16	*Penicillium* sp.	*P. oxalicum*	–	*P. oxalicum*	16.131
19	9	*P. crustosum*	*P. crustosum*	*P. crustosum*	*P. crustosum*	16.132
20	8	*Penicillium* sp.	*P. citrinum*	*P. corylophilum*	Uncertain	16.133
21	8	*P. nalgiovense*	*P. solitum*	*P. solitum*	*P. solitum*	16.135
22	17	*Penicillium* sp.	*P. adametzioides*	*P. jugoslavicum*	Uncertain	16.136
23	17	*Penicillium* sp.	*P. adametzii*	*P. charlesii*	*P. charlesii*	16.137
24	17	*P. brevicompactum*	*P. brevicompactum*	*P. brevicompactum*	*P. brevicompactum*	16.15
25	8	*P. nalgiovense*	*P. solitum*	*P. solitum*	*P. solitum*	16.138
26	8	*P. nalgiovense*	*P. solitum*	*P. solitum*	*P. solitum*	16.139
27	16	*P. nalgiovense*	*P. solitum*	*P. solitum*	*P. solitum*	16.140

–: no sequence available.

therefore, data from literature are reasonably in agreement with those reported in this study.

CIT in cheese was reported by Jarvis (1983) who investigated 44 cheese samples and found this mycotoxin in 17 of them (39%) in concentrations up to 50 μg/kg; thus, also these data are comparable with our results, with 22% of samples positive and contamination between 42 and 97 μg/kg.

CIT, penitrem A and roquefortine C (Samson and Frisvad, 2004) could be detected in cheese, based on *P. citrinum* and *P. crustosum* fungi isolation in this study, in agreement with literature (Kokkonen et al., 2005; Richard and Arp, 1979). It is not clear which fungus is responsible for OTA contamination, because of the lack of isolation of the candidates *P. verrucosum* and *P. nordicum*. Therefore, this must be considered in future studies.

5. Conclusions

This work has provided for the first time information about Penicillia growing on grana cheese crust during ripening. Two dominant species, *P. crustosum* and *P. solitum*, emerged clearly from this survey, but neither is reported as an OTA-producer. Thus, the origin of OTA in all the cheese crust samples remains unknown. Ochratoxigenic species, although present to a lesser extent than the dominant fungi, must be considered and detecting methods other than the classic media-dependent isolation approach should be considered: since the latter is time-consuming and sometimes unreliable, support of molecular approaches is mandatory.

In conclusion, further studies on a broader set of strains are required to corroborate these data, contribute to better identification of the mycobiota and provide input for effective strategies to mitigate mycotoxin contamination.

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