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Polyphasic identification of Penicillia and Aspergilli isolated from Italian grana cheese

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ABSTRACT

Penicillium and *Aspergillus* genera, both including mycotoxin producing species, were reported as associated to cheese and cheese working environment, but never studied in an extensive way in Italian grana cheese (Grana Padano and Parmigiano Reggiano). The aim of this work was to address the identification of Aspergilli and Penicillia associated to grana cheese in order to lay down the basis for risk assessment and safe processing for a high quality production. One hundred and four strains belonging to *Aspergillus* and *Penicillium* genera were obtained from cheese crust and from ripening room air (with the latter largely dominant), and identified following a polyphasic approach, strongly required for the identification at the species level. Morphological observation was used along with molecular techniques, RAPD-PCR fingerprinting and calmodulin gene sequencing (*CaM*), the former aimed to limit as much as possible the latter sequencing effort. Seventy four percent of the strains were assigned to *Penicillium* subgenus *Penicillium*, section *Fasciculata*. Main mycotoxin producing species identified were *A. flavus*, *P. crustosum* and *P. verrucosum*, while the dominant species in both air and cheese crust was *P. solitum*, which has never been so far reported as mycotoxigenic. Results obtained in this study confirmed that mycotoxin contamination is a possible issue to face during grana cheese making.

1. Introduction

The well-known “Grana Padano” and “Parmigiano Reggiano” are called collectively grana cheeses and are produced in Italy. They obtained the Protected Designation of Origin (PDO) certification from the European Union and the production process is managed according to guidelines established by their respective consortia (www.granapadano.it; www.parmigianoreggiano.it). In compliance with PDO certification, grana cheeses have a long ripening period; from 9 months for standard, up to several years for premium goods.

Grana cheese production relies on the action of microorganisms, including bacteria, yeasts and filamentous fungi (ff) which convert the processed milk into cheese, contributing to its final characteristics like consistency, taste and flavour: while the primary microbiota is mainly composed of starter lactic acid bacteria, the secondary microbiota includes salt-tolerant bacteria, yeasts, and ff which perform degradation of proteins, sugars and lipids. In this type of cheese, the development of ff usually occurs during ripening and it is only limited to the crust like the most of cheeses, if we exclude blue ones where fungal inoculum is intentionally added to obtain mould growth internally (Ropars et al., 2012). However, ff belonging to *Aspergillus*, *Cladosporium*, *Fusarium*, *Geotrichum*, *Mucor*, *Penicillium* and *Trichoderma* can cause cheese spoilage (Lund et al., 1995; Montagna et al., 2004; Northolt and Bullerman, 1980) with many undesired organoleptic effects (Ropars et al., 2012).

Penicillium species are commonly reported as the dominant contaminant fungi in cheese (Lund et al., 1995) and among them *P. commune*, *P. roqueforti*, *P. crustosum* (Frisvad and Samson, 2004), *P. citrinum* (Manabe, 2001) and also *P. nordicum* and its closely related species *P. verrucosum* (Frisvad and Samson, 2004) can be mentioned. In particular, *P. commune* is considered a separated species from *P. camemberti* and *P. caseifulvum* only under a technological point of view: in fact, Visagie et al. (2014a) found that these three species showed identical ribosomal RNA internal transcribed spacer (*ITS*), β-tubulin (*BenA*) and calmodulin (*CaM*) sequences, confirming the results obtained by Giraud et al. (2010) for translation elongation factor 1-alpha (*TEF-1α*). Some *Aspergillus* spp are also included: for instance *A. versicolor*, which was

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reported in past years as cheese contaminant (Lund et al., 1995), and also *A. flavus*, *A. fumigatus* and *A. niger* (Hayaloglu and Kirbag, 2007).

Several mycotoxins, which are toxic secondary metabolites produced by some fungi with adverse health effects on humans and animals (Paterson and Lima, 2010), produced by Penicillia have been detected in cheese including citrinin, cyclopiazonic acid, isofumiglacavine A, mycophenolic acid, penicillic acid, patulin, PR-toxin, roquefortine C, sterigmatocystin and ochratoxin A (Hymery et al., 2014). In addition, sterigmatocystin produced by *A. versicolor* and some other closely-related species (*A. tennesseensis*, *A. venenatus* and, possibly, *A. puulaauensis*) was also detected in cheese (Hymery et al., 2014; Jurjević et al., 2012). Aflatoxin (AF) M_1 was reported in milk, as a carry over by dairy animals fed with AFB_1-contaminated feed and, consequently, in Parmigiano Reggiano (Pietri et al., 2003) and in Grana Padano cheeses (Pietri et al., 1997).

Despite grana cheese being important in Italian food, information on the associated mycobiota is infrequent, with only two reports published in the last four decades (Dragoni et al., 1983; Decontardi et al., 2017). The fungi identified included several *Penicillium* spp (*P. brevicompactum*, *P. citrinum*, *P. crustosum*, *P. expansum* and *P. solitum*). So, there is a need to contribute with more consistent knowledge.

The identification of *Penicillium* and *Aspergillus* strains at species level is difficult and the best approach to get a precise and reliable identification is polyphasic, merging the morphological, chemical and molecular trait results in the final identification (Frisvad and Samson, 2004; Geiser et al., 2007; Houbraken et al., 2011; Rodrigues et al., 2009; Silva et al., 2015; Simões et al., 2013).

The present work addressed the identification of Penicillia and Aspergilli associated with Italian grana cheese through a polyphasic approach, keeping in mind mycotoxin producers, in order to lay down the basis for risk assessment and safe processing for high quality production.

2. Materials and methods

2.1. Sampling

2.1.1. Cheese sampling

Fifty four cheese crust samples were delivered from 9 over 12 storehouses considered from 5 locations in Italy. The sampling consisted of 10 g of cheese crust, scratched up to 0.5 mm depth, from randomly selected cheese, as previously described by Decontardi et al. (2017). Different ripening times, from 2 to 16 months, were considered. Water activity (a_w) of the samples was measured using an Aqualab LITE (version 1.3 © Decagon devices Inc., WA, USA). The details of sampling, including a_w values, are listed in Table 1s.

2.1.2. Storehouse air sampling

In each of the 12 cheese factories, 10 potato dextrose agar (PDA, Himedia) plates with streptomycin (1 g/L, Sigma-Aldrich), randomly distributed in ripening rooms, were opened, placed close to cheese wheels and exposed to the air (Battilani et al., 2007) for 20 min, then closed and sealed in plastic bags.

2.2. Mycological analysis

2.2.1. Ripening cheese

One g of crust was suspended in 9 mL of physiological solution (1% peptone, BioLab). The suspension was mixed using a stirrer and serially diluted to 10^{-7}, plated in triplicate using dichloran rose Bengal chloramphenicol agar (DRBC, Oxoid) and incubated for 7 days in the dark at 25 °C. Colonies were counted and fungi were identified to genus level according to Samson and Frisvad (2004) and Samson et al. (2010) using a stereomicroscope (Motic SMZ 140-2L). Results were expressed as colony forming units (CFU) per g of cheese crust.

2.2.2. Storehouse air

PDA plates exposed in the ripening rooms were incubated at 25 °C in the dark for 7 days (Battilani et al., 2007). Colonies were counted and fungi identified to genus level as above described. The results were expressed both as CFU/cm^2 of exposed area and as CFU/m^3 of air according to the Omeliansky formula (Abdel Hameed and Adel Malwa, 2012; Abdel Hameed et al., 2012).

Some representative colonies of *Aspergillus* and *Penicillium* were selected based on their appearance on culture media (3–5 for each morphotype observed), repeatedly transferred on Czapek Dox agar (CZ, Oxoid) and managed to obtain monosporic cultures: spores were picked from each colony, suspended in 1 mL of physiological solution and serially diluted to 10^{-5}; then, 10^{-4} and 10^{-5} dilutions were plated in triplicate on Water Agar (WA, microbiological grade agar, 20 g; bidistilled water, 1 L) and incubated at 25 °C in the dark for 2–3 days. Two growing colonies were subsequently cut from the plate and grown at 25 °C in the dark on Malt Extract Autolysate Agar (MEA, malt extract 20 g; mycological peptone 1 g; glucose 20 g; agar, 15 g; bidistilled water, 1 L). The serial dilutions and the growing on MEA were repeated twice; one monosporic colony was then chosen for each isolate and eight plugs were removed, put in vials containing bidistilled sterile water and stored at 4 °C before the identification to species level.

2.3. Morphological characterisation

2.3.1. Macroscopic features observation

Monosporic cultures were three-point inoculated using a soft agar spore suspension (0.3% agar, 0.1% Tween 80®) on three media: MEA, Creatine Sucrose Neutral Agar (CSN, KCl 0.5 g; sucrose 10 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; $FeSO_4 \cdot 7H_2O$ 0.01 g; $ZnSO_4$ 0.01 g $CuSO_4 \cdot 5H_2O$ 0.005 g; creatine 5 g; KH_2PO_4 1 g; bromocresol purple 0.05 g; agar, 15 g; bidistilled water to 1 L) and Czapek Yeast Autolysate (CYA, 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; agar 15 g; bidistilled water 1 L) according to Pitt (1979); moreover, presumptive *P. nordicum*/*P. verrucosum* isolates were also inoculated on Yeast Extract Sucrose (YES, yeast extract 20 g; sucrose 15 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; $CuSO_4 \cdot 5H_2O$ 0.005 g; $ZnSO_4 \cdot 7H_2O$ 0.01 g; agar 20 g; bidistilled water 1 L) in order to observe the reverse colour of the colonies as contribute to their identification at species level, according to Samson and Frisvad (2004). Inoculated plates were incubated at 25 °C in the dark for 7 days and two perpendicular diameters were measured for each colony at the end of incubation. Medium and colony reverse reaction were also observed for the CSN inoculations and classified according to Pitt and Hocking (1997). Using a Canon EOS 5D Mark II digital camera, obverse and reverse of colonies grown on MEA and CYA media were photographed as described in Simões et al. (2013).

2.3.2. Microscopic features observation

MEA 60 mm diameter plates were centrally inoculated with a drop of the spore suspension previously mentioned and incubated at 25 °C in the dark for 7 days. Microscope slides were prepared using 96% ethanol as the mounting fluid; excess of spores was washed out before staining with 50% cotton blue and 50% lactic acid solution. Morphologies were observed using an optical microscope (Leica DM5000B). Length and width of rami, metulae, phialides and two perpendicular diameters of the spores were measured for the

majority of Penicillia except for the monoverticillates, while for Aspergilli and the monoverticillate Penicillia belonging to Subg. *Aspergilloides* (Visagie et al., 2014b), the diameter of the vesicula and the width of the stem along with measurements of phialides and spores were determined. These characters were compared with those of reference strains listed in Table 1.

2.4. Molecular characterisation of the strains

2.4.1. DNA extraction

Strains were inoculated in 20 mL of malt extract broth (MEB, malt extract 20 g; mycological peptone 1 g; glucose 20 g; bidistilled water, 1 L) and incubated at room temperature for 7 days in 50 mL Falcon® tubes on an orbital shaker (100 rpm). Genomic DNA was extracted using a "FastDNA® SPIN Kit" according to the manufacturer's handbook. The DNA was subjected to quality assessment by electrophoresis (70 V/cm for 30 min) on 1% (W/V) agarose in 0.5× TAE buffer gel (50× TAE buffer gel: tris base 242 g; acetic acid glacial 57.1 mL; 0.5 M EDTA pH 8.0100 mL; bidistilled water to 1 L). GreenSafe Premium was added and photographs taken with a Molecular Imager® ChemiDoc™ XRS + Imaging System (BioRad Laboratories). NZYDNA ladder III (1Kbp) was used as a DNA molecular weight marker. In addition, DNA quantity and quality was measured by reading the whole absorption spectrum (220–750 nm) with Micro-Spectrophotometer NanoDrop ND-1000 and calculating DNA concentration and absorbance ratio at both 260/280 and 230/260 nm. The machine was calibrated and cleaned according to the calibration check procedure.

2.4.2. PCR fingerprinting amplification

2.4.2.1. Amplification. DNA concentrations between 10 and 20 ng/μL were used as a template for RAPD-PCR fingerprinting with the M13 primer (sequence: 5′-GAGGGTGGCGGTTCT-3′) (Schlick et al., 1994). PCR reactions were performed in 25 μL reaction mixtures containing NZYTaq 2× Green master Mix (12.5 μL), genomic DNA (1 μL), the primer (2 μL; 0.4 mM) and 9.5 μL of bidistilled sterile water. Amplification conditions used for RAPD-PCR reaction with M13 primer were published by CBS-KNAW (full details at http://www.scedosporium-ecmm.com/Protocols/Fingerprinting%20Random%20Amplified%20Polymorphic%20DNA%20with%20M13.pdf).

Amplified products were resolved by electrophoresis (50 V/cm for 2 h) on 0.8% (W/V) agarose in 0.5× TAE buffer gels.

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

Species	Strain code	Origin
A. versicolor	CBS 108959	Denmark
A. versicolor	CBS 116680	Unknown
P. brevicompactum	MUM 16.15	Italy
P. camemberti	CBS 122399	USA
P. citrinum	CBS 122396	The Netherlands
P. citrinum	MUM 16.09	Italy
P. citrinum	MUM 16.10	Italy
P. crustosum	CBS 115503	The Netherlands
P. crustosum	MUM 16.11	Italy
P. crustosum	MUM 16.12	Italy
P. nalgiovense	CBS 109609	Denmark
P. nordicum	BFE 856	Italy
P. nordicum	CBS 112573	The Netherlands
P. roqueforti	CBS 221.30	USA
P. solitum	MUM 16.13	Italy
P. solitum	MUM 16.14	Italy
P. verrucosum	CBS 325.92	The Netherlands

CBS: Westerdijk Fungal Biodiversity Institute.
MUM: Micoteca da Universidade do Minho.
BFE: Federal Research Centre for Nutrition and Food.

2.4.2.2. Data analysis. Photographs were saved as TIFF files and exported into the pattern analysis software package BioNumerics (version 6.6; Applied Maths BVBA, Sint-Martens-Latem, Belgium) for processing. Calculation of similarity of the PCR fingerprinting profiles were based on the Pearson product-moment correlation coefficient (PPMC). A dendrogram was deduced from the matrix of similarities by the unweighted pair group method using arithmetic average (UPGMA) clustering algorithm (Vauterin and Vauterin, 1992).

2.4.3. Gene sequencing

CaM amplification and sequencing were performed for samples that did not show good clustering with M13 fingerprinting, and/or for those ones that rose doubts in morphological observation.

2.4.3.1. CaM gene amplification. Diluted genomic DNA (1:10) was employed for PCR amplification using the primers pair with the sequences 5′-GA(GA)T(AT)CAAGGAGGCCTTCTC-3′ for CL_1 (forward) and 5′-TTTTTGCATCATGAGTTGGAC-3′ for CL_2a (reverse) (O'Donnell et al., 2000). PCR reactions were performed in 50 μL reaction mixtures containing NZYTaq 2× Green master Mix (25 μL), genomic DNA (2 μL), 1 μL (10 mM) of each primer and 21 μL of bidistilled sterile water. Amplification conditions (Rodrigues, 2010) were the following: initial denaturation: 600 s at 95 °C; denaturing: 50 s at 95 °C; annealing: 50 s at 55 °C and extension: 60 s at 72 °C repeated for 35 cycles. Final extension: 420 s at 72 °C, resting: 4 °C. Alternative conditions (initial denaturation: 300 s at 94 °C; denaturing: 45 s at 94 °C; annealing: 45 s at 52 °C; extension 60 s at 72 °C repeated for 35 cycles; final extension: 420 s at 72 °C; resting: 10 °C) were also tested according to Visagie et al. (2014b).

Amplified products were resolved by electrophoresis (70 V/cm for 30 min) on 1% (W/V) agarose in 0.5× TAE buffer gels, to which GreenSafe Premium was added. Amplification success was checked. One kbp NZYDNA ladder III was used as a DNA molecular weight marker.

2.4.3.2. PCR product cleaning and sequencing. Cleaning of the PCR product was performed using a NZYGelpure kit according to the manufacturer's protocol.

Sequencing of the cleaned amplified PCR products was performed by Stab Vida LDA (Madan Parque, Caparica, Portugal). Ten μL of purified PCR product were put in a labelled 1.5 mL tube along with 3 μL of primer (one tube was prepared including the forward primer, the other including the reverse primer) and analysed by Stab Vida Oporto laboratories (Centro de Testagem Molecular, Vairão, Portugal).

2.4.3.3. Data analysis. Gene sequences (forward and reverse) were provided in AB1 format file. Every forward and reverse AB1 file was opened into the pattern analysis software package Bioedit Sequence Alignment Editor v.7.2.5.0 (Alzohairy, 2011) and then processed until the global sequence was obtained. Dendrograms were then deduced, after BLAST (Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.gov/) search, opening the sequences into the pattern analysis software package Mega7 (Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets; Kumar et al., 2016) and using the Neighbour-Joining method (Saitou and Nei, 1987). The optimal tree with the minimum sum of branch length was shown and the percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches (Felsenstein, 1985). The tree is drawn with branch lengths in the same unit as those of the evolutionary distances used to infer the phylogenetic tree. 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. All positions containing gaps and missing data were eliminated. Sequences of the reference strains, also included for clustering, were obtained by direct sequencing or by searching on the website of the fungal collection CBS-KNAW; also GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) was consulted when required sequences were not available. *Trichocoma paradoxa* CBS 788.83 was used as outgroup for all the dendrograms performed and *Rasamsonia argillacea* CBS 101.69 was also included in the dendogram containing mono- and biverticillate Penicillia, enhancing statistical differences between them and the outgroups; that was necessary because of the poor number of available sequences.

3. Results

3.1. Storehouses

In 2014, 12 storehouses placed in different districts, located within the areas of production of grana cheese in northern Italy, were considered according to section 2.2. All of them were air-conditioned and temperature and relative humidity were as reported in Table 2.

3.2. Fungal bioload

Bioload in ripening cheese ranged for Penicillia between nil to 3.27×10^7 CFU/g, with a mean of 3.85×10^6 CFU/g, while for all the other fungi, including Aspergilli, it ranged from nil to 1.20×10^8 with a mean value of 9.77×10^6 CFU/g. Samples coming from storehouses N. 5 and 4 were the most contaminated by Penicillia (about 10^7 CFU/g), followed by N. 3 (about 10^6 CFU/g), N. 7, 8, 2, and 12 (about 10^5 CFU/g) and N. 1 and 10 (about 10^3 CFU/g). The highest amount of all ff were detected in samples coming from storehouses N. 5, 8 and 3 (about 10^7 CFU/g), followed by N. 1, 7, 4 and 2 (about 10^6 CFU/g) and N. 12 and 10 (about 10^5 CFU/g). Results are listed in Tables 1s and 2s

Regarding the storehouse air, the number of *Penicillium* spp ranged from nil to 0.34 CFU/cm^2, with a mean value of 0.10 CFU/cm^2. The mean CFU/cm^2 for all the other ff including Aspergilli was approximately 1.08. Plates coming from storehouses N. 11, 8, 7 and 3 were the most contaminated by Penicillia (0.15–0.34 CFU/cm^2), followed by N. 12, 4, 2 and 9 (0.03–0.08 CFU/cm^2) and N. 6,1 and 5 (0–0.005 CFU/cm^2). The most contaminated environments by all ff were storehouses N. 1, 8 and 12 (1.50–4.22 CFU/cm^2), followed by N. 2, 6, 7, 4, 9, and 5 (0.12–0.95 CFU/cm^2) and N. 11 and 3 (0.03–0.10).

In addition, the fungal bioload of air ranged from nil to 8.65×10^2 CFU/m^3, with a mean value of 2.52×10^2 CFU/m^3 for Penicillia, and the mean value for all the other ff was approximately 5.56×10^3 CFU/m^3. Results are listed in Table 3s.

Plates from storehouse N. 10 were excluded from those calculations as they were completely covered by *Penicillium* spp colonies and were therefore uncountable.

Other ff detected belonged to *Cladosporium*, *Trichoderma* and *Rhizopus*; they were not processed for identification to species level because the focus of the study only concerned Penicillia and Aspergilli.

No correlation was observed between the amount of fungal bioload in environmental air and in cheese crust, so as with ecological conditions registered in the environments (data not shown).

One hundred and four strains were selected from the fungal bioload monitoring programme for the identification at species level, 94 Penicillia and 10 Aspergilli; an almost equally distribution between air (49 strains, 47%) and cheese crust (55 strains, 53%) was used.

3.3. Morphological characterisation

Fifty-four out of 94 *Penicillium* spp isolates were identified based on their morphology as follows: *P. solitum* (30 strains), *P. nordicum*/*P. verrucosum* (11), *P. crustosum* (8), *P. chrysogenum* (3) *P. brevicompactum* and *P. citreonigrum* (1 each) (Tables 3s and 4s; Fig. 1s). The majority of *P. solitum* isolates showed weak acid-margin neutral reaction in the CSN medium (18 over 30) and weak acid or alkaline reaction in the colony reverse (28 over 30). *P. nordicum*/*P. verrucosum* isolates showed a weak acid-margin neutral (6 over 11) or neutral (5 over 11) reaction in the medium and a brown colony reverse (11 over 11). All *P. crustosum* isolates showed acid or strong acid reactions either in growth medium or in colony reverse, while all *P. chrysogenum* isolates showed weak acid colony reverse reactions and strong acid medium reactions.

The observation of *P. nordicum*/*P. verrucosum* isolates' reverse colony colour on YES suggested to assign them to *P. verrucosum* species because they all showed a terracotta-brown pigmentation, according to Samson and Frisvad (2004).

Forty *Penicillium* isolates out of 94 were not identified to species level at this stage (Table 3). Regarding the Genus *Aspergillus*, *Aspergillus flavus* (5 strains) and *A. versicolor* (5 strains) were also identified.

3.4. Molecular characterisation

3.4.1. PCR fingerprinting

M13 RAPD-PCR was performed to provide characters for cluster analysis of the studied strains (Figs. 1–3; Table 3); dendrograms were obtained for (a) Aspergilli, (b) Penicillia subgenus *Aspergilloides* (mono and biverticillate) and (c) Penicillia subgenus *Penicillium* (terverticillate).

All the strains identified morphologically as *A. flavus* clustered together except one, N. 68, which was placed near the *A. versicolor* cluster; 4 out of 5 strains identified as *A. versicolor* clustered together, except strain N. 60. The two reference strains of *A. versicolor* (CBS 108959 and CBS 116680) were placed near the outlier, but not joined in a cluster (Fig. 1).

For the mono and biveriticillate Penicillia, the M13 RAPD-PCR dendrogram was less informative, since the majority of them were single isolates: only 2 unidentified strains (N. 77 and N. 91) with similar morphological features formed a cluster, while the others were distributed (Fig. 2).

Table 2
Environmental temperature (T) and relative humidity (RH) conditions of the 12 cheese storehouses considered in this study.

Storehouse	District	T (°C)	RH (%)
1	Bergamo (BG)	15.0	72.5
2	Bergamo (BG)	–	–
3	Brescia (BS)	15.5	82.5
4	Brescia (BS)	–	–
5	Cremona (CR)	15.0	82.5
6	Mantua (MN)	17.0	79.5
7	Piacenza (PC)	15.5	82.5
8	Reggio Emilia (RE)	20.0	72.0
9	Reggio Emilia (RE)	16.5	80.0
10	Reggio Emilia (RE)	16.2	83.0
11	Reggio Emilia (RE)	15.4	82.5
12	Reggio Emilia (RE)	17.0	80.0

-: no data available.

Table 3
Morphological identification, M13 fingerprinting clustering, *CaM* gene sequencing results, final identification (Final ID) and MUM code assigned to every strain. **Storehouse** ("Stor."): 1–12 according to Table 1; **matrix**: cheese crust, storehouse air.

Isolate N.	Stor.	Matrix	Morphology	Fingerprinting	*CaM* gene sequencing	Final ID	MUM code
31	1	crust	*P. solitum*	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.16
32	5	air	*P. solitum*	*P. solitum*	*ND*	*P. solitum*	MUM16.17
33	3	air	*Penicillium* sp	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.18
34	4	air	*Penicillium* sp	*P. solitum*	*ND*	*P. solitum*	MUM16.19
39	1	air	*P. chrysogenum*	*P. chrysogenum*	*P. chrysogenum*	*P. chrysogenum*	MUM16.20
48	4	air	*P. solitum*	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.21
49	3	air	*P. solitum*	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.22
51	4	air	*Penicillium* sp		*P. charlesii*	*P. charlesii*	MUM16.23
53	1	crust	*A. versicolor*	*A. versicolor*	*A. puulaauensis*	*A. puulaauensis*	MUM16.24
54	3	air	*Penicillium* sp	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.25
55	3	air	*Penicillium* sp	*P. solitum*	*ND*	*P. solitum*	MUM16.26
57	3	air	*P. citreonigrum*		*P. citreonigrum*	*P. citreonigrum*	MUM16.28
58	2	crust	*A. flavus*	*A. flavus*	*ND*	*A. flavus*	MUM16.29
59	4	air	*Penicillium* sp	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.30
60	1	crust	*A. versicolor*		*A. puulaauensis*	*A. puulaauensis*	MUM16.31
61	7	air	*A. versicolor*	*A. versicolor*	*A. puulaauensis*	*A. puulaauensis*	MUM16.32
62	2	air	*A. flavus*	*A. flavus*	*ND*	*A. flavus*	MUM16.33
63	4	air	*P. verrucosum*	*P. verrucosum*	*P. verrucosum*	*P. verrucosum*	MUM16.34
64	2	air	*P. verrucosum*	*P. verrucosum*	*ND*	*P. verrucosum*	MUM16.35
65	3	air	*Penicillium* sp	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.36
66	2	air	*P. verrucosum*	*P. verrucosum*	*ND*	*P. verrucosum*	MUM16.37
67	1	air	*A. versicolor*	*A. versicolor*	*A. puulaauensis*	*A. puulaauensis*	MUM16.38
68	7	air	*A. flavus*		*ND*	*A. flavus*	MUM16.39
69	2	air	*P. chrysogenum*	*P. chrysogenum*	*P. chrysogenum*	*P. chrysogenum*	MUM16.40
70	2	air	*Penicillium* sp		*P. commune*	*P. commune*	MUM16.41
71	2	air	*P. verrucosum*	*P. verrucosum*	*ND*	*P. verrucosum*	MUM16.42
72	3	air	*Penicillium* sp	*P. solitum*	*ND*	*P. solitum*	MUM16.43
73	3	air	*P. solitum*	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.44
74	1	air	*P. brevicompactum*		*P. bialowiezense*	*P. bialowiezense*	MUM16.45
75	3	crust	*Penicillium* sp	*P. solitum*	*ND*	*P. solitum*	MUM16.46
76	5	crust	*P. chrysogenum*		*P. chrysogenum*	*P. chrysogenum*	MUM16.47
77	4	crust	*Penicillium* sp	*Penicillium* sp	*P. jugoslavicum*	*P. jugoslavicum*	MUM16.48
78	5	crust	*P. solitum*	*P. solitum*	*ND*	*P. solitum*	MUM16.49
79	4	crust	*P. solitum*	*P. solitum*	*ND*	*P. solitum*	MUM16.50
80	4	crust	*P. solitum*	*P. solitum*	*ND*	*P. solitum*	MUM16.51
81	5	crust	*Penicillium* sp	*P. solitum*	*ND*	*P. solitum*	MUM16.52
82	5	crust	*Penicillium* sp	*Penicillium* sp	*P. commune*	*P. polonicum*	MUM16.53
83	4	crust	*Penicillium* sp	*Penicillium* sp	*ND*	*P. polonicum*	MUM16.54
84	1	crust	*Penicillium* sp		*P. commune*	*P. commune*	MUM16.55
85	1	crust	*Penicillium* sp	*P. solitum*	*P. commune*	*P. commune*	MUM16.56
86	7	crust	*P. solitum*	*P. solitum*	*ND*	*P. solitum*	MUM16.57
87	1	crust	*P. crustosum*	*P. crustosum*	*ND*	*P. crustosum*	MUM16.58
88	5	crust	*P. solitum*	*P. solitum*	–	*P. solitum*	MUM16.59
89	7	crust	*Penicillium* sp	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.60
90	2	crust	*P. solitum*	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.61
91	1	crust	*Penicillium* sp	*Penicillium* sp	*P. jugoslavicum*	*P. jugoslavicum*	MUM16.62
92	1	crust	*Penicillium* sp		*P. commune*	*P. commune*	MUM16.63
93	3	crust	*P. solitum*	*P. solitum*	*ND*	*P. solitum*	MUM16.64
94	5	crust	*Penicillium* sp		*P. glabrum*	*P. glabrum*	MUM16.65
97	4	crust	*Penicillium* sp	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.66
98	7	crust	*Penicillium* sp	*P. solitum*	*P. commune*	*P. commune*	MUM16.67
99	1	crust	*P. crustosum*	*P. crustosum*	*ND*	*P. crustosum*	MUM16.68
100	1	crust	*A. versicolor*	*A. versicolor*	*A. puulaauensis*	*A. puulaauensis*	MUM16.69
101	5	crust	*Penicillium* sp	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.70
102	7	crust	*P. solitum*	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.71
103	7	crust	*P. solitum*	*P. solitum*	*ND*	*P. solitum*	MUM16.72
104	2	crust	*P. solitum*	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.73
105	11	air	*P. solitum*	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.74
106	8	air	*Penicillium* sp	*P. solitum*	*ND*	*P. solitum*	MUM16.75
107	10	air	*P. verrucosum*	*P. verrucosum*	*ND*	*P. verrucosum*	MUM16.76
108	6	air	*Penicillium* sp	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.77
111	9	air	*P. verrucosum*	*P. verrucosum*	*ND*	*P. verrucosum*	MUM16.78
112	9	air	*P. verrucosum*	*P. verrucosum*	*ND*	*P. verrucosum*	MUM16.79
113	11	air	*P. solitum*	*P. solitum*	*ND*	*P. solitum*	MUM16.80
117	6	air	*Penicillium* sp		*P. paneum*	*P. paneum*	MUM16.81
118	12	air	*P. solitum*	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.82
119	6	air	*P. solitum*	*P. solitum*	*ND*	*P. solitum*	MUM16.83
120	12	air	*P. solitum*	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.84
121	8	air	*P. solitum*	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.85
122	8	air	*Penicillium* sp		*P. corylophilum*	*P. corylophilum*	MUM16.86
124	11	air	*Penicillium* sp		*P. charlesii*	*P. charlesii*	MUM16.87
125	11	air	*P. solitum*	*P. solitum*	*ND*	*P. solitum*	MUM16.88

(*continued on next page*)

Table 3 (*continued*)

Isolate N.	Stor.	Matrix	Morphology	Fingerprinting	*CaM* gene sequencing	Final ID	MUM code
126	11	air	*P. solitum*	*P. solitum*	*ND*	*P. solitum*	MUM16.89
127	9	air	*P. solitum*	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.90
128	6	air	*P. solitum*	*P. solitum*	*ND*	*P. solitum*	MUM16.91
129	12	air	*P. solitum*	*P. solitum*	*ND*	*P. solitum*	MUM16.92
130	10	air	*P. verrucosum*		*ND*	*P. verrucosum*	MUM16.93
131	12	air	*P. verrucosum*	*P. verrucosum*	*ND*	*P. verrucosum*	MUM16.94
132	10	air	*P. verrucosum*	*P. verrucosum*	*ND*	*P. verrucosum*	MUM16.95
133	10	air	*P. verrucosum*	*P. verrucosum*	*ND*	*P. verrucosum*	MUM16.96
134	9	air	*P. solitum*	*P. solitum*	*ND*	*P. solitum*	MUM16.97
135	12	crust	*P. crustosum*	*P. crustosum*	*P. commune*	*P. crustosum*	MUM16.98
136	8	crust	*P. crustosum*	*P. crustosum*	*ND*	*P. crustosum*	MUM16.99
137	10	crust	*P. solitum*	*P. solitum*	*ND*	*P. solitum*	MUM16.100
138	8	crust	*P. solitum*	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.101
139	12	crust	*P. crustosum*	*P. crustosum*	*ND*	*P. crustosum*	MUM16.102
141	10	crust	*P. crustosum*	*P. crustosum*	*ND*	*P. crustosum*	MUM16.103
143	10	crust	*P. crustosum*	*P. crustosum*	*P. commune*	*P. crustosum*	MUM16.104
144	10	crust	*P. crustosum*	*P. crustosum*	*ND*	*P. crustosum*	MUM16.105
145	8	crust	*A. flavus*	*A. flavus*	*A. flavus*	*A. flavus*	MUM16.106
146	8	crust	*A. flavus*	*A. flavus*	*ND*	*A. flavus*	MUM16.01
147	10	crust	*Penicillium* sp		*P. antarcticum*	*P. antarcticum*	MUM16.107
148	8	crust	*Penicillium* sp		*P. paneum*	*P. paneum*	MUM16.108
149	8	crust	*Penicillium* sp		*P. paneum*	*P. paneum*	MUM16.109
150	12	crust	*Penicillium* sp	*P. solitum*	*ND*	*P. solitum*	MUM16.110
151	12	crust	*Penicillium* sp	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.111
152	8	crust	*Penicillium* sp	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.112
153	8	crust	*P. solitum*	*P. solitum*	*ND*	*P. solitum*	MUM16.113
154	10	crust	*Penicillium* sp	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.114
155	8	crust	*Penicillium* sp	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.115
156	8	crust	*Penicillium* sp	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.116
157	8	crust	*Penicillium* sp	*P. solitum*	*ND*	*P. solitum*	MUM16.117
158	12	crust	*Penicillium* sp	*P. solitum*	*ND*	*P. solitum*	MUM16.118
159	12	crust	*Penicillium* sp	*P. solitum*	*P. solitum*	*P. solitum*	MUM16.119

"ND": not done; "-": sequencing reaction failed.

Regarding *P.* subgenus *Penicillium*, the strains morphologically identified as *P. solitum* were placed in 5 clusters with other, morphologically similar, unidentified isolates, indicating they might all be the same species: thus, those unidentified fungal strains were tentatively attributed to *P. solitum* species too. *P. verrucosum* strains were distributed in two clusters, while all *P. crustosum* strains were grouped into one cluster, except the two MUM reference strains (Fig. 3A and B).

3.4.2. Gene sequencing

60 strains out of 104 were selected for gene sequencing. The identification of strains at species level based on *CaM* sequencing is

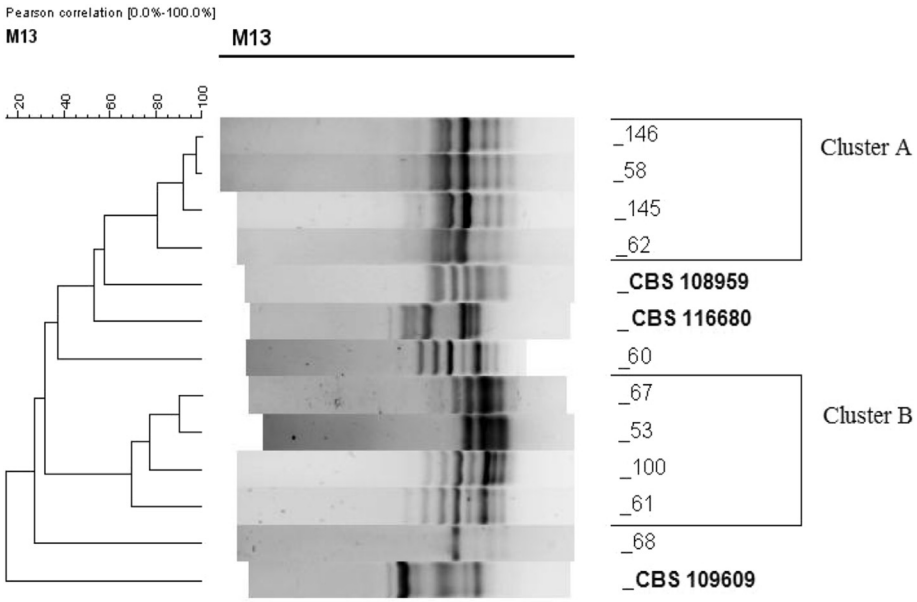

Fig. 1. M13 RAPD-PCR relatedness dendrogram for *Aspergillus flavus* and *A. versicolor* isolates. *A. flavus* morphotypes grouped in cluster A (except N. 68) while *A. versicolor* morphotypes grouped in cluster B (except N. 60). *A. versicolor* CBS 108959 and CBS 116680 reference strains are highlighted in bold. *P. nalgiovense* CBS 109609 was included as outgroup.

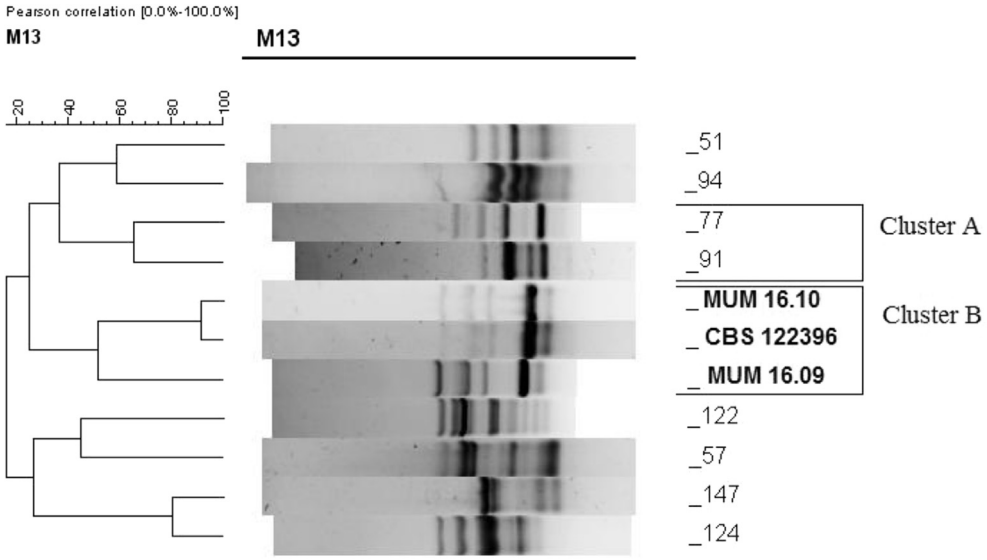

Fig. 2. M13 RAPD-PCR relatedness dendrogram for *Penicillium* subgenus *Aspergilloides* isolates. Strains morphologically similar but not identified to species level are grouped in cluster A while *P. citrinum* reference strains, highlighted in bold, are grouped in cluster B.

reported in Table 3. Evolutionary relationships of taxa for *CaM* gene are shown in Fig. 4 for *Aspergillus*, Fig. 5 for *Penicillium* subgenus *Aspergilloides* and Fig. 6 for *Penicillium* subgenus *Penicillium*.

Forty-one *Aspergillus* spp sequences were included in Fig. 4; 8 (7 *A. versicolor* and 1 *A. flavus*) of them were coming from sequencing of strains object of this study, while the others came from GenBank database. Clustering indicated that putative *A. versicolor* strains (N. 50, 53, 60, 61, 100), based on morphology, belonged to the closely related species *A. puulaauensis* (Jurjević et al., 2012), while strain 145 was confirmed as *A. flavus*.

The 24 sequences of *Penicillium* species belonging to subgenus *Aspergilloides* are shown in Fig. 5; 8 were obtained from direct sequencing of strains isolated from cheese and air monitoring in this study, the other 18 from GenBank database. Clustering was well supported by bootstrap values because almost all of them were above 70% (Hall, 2013), and all strains coming from this study grouped well with their respective reference strain.

Fig. 6 reports the dendrogram for *Penicillium* subgenus *Penicillium* with 83 sequences, 50 obtained from this study and 33 from the GenBank database. The overall clustering was good and quite well supported by bootstrap values (commonly above 70%), but sometimes closely related species were not discriminated.

The dendrogram showed a big cluster containing *P. solitum* isolates (belonging to section *Fasciculata*; Houbraken et al., 2016), including some of those tentatively assigned to this species in fingerprinting. Along with a *P. echinulatum CaM* sequence (ATCC10434) coming from GenBank, a closely related species to *P. solitum* (Samson and Frisvad, 2004), they formed a bigger cluster supported by a node with bootstrap value of 78%.

Penicillium camemberti and *P. commune* (belonging to section *Fasciculata*) clustered together, along with *P. palitans*, with a bootstrap value of 94%. It happened also for *P. carneum*, *P. paneum* and *P. roqueforti* (section *Roquefortorum;* Houbraken et al., 2016) with a bootstrap value of 98% obtained and for *P. bialowiezense* and *P. brevicompactum*, both belonging to section *Brevicompacta* (Houbraken et al., 2016) and clustering together with a bootstrap value of 98% along with *P. neocrassum*.

The presence of some outliers must also be remarked; in particular, sequences of *P. nordicum* reference strains were mixed in the dendrogram, clustering with other species such as *P. roqueforti*. The strain N. 135, identified as *P. crustosum* by morphology due to its similarity with *P. crustosum* reference strains (CBS 115503, MUM 16.11 and MUM 16.12) and M13 RAPD-PCR relatedness profile (cluster H), clustered instead with *P. solitum* strains.

3.5. Identification

Results coming from the polyphasic approach gave a sound identification at species level and, after that, all the samples were deposited in MUM culture collection and a MUM code was attributed (Table 3).

Two species belonging to the genus *Aspergillus* and 14 species belonging to the genus *Penicillium* were identified, for a total amount of 104 strains isolated. Ninety-four strains, out of the 104 collected, belonged to the genus *Penicillium* and 10 to the genus *Aspergillus. P. solitum* was the most common species (51%), followed by *P. verrucosum* (11%), *P. crustosum* (8%), *A. flavus*, *A. puulauensis* and *P. commune* (5% each), *P. chrysogenum* and *P. paneum* (3% each), *P. charlesii* and *P. jugoslavicum* (2% each) and others (such as *E. pseudoglaucum*, *P. antarcticum, P. bialowiezense*, *P. corylophilum* and *P. glabrum*) detected as single strains.

Seventy-seven strains (74%) were assigned to *Penicillium* subgenus *Penicillium,* section *Fasciculata*, while other sections of the subgenus *Penicillium* (*Roquefortorum* and *Chrysogena*) were far less represented along with species belonging to the subgenus *Aspergilloides*.

Forty-seven strains (about 45%) were straightforwardly assigned to their respective species based on morphology. In 23 strains out of 104 (about 22%) morphological identification was more difficult, but M13 RAPD-PCR profiles suggested their identification as *P. solitum,* confirmed by *CaM* sequencing, managed in 14 of the 23 strains (65% of the strains).

In 16 strains (about 15%), the morphological identification (*A. versicolor* and *P. verrucosum*) was well supported by M13 RAPD-PCR results. *CaM* sequencing revealed that *A. versicolor* belonged to very closely related species *A. puulaauensis*.

For 10 strains (about 10%) a putative morphological identification was not achieved, and also M13 RAPD-PCR results were not conclusive: so, only *CaM* sequencing was useful for their identification as *P. commune* (5 strains), *P. paneum* (3 strains), and *Penicillium polonicum* (2 strains).

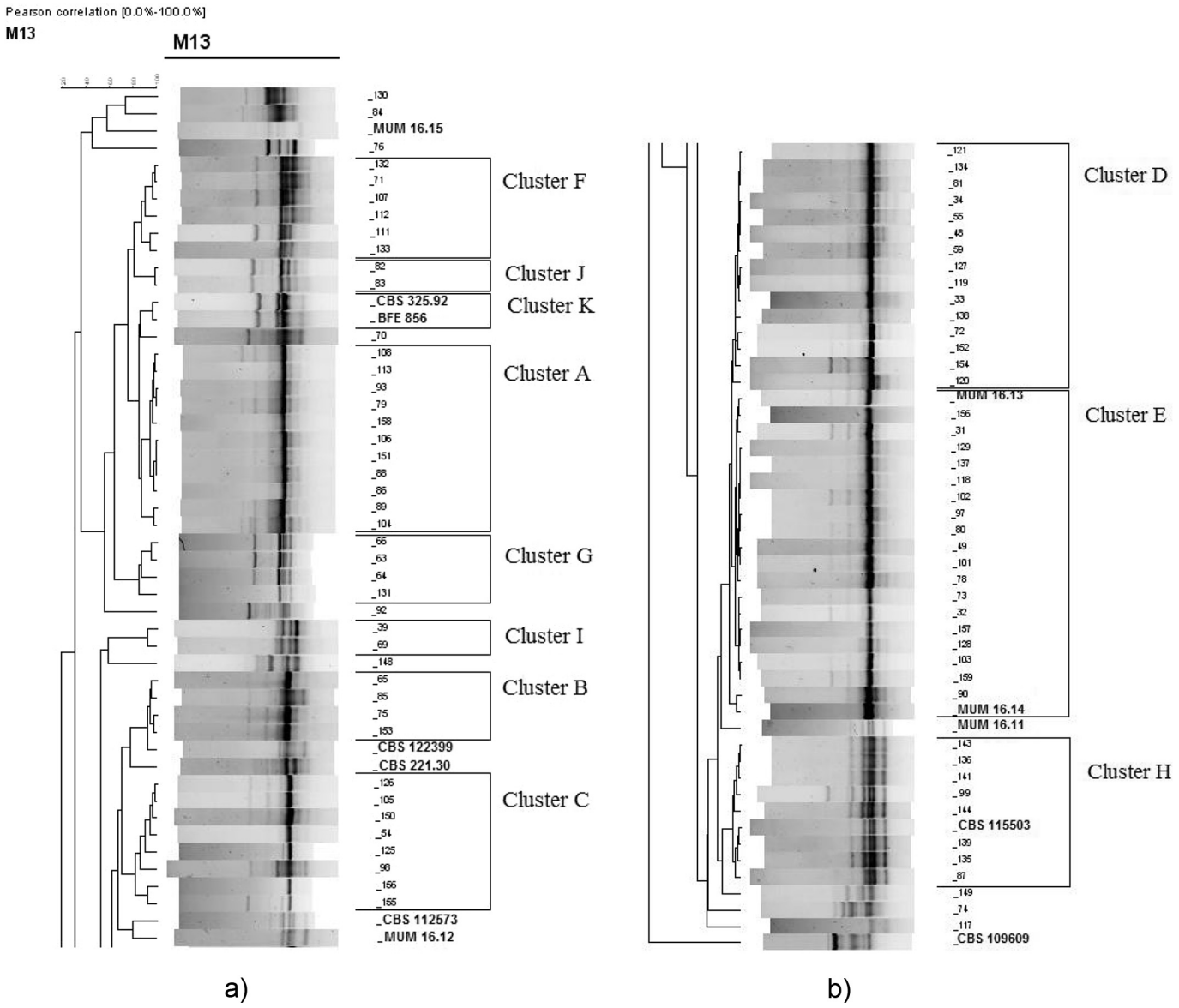

Fig. 3. A - M13 RAPD-PCR relatedness dendrogram for *Penicillium* subgenus *Penicillium* isolates. Morphotypes of *P. solitum* strains (along with similar-looking but unidentified strains) are grouped in clusters A, B and C while morphotypes of *P. verrucosum* strains are grouped in clusters F and G; clusters I and J contains *P. chrysogenum* and *Penicillium* sp. strains respectively. Cluster K contains one *P. nordicum* (BFE 856) and one *P. verrucosum* (CBS 325.92) reference strains. Reference strains *P. roqueforti* CBS 221.30, *P. nordicum* CBS 112573, *P. camemberti* CBS 122399, *P. crustosum* MUM 16.12 and *P. brevicompactum* MUM 16.15 are highlighted in bold.
B - M13 RAPD-PCR relatedness dendrogram for terverticillate *Penicillium* isolates. Morphotypes of *P. solitum* strains (along with similar-looking but unidentified isolates) are grouped in clusters D and E while morphotypes of *P. crustosum* are grouped in cluster H. Reference strains *P. nalgiovense* CBS 109609, *P. crustosum* CBS 115503 and MUM 16.11, and *P. solitum* MUM 16.13 and MUM 16.14 are highlighted in bold.

4. Discussion

Penicillia were commonly isolated both in cheese crust and in storehouse air in this study, and in some cases they also dominated the fungal mycobiota associated to Italian grana cheese. Previous studies reported *Penicillium* as the prevailing genus in the mycobiota associated to many types of cheese, but in those studies results referred to the number of identified strains and/or to the number of cheese samples with fungal presence, while the fungal bioload ("airborne fungal propagules load", roughly expressed here as CFU/g) was reported for all fungi and not shared for different genera (Barrios et al., 1998; Båth et al., 2012; Hayaloglu and Kirbag, 2007; Lund et al., 1995; Montagna et al., 2004). However, average CFU/g for Penicillia (about 3.85×10^6) and for other fungi (9.77×10^6) obtained in this study are comparable to the results obtained by Hayaloglu and Kirbag (2007) with 10^6 CFU/g of cheese and Montagna et al. (2004) with 10^3 - 10^5 CFU/g of cheese. Irrespective of dominance or comparable presence with other fungi, the relevance of *Penicillium* spp in cheese is not under discussion due to their possible mycotoxin production.

A lack of correlation between the amount of fungal bioload in storehouse air and in cheese crust was noticed in this study; this is probably due to the fact that, during ripening, the cheese wheels repeatedly undergo surface clean-up operations with brushes and water jets. Superficial contamination is therefore removed, but fungal spores are also airborne spread, increasing the bioload in the storehouse air. No details on cheese wheel cleaning were released by the storehouse operators and it is consequently impossible to

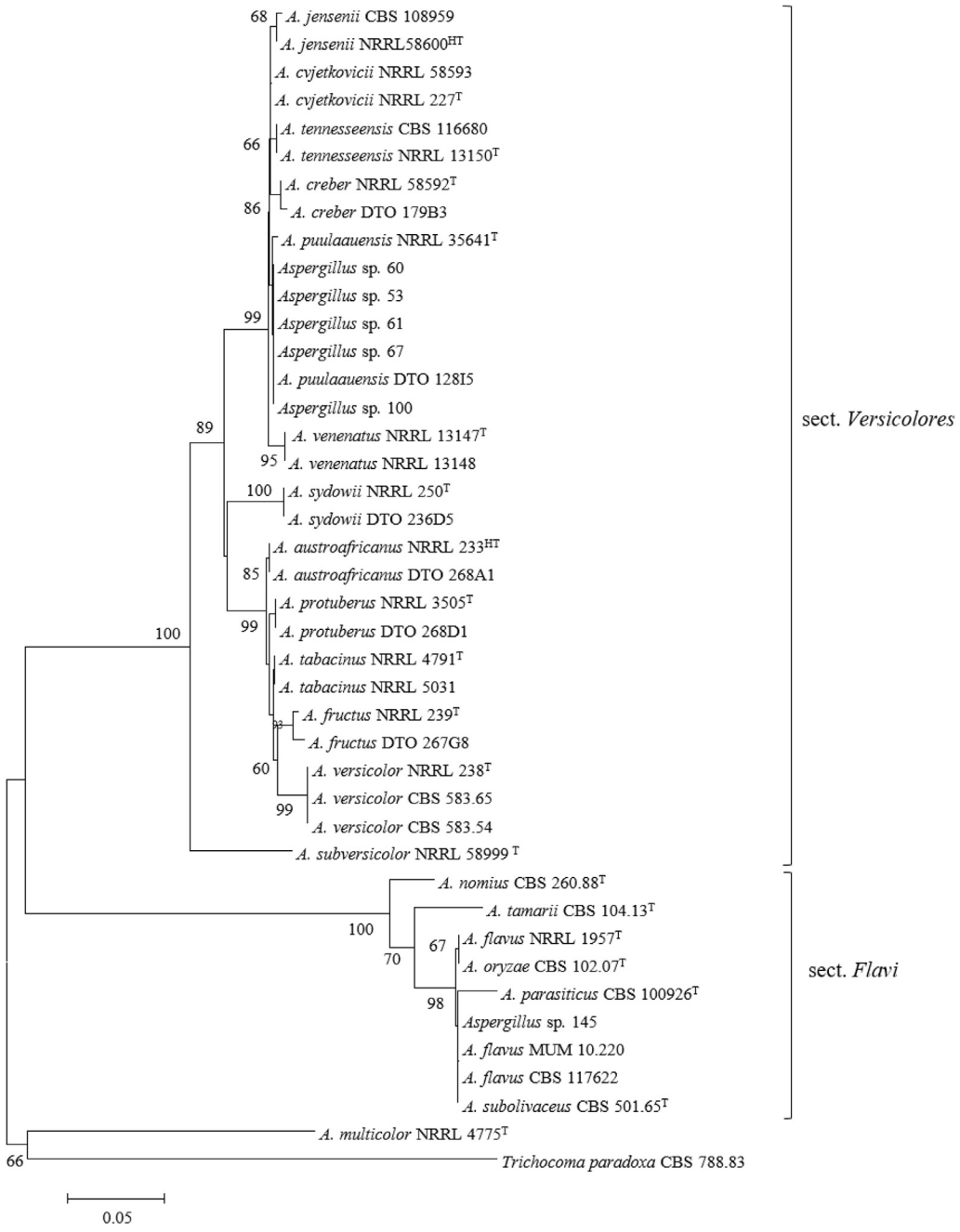

Fig. 4. Evolutionary relationships of Aspergilli isolates (*CaM*): The optimal tree with the sum of branch length = 1.02367701 is shown. The analysis involved 42 nucleotide sequences. There were a total of 350 positions in the final dataset. "T": type strain; "HT": holotype strain.

further comment on this topic. Similar results were obtained by Battilani et al. (2007); they found no correlation between fungal mycobiota isolated from Italian dry cured ham and from the air of the ripening storehouses.

Moving to the identification to species level of Aspergilli and Penicillia collected in this study, only 45% of morphological identifications were confirmed by the other approaches applied: phenotypic and biochemical features of the studied strains sometimes varied considerably from what is reported in literature, and this might have been due not only to the intraspecific variation, but also to abiotic factors like ingredients used for growth media preparation (Abbott et al., 2015; Pitt, 1979). In particular, different *P. solitum* morphotypes were observed. Besides, many species belonging to *Penicillium* subgenus *Penicillium* showed irregular appearance of the penicillus and/or co-presence of biverticillate and terverticillate structures. The morphological identification of *P. verrucosum* strains was quite straightforward, but only the observation of the colony reverse on YES was conclusive in this sense to discriminate them from *P. nordicum* species.

As a general comment, even if frequently not conclusive, morphological observation highlights intraspecific variability, so it should not be neglected.

The fingerprinting allowed to group strains with different macroscopic, but similar microscopic features, as occurred for instance for *P. solitum* strains and to confirm or reject the hypotheses of identification made after morphological observation as, for

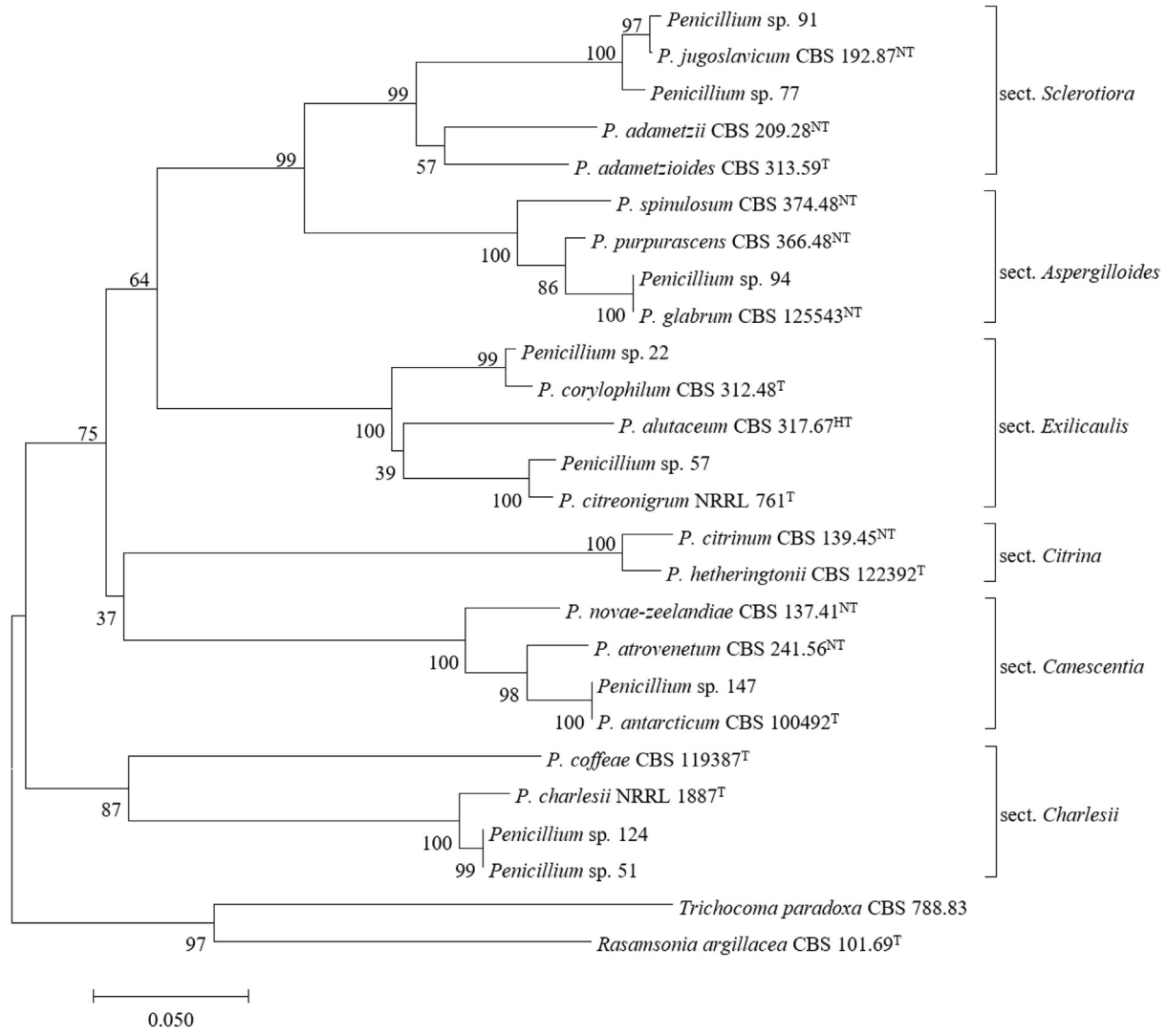

Fig. 5. Evolutionary relationships of taxa for Penicillia subgenus *Aspergilloides* strains (*CaM*): the optimal tree with the sum of branch length = 1.73514504 is shown. The analysis involved 26 nucleotide sequences. There were a total of 256 positions in the final dataset. "T": type strain; "HT": holotype strain; "NT": neotype strain.

instance, of *P. verrucosum*. It was therefore applied in this study, despite the limits of this technique are well known (Ghazi et al., 2013), to select representative strains and limit the efforts in gene sequencing. Thus, fingerprinting represented an intermediate step between massive morphological observation and focused gene sequencing.

Gene sequencing is supported by several authors as the gold standard for identification at species level (Geiser et al., 2007; Houbraken et al., 2011; Samson et al., 2004; Skouboe et al., 1999) and was the last step of the polyphasic identification process. Sequencings performed in a previous study by Decontardi et al. (2017) showed that results obtained with *BenA* and *COI* genes are generally in agreement; therefore, some of the strains identified in that study were used as references in the present work and *CaM* gene was chosen as barcode for identification. Optimal results in clustering were obtained for Aspergilli and Penicillia. Gene sequencing was crucial to correctly attribute all putative *A. versicolor* strains to the very closely related *A. puulaauensis* species (Jurjević et al., 2012), so as to identify *P. commune* strains and confirm the hypothesis of identification made for the unidentified *P. solitum* strains after fingerprinting.

However, along with the sound results, also some drawbacks need to be considered: the species belonging to *P.* section *roqueforturum* (*P. carneum, P. paneum* and *P. roqueforti*), so as some species included in *P.* section *Fasciculata* (*P. camemberti* and *P. commune*), clustered together and were not clearly separated, even with gene sequencing. Moreover, *P. nordicum* and *P. verrucosum* reference strains were, randomly placed in the dendrogram and not clearly grouped together with the only *P. verrucosum* strain sequenced (N. 63), which clustered well with a *P. verrucosum* sequence obtained from GenBank. This seems to indicate that sequencing of *CaM* gene may not be the best choice for the molecular identification of those species and, in general, sequencing more than one gene is necessary to obtain robust identification.

Some of the species identified in this work are reported as mycotoxin producers. Among them, *A. flavus* produces aflatoxins and sterigmatocystin (Hedayati et al., 2007), *A. puulaauensis* is a possible sterigmatocystin producer (Jurjević et al., 2012), *P. chrysogenum* produces PR-toxin and roquefortine C, *P. paneum* produces patulin and roquefortine C and (Frisvad and Samson, 2004). *P. bialowiezense* and *P. brevicompactum* may cause spoilage of milk-derived products (Frisvad and Samson, 2004) and intoxications from yogurts were reported in literature; so these

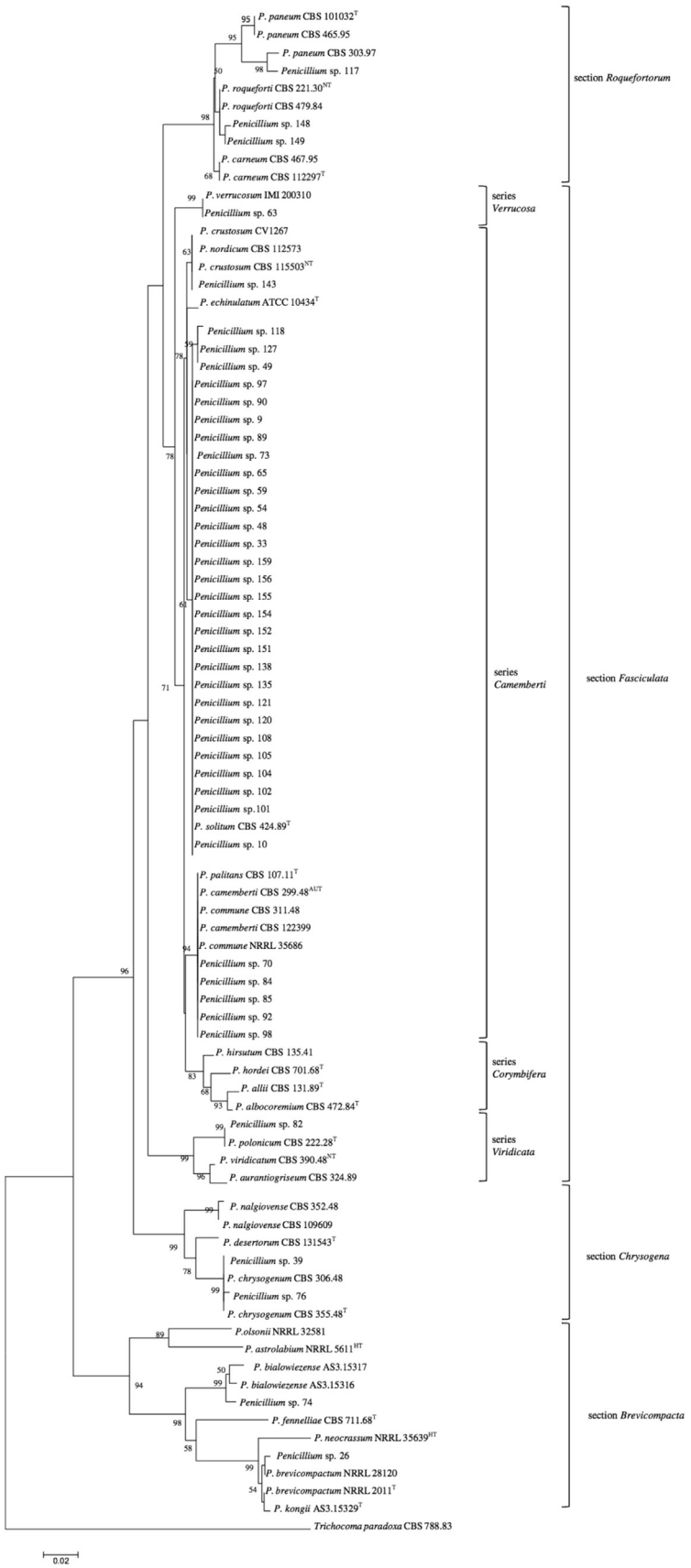

Fig. 6. Evolutionary relationships of taxa for Penicillia subgenus *Penicillium* strains (*CaM*): the optimal tree with the sum of branch length = 0.96098926 is shown. The analysis involved 84 nucleotide sequences. There were a total of 321 positions in the final dataset. "T": type strain; "HT": holotype strain; "NT": neotype strain.

species should not be ruled out although the molecules responsible for that are still not known (Samson and Frisvad, 2004). Comparing this work with the previous work conducted by Decontardi et al. (2017), a broader set of crust samples was used (54 *versus* 18), obtaining a larger number of strains (104 *versus* 27); in addition, air monitoring was added in the present work to have a better overview of the mycobiota associated to Italian grana cheese working environment. In both cases, *P. crustosum* and *P. solitum* were the dominant species, but while *P. crustosum* was dominant in the former work, *P. solitum* is the most isolated species in this study. *P. solitum* has never been signalled as mycotoxin producer, while *P. crustosum* is able to produce penitrem A (Samson and Frisvad, 2004). Moreover, based on Camardo Leggieri et al. (2017) study, mycotoxin contamination of Italian grana cheese may be not only a potential risk, but also a practical issue to cope with: in fact, ripening grana conditions are suitable for mycotoxin production by these fungi.

5. Conclusions

This study confirms that *Aspergillus* spp and *Penicillium* spp are important members of the bioload of grana cheese; a polyphasic approach is mandatory for their correct identification at species level. The molecular approach, in particular gene sequencing, commonly gives the conclusive contribute for the identification, even if more than one gene should be considered to get more robust results. Available data on gene sequencing are still poor: it is therefore strongly suggested the scientific community contribute with research on this topic.

Based on the bioload identified, Italian grana cheese could be at risk of mycotoxin contamination, and it is therefore mandatory to consider this important food safety aspect in cheese production management devoting efforts to predict the eventual consumer exposure, managing surveys to describe the real contamination and finding out possible preventive actions to mitigate mycotoxin contamination risk.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2018.01.012.

References

Abbott, S., Spero, D., Phillips, W., Hsiung, A.Y., Hardy, J., 2015. Variation in colonial morphology of fungal isolates exhibited by different malt extract products in agar media. http://www.hardydiagnostics.com/pdf/sc_posters/MaltExtractAgar_Q058.pdf. (Accessed 24 August 2016).

Abdel Hameed, A.A., Adel Malwa, H.F., 2012. Sedimentation with the Omeliansky formula as an accepted technique for quantifying airborne fungi. Pol. J. Environ. Stud. 6, 1539–1541.

Abdel Hameed, A.A., Ayesh, A.M., Abdel Razik Mohamed, M., Adel Malwa, H.F., 2012. Fungi and some mycotoxins producing species in the air of soybean and cotton mills: a case study. A. P. R. 3, 126–131.

Alzohairy, A.M., 2011. BioEdit: an important software for molecular biology. GERF B. B. 2, 60–61.

Barrios, M., Medina, L., Lopez, M., 1998. Fungal biota isolated from Spanish cheeses. J. Food Saf. 18, 151–157.

Båth, K., Persson, K.N., Schnürer, J., Leong, S.I.L., 2012. Microbiota of an unpasteurized cellar-stored goat cheese from northern Sweden. Agric. Food Sci. 21, 197–203.

Battilani, P., Pietri, A., Giorni, P., Formenti, S., Bertuzzi, T., Toscani, T., Virgili, R., Kozakiewicz, Z., 2007. *Penicillium* population in dry-cured ham manufacturing plants. J. Food Protect. 70, 975–980.

Camardo Leggieri, M., Decontardi, S., Bertuzzi, T., Pietri, A., Battilani, P., 2017. Modeling growth and toxin production of toxigenic fungi signaled in cheese under different temperature and water activity regimes. Toxins 9 (4). https://doi.org/10.3390/toxins9010004.

Decontardi, S., Mauro, A., Lima, N., Battilani, P., 2017. A first survey on Penicillia associated to the Italian grana cheese. Int. J. Food Microbiol. 246, 25–31.

Dragoni, I., Cantoni, C., Corti, S., 1983. Inquinamento fungino di Grana Padano da specie tossigene. Latte 8, 605–607.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Frisvad, J.C., Samson, R.A., 2004. Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. Stud. Mycol. 49, 1–174.

Geiser, D.M., Klich, M.A., Frisvad, J.C., Peterson, S.W., Varga, J., Samson, R.A., 2007. The current status of species recognition and identification in *Aspergillus*. Stud. Mycol. 59, 1–10.

Ghazi, F., Benmechernene, Z., Kihal, M., Candan Gurakan, G., 2013. The reproducibility of random amplified polymorphic DNA (RAPD) profiles of *Streptococcus thermophiles* strains with XD9, M13 and OPI-02 MOD primers. Afr. J. Biotechnol. 12, 6245–6252.

Giraud, F., Giraud, T., Aguileta, G., Fournier, E., Samson, R., Cruaud, C., Lacoste, S., Ropars, J., Tellier, A., Dupont, J., 2010. Microsatellite loci to recognize species for the cheese starter and contaminating strains associated with cheese manufacturing. Int. J. Food Microbiol. 137, 204–213.

Hall, B.G., 2013. Building phylogenetic trees from molecular data with MEGA. Mol. Biol. Evol. 30, 1229–1235.

Hayaloglu, A.A., Kirbag, S., 2007. Microbial quality and presence of moulds in Kuflu cheese. Int. J. Food Microbiol. 115, 376–380.

Hedayati, M.T., Pasqualotto, M.C., Warn, P.A., Bowyer, P., Denning, D.W., 2007. *Aspergillus flavus*: human pathogen, allergen and mycotoxin producer. Microbiology 153, 1677–1692.

Houbraken, J., Frisvad, J.C., Samson, R.A., 2011. Taxonomy of *Penicillium* section citrina. Stud. Mycol. 70, 53–138.

Houbraken, J., Wang, L., Lee, H.B., Frisvad, J.C., 2016. New sections in *Penicillium* containing novel species producing patulin, pyripyropens or other bioactive compounds. Persoonia 36, 299–314.

Hymery, N., Vasseur, V., Coton, M., Mounier, J., Jany, J.-L., Barbier, G., Coton, E., 2014. Filamentous fungi and mycotoxins in cheese: a review. Compr. Rev. Food. Sci. F 13, 437–456.

Jurjević, Z., Peterson, S.W., Horn, B.W., 2012. *Aspergillus* section *Versicolores*: nine new species and multilocus DNA sequence based phylogeny. IMA Fungus 3, 59–79.

Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary Genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. https://doi.org/10.1093/molbev/msw054.

Lund, F., Filtenborg, O., Frisvad, J.C., 1995. Associated mycoflora of cheese. Food Microbiol. 12, 173–180.

Manabe, M., 2001. Fermented foods and mycotoxins. Mycotoxins 51, 25–28.

Montagna, M.T., Santacroce, M.P., Spilotros, G., Napoli, C., Minervini, F., Papa, A., Dragoni, I., 2004. Investigation of fungal contamination in sheep and goat cheeses in southern Italy. Mycopathologia 158, 245–249.

Northolt, M.D., Bullerman, L.B., 1980. Prevention on mold growth and toxin production through control of environmental conditions. J. Food Protect. 45, 519–523.

O'Donnell, K., Nirenberg, H.I., Aoki, T., Cigelnik, E., 2000. A multigene phylogeny of the Gibberella fujikuroi species complex: detection of additional phylogenetically distinct species. Mycoscience 41, 61–78.

Paterson, R., Lima, N., 2010. Toxicology of mycotoxins. In: Molecular, Clinical and Environmental Toxicology-Volume 2: Clinical Toxicology. Springer, pp. 31–63.

Pietri, A., Bertuzzi, T., Bertuzzi, P., Piva, G., 1997. Aflatoxin M1 occurrence in samples of Grana Padano cheese. Food Addit. Contam. 4, 341–344.

Pietri, A., Bertuzzi, T., Moschini, M., Piva, G., 2003. Presenza di aflatossina M1 in campioni di latte destinati alla produzione del formaggio Parmigiano Reggiano. Ital. J. Food Sci. 2, 301–306.

Pitt, 1979. The Genus *Penicillium* and its Teleomorphic States *Eupenicillium* and *Talaromyces*. Academic Press Inc., 634 p.

Pitt, J.I., Hocking, A.D., 1997. Fungi and Food Spoilage. Blackie Academic & Professional.

Rodrigues, P.C.A., 2010. Mycobiota and Aflatoxigenic Profile of Portuguese Almonds and Chestnuts from Production to Commercialization. PhD Thesis in Engenharia Química e Biológica. Universidade do Minho, Braga, 333 p.

Rodrigues, P., Venâncio, A., Kozakiewicz, Z., Lima, N., 2009. A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* Section Flavi isolated from Portuguese almonds. Int. J. Food Microbiol. 129, 187–193.

Ropars, J., Cruaud, C., Lacoste, S., Dupont, J., 2012. A taxonomic and ecological overview of cheese fungi. Int. J. Food Microbiol. 155, 199–210.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Samson, R.A., Frisvad, J.C., 2004. *Penicillium* subgenus *Penicillium*: new taxonomy schemes and mycotoxins and other extrolites. Stud. Mycol. 49, 1–266.

Samson, R.A., Seifert, K.A., Kuijpers, A.F.A., Houbraken, J.A.M.P., Frisvad, J.C., 2004. Phylogenetic analysis of *Penicillium* subgenus *Penicillium* using partial β-tubulin sequences. Stud. Mycol. 49, 175–200.

Samson, R.A., Houbraken, J., Thrane, U., Frisvad, J.C., Andersen, B., 2010. Food and Indoor Fungi. CBS KNAW Biodiversity Center, 390 pp.

Schlick, A., Kuhls, K., Meyer, W., Lieckfeldt, E., Borner, T., 1994. Fingerprinting reveals gamma-ray induced mutations in fungal DNA: implications for identification of patent strains of *Trichoderma harzianum*. Curr. Genet. 26, 74–78.

Silva, F.C., Chalfoun, S.M., Batista, L.R., Santos, C., Lima, N., 2015. Use of polyphasic approach including MALDI-TOF MS for identification of *Aspergillus* section Flavi strains isolated from food commodities in Brazil. Ann. Microbiol. 65, 2119–2129.

Simões, M.F., Pereira, L., Santos, C., Lima, N., 2013. Polyphasic identification and preservation of fungal diversity: concepts and applications. In: Malik, A., Grohmann, E., Alves, M. (Eds.), Management of Microbial Resources in the Environment. Springer, Dordrecht, pp. 91–117. Simões, M.F., Santos, C., Lima, N. 2013. Structural diversity of *Aspergillus* (section *nigri*) spores. Microsc. Microanal. 19, 1151–1158.

Skouboe, P., Frisvad, J.C., Lauritsen, D., Boysen, M., Taylor, J.W., Rossen, L., 1999. Nucleotide sequences from the ITS region of *Penicillium* species. Mycol. Res. 103, 873–881.

Vauterin, L., Vauterin, P., 1992. Computer-aided objective comparison of electrophoresis patterns for grouping and identification of microorganisms. Eur. Microbiol. 1, 37–41.

Visagie, C.M., Hirooka, Y., Tanney, J.B., Whitfield, E., Mwange, K., Meijer, M., Amend, A.S., Seifert, K.A., Samson, R.A., 2014a. *Aspergillus, Penicillium* and *Talaromyces* isolated from house dust samples collected around the world. Stud. Mycol. 78, 63–139.

Visagie, C.M., Houbraken, J., Frisvad, J.C., Hong, S.B., Hubka, V., Klaassen, C.H.W., Perrone, G., Seifert, K.A., Varga, J., Samson, R.A., 2014b. Identification and nomenclature of the genus *Penicillium*. Stud. Mycol. 78, 343–371.