Ruth Walter* and Andreas Kortekamp

Molecular identification of *Penicillium* species associated with blue mold on grapes in German vineyards

Affiliation
State Service Center (DLR) Rheinpfalz, Institute of Phytomedicine, 67435 Neustadt an der Weinstraße, Germany

Correspondence
Ruth Walter: Ruth.walter@dlr.rlp.de

Andreas Kortekamp: Andreas.kortekamp@dlr.rlp.de

Summary

Species of 788 *Penicillium* strains from grape bunches affected by blue mold in German wine growing regions were identified by PCR based molecular methods. *P. expansum* was the most common species (n = 733) on the grapes and could be identified by species specific PCR of the polygalacturonase gene certainly (Marek *et al.*, 2003). Eleven further species, *P. minoluteum, P. crustosum, P. commune, P. purpurogenum, P. solitum, P. spinulosum, P. brevicompactum, P. chrysogenum* (on flower residues only), *P. aurantiogriseum, P. thomii/P. purpurescens,* and *P. janthinellum/P. griseovulvum*, could be identified by different molecular methods. However, the identification of these species and the discrimination of closely related species by ITS sequencing was time consuming and not possible in each case. Therefore, a new molecular method based on an amplification of a *cytochrome oxidase gene (cox1)* sequence by PCR followed by a single restriction with *Hpy*F3I or triple digestion with *Hpy*F3I*, Bsp*T1, and *Bsm*I was established. This method allows a fast and reliable distinction of the most common *Penicillium* species occurring on affected grape bunches in German wine growing regions, and allowed a discrimination of closely related species such as *P. crustosum and P. commune,* respectively.

Keywords

PCR, digestion, discrimination of *P. crustosum* and *P. commune*

1 Introduction

An increased occurrence of blue mold on grapes was observed in German vineyards since the end of the 1990s. This fruit disease is caused by species of the genus *Penicillium*. Among them, *Penicillium expansum* is the major causal agent of this type of decay on grapes in German vine growing regions (Walter, 2008). This pathogen requires wounds in the epidermal layer of grapes, e.g. caused by rain, hail, and even wind, as well as by biotic factors such as insect damage to enter fruit tissues and to initiate infection. Even cracks and microcracks that appear during berry ripening and *Botrytis* infections (Becker *et al.*, 2011) can be colonized by *Penicillium*. Main consequence of soft rot caused by *P. expansum* is the reduction of wine quality due to the production of toxic compounds and also volatiles, in particular geosmin, 2-methylisoborneol (2-MIB), and 2- isopropyl-3-methoxypyrazine (IPMP), which are aromatic compounds leading to persistent off-flavours in wine (La Guerche, 2004; La Guerche *et al.*, 2003). Thus, blue mold severely affects the quality properties of infected bunches making them unmarketable and, therefore, leading to considerable economic losses. Similar to other *Penicillium* species, *P. expansum* is also of public health interest, since the fungus is able to colonise different hosts and to produce the mycotoxins patulin and citrinin as well as other secondary metabolites that are toxic to humans and animals (Abramson, 1997; Andersen *et al.*, 2004). Furthermore, strains isolated from grapes seem to be the most aggressive and strongest patulin producers compared to *P. expansum* strains originating from other hosts (Sanzani *et al.*, 2013). Patulin has not only been reported in grapes but also in processed grape juice (Scott *et al.*, 1977) and fermenting wine (Majerus *et al.*, 2008). However, patulin could not be detected in ripened wine since it is degraded when sulphur dioxide is added (Trucksess and Tang, 2001; Altmayer *et al.*, 1985), or decomposed to non-toxic E-aslcadiol by *S. cerevisiae* (Moss and Long, 2002). Contamination with other mycotoxins has also been observed depending on the spectrum of *Penicillium* and also *Aspergillus* species that affect grape bunches.

In most years, grapes are mainly affected by grey mold (*Botrytis cinerea*) due to low night temperatures during autumn or late summer. However, warmer weather periods in recent years and also climate change may favour the growth of *P. expansum* which is more adapted to higher temperatures, dry environmental conditions, and low water activity compared to *B. cinerea* (Judet-Correia *et al.*, 2010). Furthermore, *Penicillium* species with higher incidences in warmer vine growing areas may be stimulated such that grapes become infested with different potential mycotoxin producers. An increased infestation with underrepresented but puta-

Submitted/accepted for publication: September 21, 2022/December 21, 2022

tively mycotoxin-producing *Penicillium* species will have an impact on quality of juice, must, and wine and may also raise health issues. Therefore, the monitoring of *Penicillium* species requires fast and easy methods for species identification and to reveal alterations in species composition over time and regions.

The reliable discrimination of *Penicillium* species on grape bunches based on morphological features such as shape and size of conidia or stipes and metulae (Samson *et al.*, 2010; Samson and Frisvad, 2004) is difficult and time consuming due to high morphological similarities and thus requires profound knowledge and practical experience. Species identification in general is also possible by using selective media, on which growth and degradation of different carbon sources is analysed. However, a systematic analysis of metabolic profiles, as it can be achieved by Fourier transform infrared (FTIR) spectroscopy, may also be expensive and many *Penicillium* isolates on grapes cannot be identified precisely due to isolate-specific differences within one single species and also unknown environmental factors. Even though FTIR was successfully used in fungal taxonomy, many reference strains reliably identified by other appropriate (molecular) methods are needed to overcome large intraspecific variability in phenotypic traits (Wenning and Scherer, 2013).

In many cases well established molecular methods such as an analysis of sequences within the ITS region or the ß-tubulin gene are used for the identification of different fungi including *Penicillium* species (Samson *et al.*, 2004; White *et al.*, 1990). Unfortunately, closely related species such as *P. crustosum* and *P. commune* cannot be discriminated with this method, since the nucleotide sequences of the PCR products are nearly identical/very similar (Frisvad and Samson, 2004, Walter, 2008). Recently Ollinger *et al.*, 2020 reported about DNA barcoding, targeting specific DNA regions of mold species occurring in bakery plants, including some *Penicillium* species. Other laboratories/working groups focus on biochemical methods for species identification and discrimination (Reeve *et al.*, 2019). Even MALDI-TOF MS based identification of fungal isolates to the species level is used in many laboratories (Quéro *et al.*, 2019). However, the methods are addressed to selected species or require suitable equipment.

Since the identification of *Penicillium* species on grape bunches was either time consuming, or expensive and not successful in case of closely related species, the aim of the presented work was to develop a fast and easy molecular method that allows the identification and discrimination of closely related *Penicillium* species. For this purpose, cytochrome C oxidase gene (*cox*1) sequences were selected and analysed to reveal differences even at a single nucleotide level. Even though *cox*1 sequences were used for fungal identification using barcode oligonucleotide arrays (Seifert *et al.*, 2007; Chen *et al.*, 2009), they did not allow a discrimination of closely related species. Therefore, in a new approach a gene-specific PCR of *cox*1 sequence was followed by a restriction analysis, thus providing a simple method for discrimination of the most important pathogenic species on grapes in German viticultural regions.

2 Material and Methods

2.1 Penicillium isolates and reference strains

A total of 788 *Penicillium* isolates were collected from 10 different viticultural regions in Germany (Table 2). These isolates were collected from grape berries showing typical green mold disease symptoms at bunches of about 70 different grape varieties. For isolation, *Penicillium* spores and mycelia were transferred to malt extract agar plates (2 %) with a fine needle.

To ensure purity and homogeneity dilutions of spore suspensions were plated on agar plates and single colonies were selected by stereo microscope. All cultivation was done at 21 °C.

Reference strains from the Centraalbureau of Schimmelcultures (CBS) and Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ) were included in the investigations to ensure the results in species identification and differentiation. The collection included *P. expansum (*DSM 62841), *P. crustosum* (CBS 313.48), *P. crustosum* (CBS 548.77), *P. crustosum* (CBS 747.74), *P. commune* (CBS 216.30) *P. commune* (CBS 184.89), *P. minioluteum* (CBS 138.84), *P. minioluteum* (CBS 996.72), *P. purpurogenum* (DSM 62866), *P. brevicompactum* (DSM 3825), *P. spinulosum* (DSM 62870), *P. solitum* (CBS 147.86), and *P. chrysogenum* (DSM 62858).

2.2 DNA extraction

For DNA extraction, single spore cultivates were grown in 2 mL glass vials with liquid malt extract (2 %) for 14 days. For DNA extraction, 2 mL glass vials with liquid malt extract (2 %) were inoculated with material of single spore cultures. 14 days after inoculation fungal material was transferred into 1.5 mL Eppendorf® tubes and cooled down with liquid nitrogen. Frozen material was crushed with three steel beads (diameter 3 mm) in a Retsch® CryoMill MM 400 (45 sec., 18 Hz). DNA was extracted by adding 500 µL extraction buffer (200 mM Tris HCl pH 8.5; 25 mM NaCl; 25 mM EDTA; 0.5 % SDS; 0.1 mL/100mL 2-mercaptoethanol). After incubation at 65 °C for 30 min, samples were allowed to cool down to room temperature, followed by an addition of 500 µL of a mixture of chloroform and isoamyl alcohol (24:1), and a centrifugation step at 20,000 rcf at room temperature for 10 min. The supernatant was transferred into a new Eppendorf® tube and 10 µL RNAse (10 mg·mL^{-1}) were added. After incubation at 37 °C for 60 min, proteins were precipitated with 0.5 volume of 7.5 M ammonium acetate (pH 6,5) followed by centrifugation at 20,000 rcf for 10 min. The upper phase was transferred to a new Eppendorf® tube and the DNA was precipitated with 1/9 volume of 3 M sodium acetate (pH 6) and 0.5 volume isopropyl alcohol. After incubation for 10 minutes on ice, samples were centrifuged for 20 min at 20,000 rcf and the supernatant was discarded. The pellet was dried at room temperature, dissolved in 50 µL TE buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA) and stored at 4 °C until use.

2.3 Species specific PCR (Penicillium expansum)

Since *P. expansum* is the main causal agent of blue mold on grapes in German viticultural regions, each strain was first

analysed with the aid of a species-specific PCR by using the primer pair PEF and PER as described by Marek *et al.* (2003). This primer pair can be used to amplify a 404 bp sequence of the polygalacturonase gene. With this method, *P. expansum* could be easily identified and clearly separated from other *Penicillium* species.

In brief, primers PEF and PER (Table 1) were added to commercial PCR beads in 200 µL PCR tubes (illustra™ PuRe Taq™ Ready-To-Go™ PCR beads, GE Healthcare) that were used for all amplifications. For each PCR, 50 ng DNA, primers (25 pmol), and bidistilled sterile water were added to a final volume of 25 µL. Amplifications were carried out in a thermal cycler *(*Eppendorf Mastercycler® gradient) by an initial denaturation step at 92 °C for 5 min, followed by 30 cycles at 92 °C for 1 min, 60 °C for 45 sec, 72 °C for 45 sec, and a final extension step at 72 °C for 7 min. An aliquot of 5 µL of each reaction was analysed by gel electrophoresis (0.8 % agarose, Merck).

2.4 PCR of the ITS region and sequencing

Primers ITS4 and ITS5 (Table 1) were added to commercial PCR beads in 200 µL PCR tubes (illustra™ PuRe Taq™ Ready-To-Go™ PCR beads, GE Healthcare) that were used for all amplifications. For each PCR, 50 ng DNA, primers (25 pmol), and bidistilled sterile water were added to a final volume of 25 µL. Amplifications were carried out in thermal cycler *(*Eppendorf Mastercycler® gradient) by an initial denaturation step at 94 °C for 2 min, followed by 35 cycles at 94 °C for 40 sec, 54 °C for 1 min, 72 °C for 2 min., and a final extension step at 72 °C for 10 min. An aliquot of 5 µL of each reaction was analysed by gel electrophoresis (0.8 % agarose, Merck).

2.5 Gene specific PCR (cytochrome oxidase cox1)

The *Cox1* gene was amplified with the primer pair PenF1 and AspR1 (Table 1). PCR was set up with PCR beads (illustra™ PuRe Taq™ Ready-To-Go™ PCR beads, GE Healthcare) as described above. DNA (50 ng), the primer pair (10 pmol), and sterile water were added to a final volume of 25 µL. Amplifications were carried out in a thermal cycler (Eppendorf Mastercycler® gradient) according to Chen *et al.* (2009) with the following modifications: The initial denaturation at 95 °C for 3 min was followed by 40 cycles of 95 °C for 1 min., 56 °C for 45 sec., 72 °C for 90 sec., and a final extension step at 72 °C for 10 min. The expected PCR product is about 600 bp. An aliquot of 5 µL volume of each PCR product was analysed by gel electrophoresis (0.8 % agarose, Merck, in TAE buffer (Roth), 20 min. at 100 V).

2.6 Restriction analysis of cox1 PCR product

To obtain species-specific banding patterns, PCR products obtained after amplification of *cox1* sequences of *Penicillium* isolates were digested with the restriction enzymes *Hpy*F3I, *Bsp*T1 and/or *Bsm*I (Fermentas, Germany). In each case, 10 µL of the PCR product were mixed with 1 µL of each enzyme (10 units/µL), 4 µL 10 × tango buffer, and 10 µL bidistilled water to a final volume of 27 µL. Samples were incubated overnight at 37 °C. Fragments were separated by gel electrophoresis (3 % agarose 1000, Merck, in TAE buffer (Roth), 30 min at 100 V).

3 Results

3.1 Identified Penicillium species

Molecular methods used in this study allowed the identification and/or discrimination of at least 12 different *Penicillium* species that cause blue mold on grapes in German viticultural regions (Table 2). 733 isolates (93 %) were identified as *P. expansum*, so this specie was the most common species found to be pathogenic on the grapes. In addition to *P. expansum*, *P. minioluteum* (n = 25), *P. crustosum* (n = 9), *P. commune* (n = 6), *P. purpurogenum* (n = 5), *P. solitum* (n = 3), *P. spinulosum* (n = 3), *P. brevicompactum* (n = 3), *P. chrysogenum* (on flower residues only), *P. aurantiogriseum* (n = 1), *P. thomii*/*P. purpurescens* (n = 1), and *P. janthinellum*/*P. griseovulvum* (n = 1) could be identified. Since *P. aurantiogriseum*, *P. thomii*/*P. purpurescens* and *P. janthinellum*/*P. griseovulvum* were only identified once by ITS sequencing, and are even not common species on grapes in other countries, no further investigations were set on the discrimination or restriction profiling of these species. Within this first step, differentiation of *P. crustosum* and *P. commune* by ITS sequencing was still not possible. However, both species were discriminated successfully by distinct digestion of *cox1* gene (see chapter 3.5).

3.2 PCR with P. expansum-specific primers

PCR with the *P. expansum* specific primer pair PEF and PER (Table 1) was successfully applied for the identification of *P. expansum*, the most frequent species on symptomatic grapes (93 % of all collected isolates). Amplification of DNA isolated from reliably identified *P. expansum* and reference isolates resulted in a single PCR product of 404 bp. No amplification product was obtained with the primers when DNA of other species

Table 1: List of used primers, sequences, and references

Primer	Sequence	Reference
PEF	5´ATC GGC TGC GGA TTG AAA G 3´	Marek *et al.*, 2003
PER	5´AGT CAC GGG TTT GGA GGG A 3´	
ITS4	5´TCC TCC GCT TAT TGA TAT GC 3´	White *et al.* 1990
ITS5	5´GGA AGT AAA AGT CGT ACC AAG G 3´	
PenF1	5`-GAC AAG AAA GGT GAT TTT TAT CTT C-3`	Seifert *et al.*, 2007
AspR1	5`- GGT AAT GAT AAT AAT AAT AAT ACA GCT G-3`	

Table 2: Species identification of *Penicillium* isolates from grape berries with typical blue mold symptoms in German viticultural regions. Species, number of isolates, origins, year and used identification method.

Penicillium **spec.**	**Number of isolates**	**Origin**	**Year**	**Used method***
P. expansum	Total: 733			
	4	Palatinate	2003	1, 2
	13	Palatinate	2004	1, 2
	54	Palatinate	2004	1
	374	Palatinate	2005	1
	35	Palatinate	2006	1
	40	Palatinate	2010	1
	15	Palatinate	2011	1
	55	Palatinate	2012	1
	24	Baden	2004	1
	4	Baden	2005	1
	1	Baden	2006	1
	6	Francs	2004	1
	9	Francs	2005	1
	12	Moselle	2004	1
	1	Moselle	2005	1
	6	Moselle	2006	1
	3	Moselle	2010	1
	2	Nahe	2004	1
	3	Nahe	2005	1
	6	Rheingau	2004	1
	11	Rhine-Hesse	2004	1
	21	Rhine-Hesse	2005	1
	2	Rhine-Hesse	2006	1
	27	Württemberg	2005	1
	3	Württemberg	2004	1
	1	Middle Rhine	2004	1
	1	Ahr	2004	1
P. minioluteum	Total: 25			
	10	Palatinate	2003	2
	12	Palatinate	2004	2
	1	Palatinate	2004	2, 3
	2	Palatinate	2005	2, 3
P. crustosum	Total: 9			
	1	Baden	2005	2, 3
	5	Palatinate	2005	2, 3
	2	Palatinate	2005	2, 3
	1	Palatinate	2006	2, 3
	2	Palatinate	2012	2, 3
P. commune	6			
	2	Palatinate	2005	2, 3
	2	Palatinate	2011	2, 3
	1	Palatinate	2012	2, 3
	1	Rhine-Hesse	2005	2, 3
P. purpurogenum	5	Palatinate		
	1	Palatinate	2007	2, 3
	4	Palatinate	2010	2, 3

Table 2:Continued

Penicillium spec.	Number of isolates	Origin	Year	Used method*
P. solitum	Total: 3			
	1	Palatinate	2005	2, 3
	1	Palatinate	2011	2
	1	Palatinate	2011	2, 3
P. spinulosum	Total: 3			
	1	Palatinate	2005	2
	1	Palatinate	2010	2
	1	Rhine-Hesse	2005	2, 3
P. brevicompactum	3	Palatinate	2011	2
P. chrysogenum	1	Palatinate (flower residues)	2005	2
P. aurantiogriseum	1	Palatinate	2005	2
P. thomii/P. purpurescens	1	Palatinate	2005	2
P. janthinellum/P. griseovulvum	1	Palatinate	2005	2

* Method no.1: PCR with primers PEF and PER (see chapters 2.3 and 3.1)
* Method no.2: PCR with primers ITS4 and ITS 5, followed by sequencing and alignment (see chapter 2.4)
* Method no.3: Amplification and restriction of partial *cox1* sequence (see chapters 2.5, 2.6, 3.3, 3.4)

was used (see chapter 2.1). In this case, the species-specific PCR failed to produce a fragment of the expected size (Fig. 1).

3.3 Gene-specific PCR (cytochrome oxidase, cox1) and restriction analysis with HpyF3I, BspT1, and BsmI

Since species-specific PCR failed in case for *Penicillium* species other than *P. expansum*, a partial sequence of the cytochrome oxidase gene (*cox1*) was amplified with the primer pair PenF1 and Asp R1 (Table 1) resulting in a PCR product of about 600 bp (Fig. 2).

3.4 Triple digestion of the cox1 sequence with HpyF3I, BspT1, and BsmI

The triple digestion of PCR products of the *cox1* sequence with HpyF3I, BspT1, and BsmI revealed species specific fragmentation in the case of seven species (*P. crustosum*, *P. commune*, *P. solitum*, *P. spinulosum*, *P. brevicompactum*, *P. chrysogenum*, and *P. purpurogenum*) as shown in Fig. 3. *P. minioluteum* showed a fragmentation pattern that was identical with that obtained for *P. commune* (data not shown). However, these two species are not closely related and could be differentiated easily by ITS sequencing as well as culture morphology and coloration on malt extract agar (Fig. 4).

3.5 Discrimination of P. crustosum and P. commune

If only the closely related *P. crustosum* and *P. commune* should be discriminated, a single restriction of *cox1* gene PCR product with only HpyF31 was also sufficient. The re-

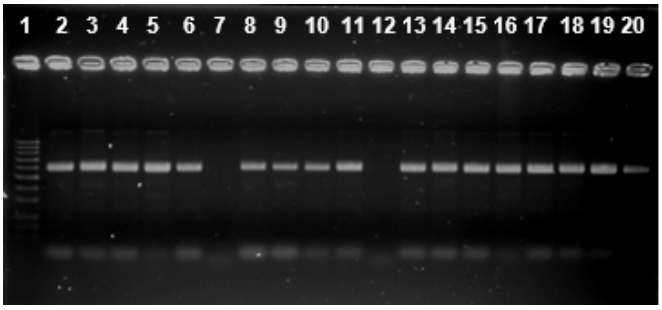

Fig. 1: Electrophoresis of PCR products obtained from DNA of pathogenic *Penicillium* samples isolated from affected grapes in the field and *P. expansum* specific primers PEF and PER (Marek *et al.*, 2003). 1 = 100 bp DNA marker (Roth); 2 = *P. expansum* (DSM 62841); 3-6, 8-11, 13-20 = *P. expansum*; 7 = *P. aurantiogriseum* (identification method see chapter 2.4); 12 = *P. brevicompactum* (identification method see chapter 2.4).

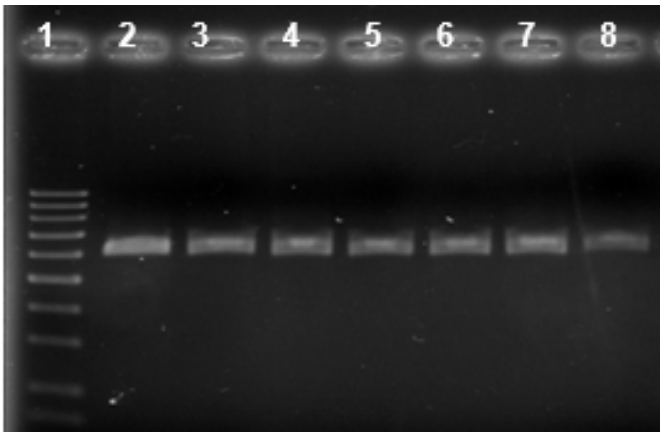

Fig. 2: Electrophoresis of cox1 gene products obtained after PCR with the primer pair PenF1 and AspR1 and DNA of relevant *Penicillium* species. 1 = 100 bp DNA marker (Roth); 2 = *P. crustosum* (DSM 313.48); 3 = *P. commune* (CBS 216.30); 4 = P. solitum (CBS 147.86); 5 = *P. spinulosum* (DSM 62870); 6 = *P. brevicompactum* (DSM 3825); 7 = *P. chrysogenum* (DSM 62858); 8 = *P. purpurogenum* (DSM 62866).

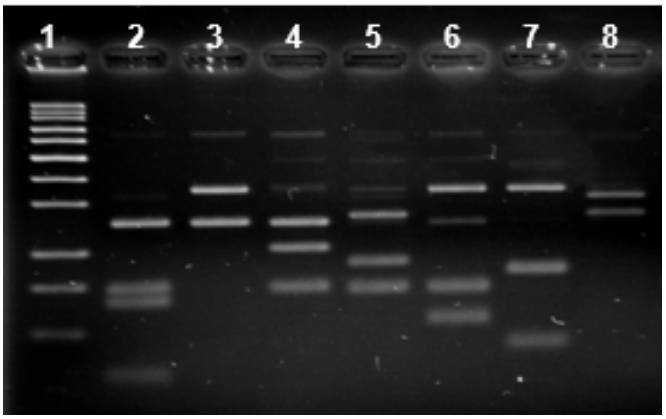

Fig. 3: Profiles obtained after restriction of the cox1 PCR product obtained with DNA of different *Penicillium* species with the enzymes HpyF3I, BspT1 und BsmI. 1 = 100 bp DNA marker (Roth), 2 = *P. crustosum* (CBS 313.48): 3 = *P. commune* (CBS 216.30); 4 = *P. solitum* (CBS 147.86); 5 = *P. spinulosum* (DSM 62870), 6 = *P. brevicompactum* (DSM 3825); 7 = *P. chrysogenum* (DSM 62858); 8 = *P. purpurogenum* (DSM 62866).

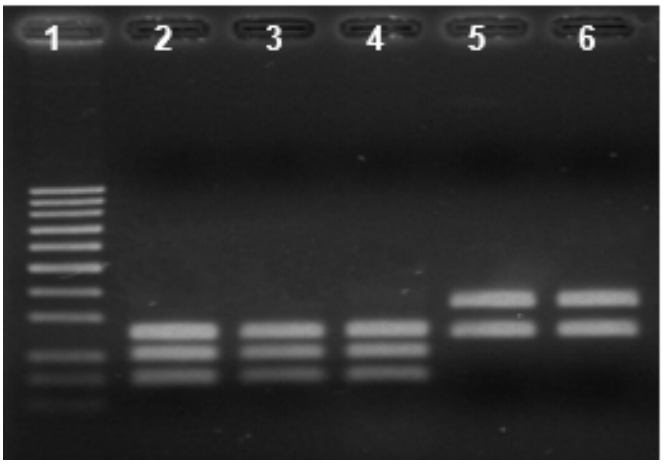

Fig. 5: Profiles obtained after restriction of the cox1 PCR product obtained with DNA of different strains of *P. crustosum* and *P. commune* with the enzyme HpyF3I. 1 0 100 bp DNA marker (Roth); 2 = *P. crustosum* (CBS 313.48); 3 = *P. crustosum* (CBS 548.77); 4 = *P. crustosum* (CBS 747.74); 5 = *P. commune* (CBS 216.30); 6 = *P. commune* (CBS 184.89).

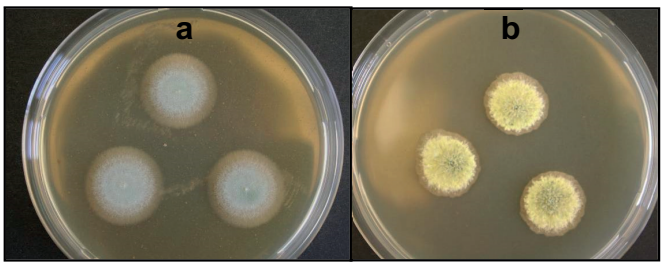

Fig. 4: Culture morphology and coloration of (a) *P. commune* (CBS 216.30) and (b) *P. minioluteum* (CBS 138.84) on malt extract agar 7 days after inoculation and incubation at 21 °C.

striction resulted in species-specific fragmentation where three fragments appeared in case for *P. crustosum* and two fragments if DNA of *P. commune* was amplified and digested (Fig. 5).

4 Discussion

*Cox*1 gene analysis revealed distinct variations in the nucleotide sequence of the *Penicillium* species investigated. These gene sequences show promise for the molecular differentiation of *Penicillium* species growing on berries. Moreover, the established method allows a distinction of even closely related species such as *P. crustosum* and *P. commune*. The triple digestion of the *cox1* gene sequence with HpyF3I, BsmI and BspT1 was a useful tool for a quick and reliable identification of all *Penicillium* species responsible for blue mold on grapes in German vineyards. However, species not common in German viticultural regions, such as *P. chrysogenum*, but also included in the experiment because of their high frequency in other countries (Felšöciová *et al.*, 2013; Sage *et al.*, 2002), could also be successfully discriminated from other species. An adaption of the established method should allow the identification of further species that may occur on grapes.

In most countries *P. expansum*, *P. chrysogenum*, *P. crustosum*, *P. commune*, *P. spinulosum*, *P. minioluteum*, *P. brevicompactum, P. solitum*, *P. glabrum*, and P. *funiculosum*, are the species occurring on grape berries (Felšöciová *et al.*, 2013; Sage *et al.*, 2002; Borgo *et al.*, 2005; Battilani *et al.*, 2003; Doaré-Lebrun, 2005; Magnoli *et al.*, 2003; Da Rocha Rosa *et al.*, 2002). Based on a bunch rot survey that was regularly conducted since 2003, most of these species are also found in Germany (Walter, 2008). Among them, *P. expansum* is the predominant species and responsible for more than 90 % of all infestations analysed so far. This is in contrast to the frequency of occurrence in other grapevine growing regions where *P. spinulosum*, and *P. minioluteum* are the most abundant species (Diguta *et al.*, 2011).

With regard to the spectrum of species found in other viticultural regions, mainly located in the Mediterranean area, a predicted climate change might not reveal new and unknown species in Germany but rather alter the frequency of occurrence within the spectrum of *Penicillium* spp. As a result of an altered species spectrum on grapes, contaminations of berries with different types of mycotoxins are possible. *P. chrysogenum*, for instance, was found in Germany only on flower residues and at very low rates but is capable of producing PR-toxin and Penicillins (Frisvad *et al.*, 2004), and was the most common species in Slovakia (Felšöciová *et al.*, 2013) and Argentina (Magnoli *et al.*, 2003).

Since *Penicillium* species are able to produce different kinds of mycotoxins and occur worldwide, the species spectrum on grape berries is of interest in all grape growing regions. Species composition may not only be modified by abiotic conditions but may also be influenced by biotic factors. Thus, other fungal organisms that colonise the same substrate may induce or reduce the production of secondary metabolites as described for geosmin and patulin (La Guerche, 2004; Morales *et al.*, 2013) and should be included in monitoring strategies.

Several sensitive and highly sophisticated analytical methods have evolved during recent years and were successfully used

for detection of mitosporic mycotoxin producers in different substrates. Capillary isoelectric focusing (Horká *et al.*, 2011), electronic noses (Pallottino *et al.*, 2012), E-probe diagnostic nucleic acid analysis (Stobbe *et al.*, 2013), loop-mediated isothermal amplification assays (Luo *et al.*, 2012), and MALDI TOF MS analysis (Reeve *et al.*, 2019). However, some of these methods require suitable equipment. Therefore, other convenient tools are developed in addition. Elhariry *et al.* (2011) reported a successful genotypic identification of *P. expansum* in apple juice by using RAPD PCR, whereas Prat *et al.* (2009) used PCR-denaturing gradient gel electrophoresis for the detection of *P. glabrum* in corks or as an alternative DNA barcoding for mold species identification occurring in bakery plants, including some *Penicillium* species, as published by Ollinger and co-workers (Ollinger *et al.*, 2020).

One disadvantage of several publications treating the occurrence of *Penicillium* species on grape berries is that despite a comprehensive investigation of the mycoflora information about pathogenicity is missing. In these cases, and since berry surfaces exhibit a large flora of bacteria, yeasts, and other fungi, information about microbial interactions is not available and a comparison of the spectrum of those species pathogenic to grapevine is difficult. Furthermore, the fungal composition seems to be altered by cultivation techniques. Walter (2008) demonstrated that the number of spores on berry surfaces in the grape zone increases and that conidia of *Penicillium* species were dispersed from soil into air when tilling is adopted. Conidia of these species may just be attached to the berry surfaces, without provoking decay due to a lack of pathogenicity on grapes. *In vivo* experiments using grape berries of 'Riesling' revealed that at moderate temperatures *P. expansum* was the most aggressive pathogen, followed by *P. crustosum* and *P. purpurogenum*. Other species such as *P. citrinum*, usually found on citrus fruits, were not able to infect berries and to cause symptoms at all, suggesting host specificity. Moreover, pathogenicity may also differ depending on the grape variety, as demonstrated for *P. solitum*, *P. expansum*, *P. bialowiezense*, *P. echinulatum*, and even *P. citrinum* by Kim *et al.* (2007).

As mycotoxin contaminations are a critical issue not only in grapes and wine but also in fruit and food production worldwide, fast and easy methods for the detection and identification of fungal organisms, such as Aspergillus and *Penicillium* species, are very useful especially for those institutions just equipped with basic molecular tools. A molecular analysis of the *cox1* sequence of *Penicillium* species responsible for blue mold in German vineyards by PCR followed by an enzymatic digestion offers such a suitable technique allowing the detection of minor differences in nucleotide sequences even at a single nucleotide level that might not be revealed in case for ITS sequences.

5 Conclusion

For mycotoxin risk assessment, the identification of foodborne *Penicillium* species is essential. Thus, several research groups focused on the development of reliable methods for species identification. However, up to now biochemical or molecular methods are still limited since *Penicillium* species are very closely related. Beside the availability of the respective equipment, the choice of a suitable identification method, or the combination of methods, might depend on the expected species profile, source, quality, and quantity of the fungi. However, the amplification of the *cox1* sequence followed by specific restriction digestion is a further useful tool for the molecular mold species identification, especially for detecting *Penicillium* species profile on grapes.

Conflicts of interest

The authors declare that they do not have any conflicts of interest.

Acknowledgements

We thank Susanne Jausel and Janine Köckerling for excellent technical assistance. This work was financially supported by the German Federal Ministry of Food and Agriculture (BMEL) through the Federal Office for Agriculture and Food (BLE), grant number 2810HS016.

References

Abramson, D., 1997. Toxicants of the genus *Penicillium*. In: Handbook of Plant and Fungal Toxicants (D´Mello J.F.P., ed.). DRC Press, Boca Raton, NY, 303-317.

Altmayer, B., Eichhorn, K.W., Schwenk, S., 1985: Importance of mycotoxin producing fungi in viticulture. Nachrichtenblatt des Deutschen Pflanzenschutzdienstes, Braunschweig, 37, 117-122.

Andersen, B., Smedsgaard, J., Frisvad, J.C., 2004: *Penicillium* expansum: consistent production of patulin, chaetoglobosins, and other secondary metabolites in culture and their natural occurrence in fruit products. Journal of Agricultural and Food Chemistry 52: 2421-2428, DOI: 10.1021/jf035406k.

Battilani, P., Giorini, P., Pietri, A., 2003: Epidemiology of toxin-producing fungi and ochratoxin A occurrence in grape. European Journal of Plant Pathology 109: 715-722, DOI: 10.1023/A:1026030408338.

Borgo, M., Burruano, S., Faretra, F., Serra, S., Vercesi, A., 2005: Dynamics of Aspergillus and *Penicillium* species in different Italian vineyards. In: Ochratoxin A in grapes and wine: prevention and control. Marsala, 59.

Becker, T., Walter, R., Kortekamp, A., Grimm, E., Knoche, M., 2011. Das Platzen von Weinbeeren (Vitis vinifera) bei Befall mit Grauschimmel (Botrytis cinerea), Erwerbs-Obstbau (53), 85–92

Chen, W., Seifert, K.A., Levesque, C.A., 2009: A high density COX1 barcode oligonucleotide array for identification and detection of species of *Penicillium* subgenus *Penicillium*. Molecular Ecology Resources 9: 114–129, DOI: 10.1111/j.1755-0998.2009.02638.x.

Da Rocha Rosa, C.A., Palacios, V., Combina, M., Fraga, M.E., De Oliveira Rekson, A., Dalcero, A.M., 2002: Potential ochratoxin A producers from wine grapes in Argentina and Bra-

zil. Food Additives and Contaminants 19, 4: 408-414, DOI: 10.1080/02652030110092748.

Diguta, C.F., Vincent, B., Guilloux-Benatier, M., Alexandre, H., Rousseaux, S., 2011: PCR ITS-RFLP: A useful method for identifying filamentous fungi isolates on grapes. Food Microbiology 28: 1145-1154, DOI: 10.1016/j.fm.2011.03.006.

Doaré-Lebrun, E., 2005: Charactérisation de la microflore des raisins par méthodes FISH et PCR-TTGE – Application à la résolution des goûts terreux dans les vins. Thesè, Université de Technologie de Compiègne. Available at: https://irsteadoc.irstea.fr/cemoa/PUB00019322.

Elhariry, H., Bahobial, A.A., Gherbawy, Y., 2011: Genotypic identification of *Penicillium* expansum and the role of processing on patulin presence in juice. Food and Chemical Toxicology 49: 941-946, DOI: 10.1016/j.fct.2010.12.018

Felšöciová, S., Rybárik, L., Tančinová, D., Mašková, Z., 2013: Occurrence of *Penicillium* species in grapes from Nitra wine region. Journal of Microbiology, Biotechnology and Food Sciences 2, special issue 1: 1333-1345.

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. Studies in Mycology 49: 1-174.

Frisvad, J.C., Smedsgaard, J., Larsen, T.O., Samson, R.A., 2004: Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*. Studies in Mycology 49: 201-241.

Horká, M., Horký, J., Kubesová, A., Zapletalová, E., Šlais, K., 2011: The trace analysis of microorganisms in real samples by combination of a filtration microcartridge and capillary isoelectric focusing. Analytical and Bioanalytical Chemistry 400: 3133-3140, DOI: 10.1007/s00216-011-4975-6.

Judet-Correia, D., Bollaert, S., Duquenne, A., Carpentier, C., Bensoussan, M., Dantigny, P., 2010: Validation of a predictive model for the growth of Botrytis cinerea and *Penicillium expansum* on grape berries. International Journal of Food Microbiology 142: 106-113, DOI: 10.1016/j.ijfoodmicro.2010.06.009.

Kim, W.K., Sang, H.K., Woo, S.K., Park, M.S., Paul, N.C., Yu, S.H., 2007: Six Species of *Penicillium* associated with blue mold of Grape. Mycobiology 35 (4): 180-185.

La Guerche, S., 2004: Recherches sur les déviations organoleptiques des moûts et des vins associées au développement de pourritures sur les raisins. Etude particulière de la géosmine. Thesis, University of Bordeaux.

La Guerche, S., Blancard, D., Chamont, S., Dubourdieu, D., Darriet, P., 2003: Origine de la géosmine, compose reponsable d`un arome terreux dans les vins – étude des microorganismes impliqués et leur metabolisme. Enologie, 7. Symposium International dòenologie, Ed. TEC&DOC, 22-25.

Luo, J., Vogel, R.F., Niessen, L., 2012: Development and application of a loop-mediated isothermal amplification assay for rapid identification of aflatoxigenic molds and their detection in food samples. International Journal of Food Microbiology 159: 214-224, DOI:/10.1016/j.ijfoodmicro.2012.08.018.

Magnoli, C., Violante, M., Combina, M., Palacio, G., Dalcero, A., 2003: Mycoflora an ochratoxin-producing strains of Aspergillus section Nigri in wine grapes in Argentina. Letters in Applied Microbiology 37: 179-184, DOI: 10.1046/j.1472-765x.2003.01376.x.

Majerus, P., Hain, J., Köld, C., 2008: Patulin in grape must and new, still fermenting wine (Federweißer). Mycotoxin Research 24: 135-139, DOI: 10.1007/BF03032340.

Marek, P., Annamalai, T., Venkitanarayanan, K., 2003: Detection of *Penicillium* expansum by polymerase chain reaction. International Journal of Food Microbiology 89: 139-144, DOI: 10.1016/S0168-1605(03)00115-6

Morales, H., Paterson, R.R.M., Venancio, A., Lima, N., 2013: Interaction with *Penicillium* expansum enhances *Botrytis cinerea* growth in grape juice medium and prevents patulin accumulation in vitro. Letters in Applied Microbiology 56: 356-360, DOI: 10.1111/lam.12056.

Moss, M.O., Long, M.T., 2002: Fate of patulin in the presence of the yeast *Saccharomyces cerevisiae*. Food Additives and Contaminants Part A, 19, 387–399.

Ollinger, N., Lasinger, V., Probst, C., Pitsch, J., Sulyok, M., Weghuber, J., 2020: DNA barcoding for the identification of mold species in bakery plants and products. Food Chemistry 318: 126501, DOI: 10.1016/j.foodchem.2020.126501.

Pallottino, F., Costa, C., Antonucci, F., Strano, M.C., Calandra, M., Menesatti, P., 2012: Electric nose application for determination of *Penicillium digitatum* in Valencia oranges. Journal of the Science in Food and Agriculture 92: 2008-2012, DOI: 10.1002/jsfa.5586.

Prat, C., Ruiz-Rueda, O. Trias, R., Anticó, E., Capone, D., Bañeras, L., 2009: Molecular fingerprinting by PCR-denaturing gradient gel electrophoresis reveals differences in the levels of micobial diversity for musty-earthy tainted corks. Applied and Environmental Microbiology 75: 1922-1931, DOI: 10.1128/AEM.02758-08.

Quéro, L., Girard, V., Pawtowski, A., Tréguer, S., Weill, A., Arend, S., Cellière, B., Polsinelli, S., Monnin, V., van Belkum, A., Vasseur, V., Nodet, P., Mounier, J., 2019: Development and application of MALDI-TOF MS for identification of food spoilage fungi. Food Microbiology 81: 76-88.

Reeve, M.A., Bachmann, D., Caine, T.S., 2019: Identification of *Penicillium* species by MALDI-TOF MS analysis of spores collected by dielectrophoresis. Biology Methods and Protocols 4 (1): 1-15, DOI: 10.1093/biomethods/bpz018.

Sage, L., Krivobok S., Delbos, E., Seigle-Murandi, F., Creepy, E.E., 2002: Fungal flora and ochratoxin A production in grapes and musts from France. Journal of Agricultural and Food Chemistry 50: 1306-1311, DOI: 10.1021/jf011015z.

Samson, R.A., and Frisvad, J.C., 2004: *Penicillium* subgenus *Penicillium*: new taxonomic schemes, mycotoxins and other extrolites. Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.

Samson, R.A., Houbraken, J., Thrane, U., Frisvad, J.C., Andersen, B., 2010: Food and indoor fungi. Utrecht: CBS – KNAW Fungal Biodiversity Centre.

Samson, R.A., Seifert, K.A. Kuijpers, A.F.A., Houbraken, J.A.M.P., and Frisvad, J.C., 2004: Phylogenetic analysis of *Penicillium* subgenus *Penicillium* using partial β-tubulin sequences. Studies in Mycology 48: 175-200.

Sanzani, S.M., Montemurro, C., Di Rienzo, V., Solfrizzo, M., Ippolito, A., 2013: Genetic structure and natural variation associated with host of origin in *Penicillium expansum* strains causing blue mould. International Journal of Food Microbiology 165: 111-120.

Scott, P.M., Fuleki, T., Harwig, J., 1977: Patulin content of juice and wine produced from moldy grapes. Journal of Agricultural and Food Chemistry 25: 434-436, DOI: 10.1016/j.ijfoodmicro.2013.04.024.

Seifert, K.A., Samson R.A., deWaard, J.R., Houbraken, J., Lévesque C.A., Hebert, P.D.N., 2007: Prospects for fungus identification using CO 1 DNA barcodes, with *Penicillium* as a test case. Proceedings of the National Academy of Sciences (USA) 104: 3091–3906, DOI: 10.1073/pnas.0611691104.

Stobbe, A.H., Daniels, J., Espindola, A.S., Verma, R., Melcher, U., Schneider, W., 2013: E-probe diagnostic nucleic acid analysis (EDNA): A theoretical approach for handling of next generation sequencing data for diagnosis. Journal of Microbiological Methods 94 (3): 356-366, DOI: 10.1016/j.mimet.2013.07.002.

Trucksess, M.W., Tang, Y., 2001: Solid phase extraction method for patulin in grape juice and unfiltered apple juice. In: M.W. Trucksess und A. F. Pohland (eds.): Mycotoxin protocols. Humana Press, Totowa, New York, 205-213.

Walter, R., 2008: Untersuchungen zur Grünfäule an Tauben. Dissertation University of Hohenheim. http://opus.ub.uni-hohenheim.de/volltexte/2008/297/.

Wenning, M., Scherer, S., 2013: Identification of microorganisms by FTIR spectroscopy: perspectives and limitations of the method. Applied Microbiology and Biotechnology 97: 7111-7120, DOI: 10.1007/s00253-013-5087-3.

White, T. J., Bruns, T., Lee, S., Taylor, J. W., 1990: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: a guide to methods and applications (Innis M. A., Gelfand D. H., Sninsky J. J., White T. J., eds.): Academic Press, New York, 315-322.