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Development of an Efficient Fungal DNA Extraction Method To Be Used in Random Amplified Polymorphic DNA–PCR Analysis To Differentiate Cyclopiazonic Acid Mold Producers

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ABSTRACT

A variety of previously established mechanical and chemical treatments to achieve fungal cell lysis combined with a semiautomatic system operated by a vacuum pump were tested to obtain DNA extract to be directly used in randomly amplified polymorphic DNA (RAPD)–PCR to differentiate cyclopiazonic acid–producing and –nonproducing mold strains. A DNA extraction method that includes digestion with proteinase K and lyticase prior to using a mortar and pestle grinding and a semiautomatic vacuum system yielded DNA of high quality in all the fungal strains and species tested, at concentrations ranging from 17 to 89 ng/μl in 150 μl of the final DNA extract. Two microliters of DNA extracted with this method was directly used for RAPD-PCR using primer $(GACA)_4$. Reproducible RAPD fingerprints showing high differences between producer and nonproducer strains were observed. These differences in the RAPD patterns did not differentiate all the strains tested in clusters by cyclopiazonic acid production but may be very useful to distinguish cyclopiazonic acid producer strains from nonproducer strains by a simple RAPD analysis. Thus, the DNA extracts obtained could be used directly without previous purification and quantification for RAPD analysis to differentiate cyclopiazonic acid producer from nonproducer mold strains. This combined analysis could be adaptable to other toxigenic fungal species to enable differentiation of toxigenic and nontoxigenic molds, a procedure of great interest in food safety.

The ecological conditions found in dry-cured meat products during ripening favor growth of a mold population *(21).* Several strains isolated from these products have produced mycotoxins, mainly cyclopiazonic acid (CPA) *(24).* Randomly amplified polymorphic DNA (RAPD)–PCR analysis provides a powerful tool to differentiate mycotoxigenic strains, since a high correlation between RAPD-PCR profiles and mycotoxigenic production has been reported *(7, 10, 18).* However, the quality of DNA used in this analysis is of great importance, since some differences in the RAPD profiles may be generated when DNA of poor quality is used. Thus, the availability of pure DNA lacking PCR inhibitors, as well as a rapid and easy-to-perform fungal DNA extraction protocol, is essential. Conventional DNA extraction of fungal cells is very time-consuming or shows poor release of fungal DNA compared to methods of extraction of DNA of human cells or viruses *(17).*

Several fungal DNA extraction methods including mechanical disruption of conidia and hyphae in liquid nitrogen with mortar and pestle, sonication, glass bead milling, or microwaving have been reported *(3, 12, 13, 17, 25).* In addition, nonmechanical disruption protocols such as treatment with alkaline chemicals, detergents, and xanthogenates have also been used to yield large quantities of good-quality fungal DNA *(4, 22).* Recently Karakousis et al. *(15)* found higher efficiency in fungal DNA extraction by using chemical digestion with lyticase or proteinase K than by using mechanical disruption with sonication or in liquid nitrogen with mortar and pestle. To yield DNA of good quality, additional steps of DNA purification with toxic chemicals such as phenol-chloroform *(2, 14)* or guanidine thiocyanate *(5)* are usually included in fungal DNA extraction protocols. To avoid steps of DNA purification with toxic chemicals, several companies have commercialized fungal DNA isolation kits. However, no single method is appropriate for all fungi, since each species requires a specific method to efficiently extract DNA *(19).* Combinations of chemical digestion of molds and commercial extraction kits have been reported as being appropriate to yield fungal DNA. Thus, Karakousis et al. *(15)* found high efficiency in extraction of fungal DNA of clinical origin by adapting a step of chemical digestion with lyticase or proteinase K to a commercial extraction kit. Another possibility for avoiding the use of toxic chemicals in DNA purification, is the use of a semiautomatic system for extracting fungal DNA equipped with specific membranes and filters operated by a vacuum pump. This method has been applied to extract DNA from human cells *(1).* Due to the difficulty inherent in fungal cell lysis, this kind of method should be combined with a prior mechanical or chemical treatment of conidia and hyphae to achieve a good yield of DNA.

The purposes of this work were (i) to develop an efficient fungal DNA extraction method using a semiautomatic system operated by a vacuum pump combined with

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TABLE 1. *Mold strains used in this study*

Species designation	Strain reference	CPA production as detected by: MECE	HPLC-MS
A. flavus	CECT[a] 2687	Producer	Producer
A. flavus	IBT[b] 3696	Producer	Producer
A. tamarii	IBT 26855	Producer	Producer
P. palitans	IBT 15975	Nonproducer	Producer
P. camemberti	IBT 11570	Producer	Producer
P. camemberti	CBS[c] 299.48	Producer	Producer
P. camemberti	CBS 273.97	Producer	Producer
P. camemberti	CBS 112.562	Producer	Producer
P. camemberti	CECT 2267	Nonproducer	Producer
P. griseofulvum	CBS 485.84	Nonproducer	Producer
P. griseofulvum	CBS 295.97	Producer	Producer
P. griseofulvum	CBS 110.416	Nonproducer	Nonproducer
P. griseofulvum	CECT 2605	Nonproducer	Nonproducer
P. griseofulvum	CECT 2919	Nonproducer	Nonproducer
P. griseofulvum	IBT 14319	Nonproducer	Nonproducer
Penicillium dipodomyicola	IBT 26223	Nonproducer	Nonproducer
P. commune	IBT 22298	Producer	Producer
P. commune	CBS 169.44	Nonproducer	Producer
P. commune	CBS 311.48	Producer	Producer
P. commune	CBS 341.59	Nonproducer	Producer
P. commune	Pc[d] 7	Producer	Producer
P. commune	Pc 58	Producer	Producer
P. commune	CBS 247.32	Nonproducer	Nonproducer
P. commune	CBS 282.36	Nonproducer	Nonproducer
P. commune	Pc 10	Nonproducer	Nonproducer
P. commune	Pc 4	Nonproducer	Nonproducer
P. commune	Pc 20	Nonproducer	Nonproducer
P. commune	Pc 21	Nonproducer	Nonproducer
P. commune	Pc 27	Nonproducer	Nonproducer
P. commune	Pc 31	Nonproducer	Nonproducer
P. commune	Pc 36	Nonproducer	Nonproducer
P. commune	Pc 54	Nonproducer	Nonproducer

[a] CECT, Spanish Type Culture Collection.
[b] IBT, Type Culture Collection of the Department of Biotechnology, Technical University of Denmark.
[c] CBS, Centraalbureau voor Schimmelcultures (The Netherlands).
[d] Pc, strains isolated from dry-cured ham.

different previous mechanical and chemical treatments to achieve fungal cell lysis and (ii) to use DNA extracted by the most efficient method to assay samples directly by RAPD-PCR to obtain RAPD profiles in order to differentiate CPA-producing from CPA-nonproducing mold strains.

MATERIALS AND METHODS

Strains and culture conditions. Seventeen CPA producer mold strains and 15 CPA nonproducer strains belonging to seven different species from the Spanish Type Culture Collection, the Centraalbureau voor Schimmelcultures in The Netherlands, and the Type Culture Collection of the Department of Biotechnology from the Technical University of Denmark were used. In addition 10 mold strains isolated from dry-cured ham and characterized as *Penicillium commune (21)* were tested (Table 1).

All mold strains were three-point inoculated onto meat extract agar (2% malt extract, 2% glucose, 0.1% peptone, and 2% agar) and incubated for 4 days at 25°C. Production of CPA was analyzed by micellar electrokinetic capillary electrophoresis (MECE) *(20)* and high-pressure liquid chromatography–mass spectrometry (HPLC-MS) *(24).*

Fungal cell lysis and digestion. For DNA extraction, fungal cell lysis was performed using 50 mg (wet weight) of mycelium grown during 4 days using the following methods.

Method 1 consisted of liquid nitrogen freezing and mortar and pestle grinding and treatment with proteinase K: 20 ml of liquid nitrogen was added to the mycelium, which was ground to a fine powder in a −80°C prefrozen mortar and pestle for 2 min and recovered in 300 μl of nucleic acid purification lysis solution from TransPrep (Applied Biosystem, Warrington, UK) according to the user's manual. Then, 20 μl of proteinase K (1 mg/ml) (Sigma-Aldrich, St. Louis, Mo.) was added, and the extracts were incubated at 60°C for 35 min.

Method 2 consisted of liquid nitrogen freezing, mortar and pestle grinding, and treatment with TES (0.05 M Tris, 0.005 M EDTA, 0.05 M NaCl, pH 8) buffer, sodium dodecyl sulfate (SDS), and proteinase K. The fine powder of crude extract obtained after grinding the mycelium with liquid nitrogen as described above for method 1 was recovered in 300 μl of TES buffer containing 1.5% SDS. Then, 20 μl of proteinase K (1 mg/ml) (Sigma-Aldrich) was added, and the extract was incubated at 60°C for 35 min.

Method 3 consisted of liquid nitrogen freezing, mortar and pestle grinding, and lyticase digestion. The crude extract obtained after grinding the mycelium in a mortar and pestle and using liquid nitrogen was then recovered and digested with 400 U of lyticase (from *Arthrobacter luteus,* L4025, Sigma-Aldrich) in 300 μl of a sorbitol buffer containing 0.1 M sorbitol, 100 mM Tris-HCl, 100 mM EDTA, and 14 mM β-mercaptoethanol, pH 7.8 at 30°C overnight *(15).*

Method 4 consisted of lyticase digestion, liquid nitrogen freezing, and mortar and pestle grinding. The mycelium was digested with 400 U of lyticase (Sigma-Aldrich) in 300 μl of the above-described sorbitol buffer at 30°C overnight. Then, 20 ml of liquid nitrogen was added and the extract was ground in a mortar and pestle for 2 min.

Method 5 consisted of proteinase K and lyticase digestion, liquid nitrogen freezing, and mortar and pestle grinding. The mycelium was first digested with proteinase K (Sigma-Aldrich) (1 mg/ml) in 300 μl of the above-described sorbitol buffer at 60°C for 35 min and then incubated with 400 U of lyticase (Sigma-Aldrich) at 30°C overnight. Then, 20 ml of liquid nitrogen was added and the mixture was ground in a mortar and pestle for 2 min.

In all of the above methods, after the corresponding treatment, the resulting extracts were added to 10-μg/μl RNase A (Sigma-Aldrich) and incubated at 37°C for 1 h to eliminate RNA, before being applied to a semiautomatic system operated by a vacuum pump for DNA extraction.

DNA extraction. Fungal extracts obtained by the above five treatments were applied individually to a semiautomatic system operated by a vacuum pump, ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, Calif.), according to the protocol provided. The purified DNA finally was eluted in a total volume of 150 μl. Analyses were done in triplicate for all mold strains tested.

Determination of quantity and quality of DNA extracts. The quantity and quality of purified DNA were determined spectrophotometrically in a Biophotometer Eppendorf (Eppendorf AG, Hamburg, Germany) and by comparison to DNA standards, using agarose gel electrophoresis. For analysis in agarose gel electrophoresis, 5 μl of the DNA extract from each method was run on

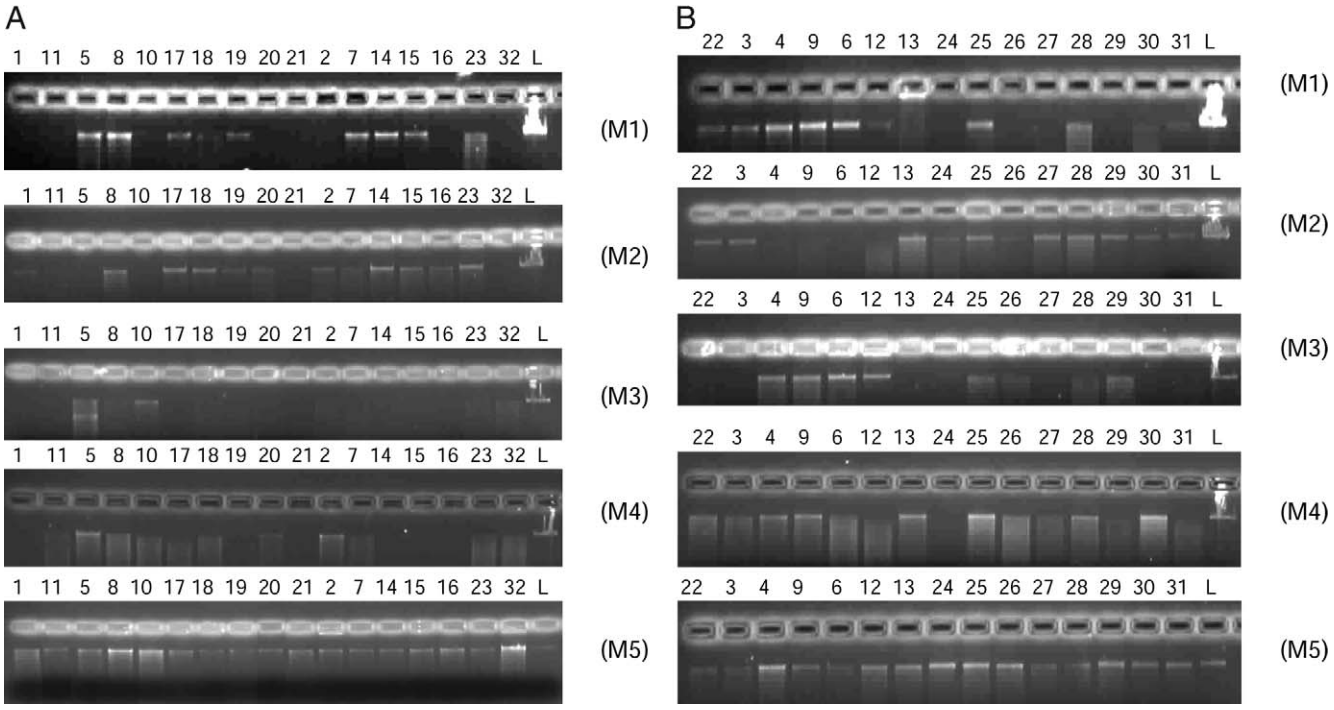

FIGURE 1. *Agarose gel analysis of DNA extracted from CPA (A) and non CPA (B) producer mold strains: M1, liquid nitrogen freezing, mortar and pestle grinding, and treatment with proteinase K; M2, liquid nitrogen freezing, mortar and pestle grinding, and treatment with TES buffer, SDS, and proteinase K; M3, liquid nitrogen freezing, mortar and pestle grinding, and lyticase digestion; M4, lyticase digestion, liquid nitrogen freezing, and mortar and pestle grinding; M5, proteinase K and lyticase digestion, liquid nitrogen freezing, and mortar and pestle grinding. CPA producers: 1,* A. flavus *CECT 2687; 11,* A. flavus *IBT 3696; 5,* A. tamarii *IBT 26855; 8,* P. palitans *IBT 15975; 10,* P. camemberti *IBT 11570; 17,* P. camemberti *CBS 299.48; 18,* P. camemberti *CBS 273.97; 19,* P. camemberti *CBS 112.562; 20,* P. griseofulvum *CBS 485.84; 21,* P. griseofulvum *CBS 295.97; 2,* P. griseofulvum *CECT 2267; 7,* P. commune *IBT 22298; 14,* P. commune *CBS 169.44; 15,* P. commune *CBS 311.48; 16,* P. commune *CBS 341.59; 23,* P. commune *Pc 7; 32,* P. commune *Pc 58. CPA nonproducers: 22,* P. griseofulvum *CBS 110.416; 3,* P. griseofulvum *CECT 2605; 4,* P. griseofulvum *CECT 2919; 9,* P. griseofulvum *IBT 14319; 6,* P. dipodomyicola *IBT 26223; 12,* P. commune *CBS 247.32; 13,* P. commune *CBS 282.36; 24,* P. commune *Pc 10; 25,* P. commune *Pc 4; 26,* P. commune *Pc 20; 27,* P. commune *Pc 21; 28,* P. commune *Pc 27; 29,* P. commune *Pc 31; 30,* P. commune *Pc 36; 31,* P. commune *Pc 54. L, DNA marker consisting of double-stranded DNA fragment of 48.5 kbp.*

a 1% (wt/vol) agarose gel. The gels were stained with ethidium bromide (0.5 μg/ml), and the products were visualized using the UV transillumination G-Box of Syngene (Synoptics group, Frederick, Md.), photographed, and analyzed by the integrated camera and software GeneSnap and GeneTools of Syngene. A DNA molecular size marker of 48.5 kbp from Roche diagnostic (Roche Pharma, Indianapolis, Ind.) was used.

RAPD-PCR. Two microliters of the DNA extract obtained with method 5 (proteinase K and lyticase digestion) was used for RAPD-PCR. The primer used for the RAPD-PCR was $(GACA)_4$ *(7).* The reaction was done in a total volume of 50 μl, 10 mM Tris-HCl, 100 μM (each) dATP, dCTP, dGTP, and dTTP, 4 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Finnzyme, Espoo, Finland), and primer (1 μM). The reaction mixtures were incubated in a programmable thermal cycler, Mastercycler epgradient from Eppendorf AG, using 30 cycles consisting of 1 min at 94°C, 1 min at 42°C, and 1.5 min at 72°C. A final step of 5 min at 72°C was carried out. The amplification products were analyzed by submerged gel electrophoresis in 2.5% agarose gels, using Tris-acetate-EDTA buffer at 100 V for 66 min. The gels were stained with ethidium bromide (0.5 μg/ml), and the products were visualized by UV transillumination G-Box and photographed with the Syngene system. DNA molecular size markers of 0.15 to 2.1 kbp from Roche diagnostic (Roche Pharma) were used to determine the size of the PCR products.

Cluster analysis. The genetic similarity of CPA-producing and non-CPA-producing mold strains tested was assessed, based on RAPD patterns, by the similarity coefficient of Dice *(6).* The distance matrices of Dice *(6)* were computed using the GeneDirectory program from Syngene (Synoptics group), and dendrograms were generated by the unweighted pair group method with arithmetic mean (UPGMA).

RESULTS

Production of CPA was analyzed in all producer strains by MECE and HPLC-MS (Table 1). In the producer strains *Penicillium palitans* IBT 15975, *Penicillium camemberti* CECT 2267, *Penicillium griseofulvum* CBS 485.84, *Penicillium commune* CBS 169.44, and *P. commune* CBS 341.59, CPA production was detected only by the HPLC-MS analysis (Table 1).

The quality of extracted fungal DNA of the CPA-producing and non–CPA-producing molds obtained from the five extraction methods showing all spectrophotometrically ratios at 260:280 above 1.7 was compared by visual analysis under the intensities of UV light of the stained DNA (Fig. 1). Methods 1, 2, and 3, which did not include previous digestion before liquid nitrogen freezing and mortar and pestle grinding, showed DNA bands for 60.4% (Fig. 1,

TABLE 2. *Amount of DNA recovered from the 32 mold strains assayed, by extraction method*

Strain	Amt of DNA (ng/μl) recovered by extraction method no.:				
	1	2	3	4	5
A. flavus CECT 2687	ND[a]	7.40	ND	ND	40.43
A. flavus IBT 3696	ND	ND	ND	3.00	22.83
A. tamari IBT 26855	29.10	ND	43.00	26.30	89.83
P. palitans IBT 15975	30.80	14.90	ND	14.10	17.33
P. camemberti IBT 11570	ND	ND	36.70	ND	27.36
P. camemberti CBS 299.48	10.10	36.00	ND	ND	51.33
P. camemberti CBS 273.97	6.00	18.90	ND	2.00	20.26
P. camemberti CBS 112.562	10.00	15.60	ND	ND	52.63
P. camemberti CECT 2267	ND	32.40	ND	14.40	33.20
P. griseofulvum CBS 485.84	ND	13.40	ND	9.20	40.83
P. griseofulvum CBS 295.97	ND	ND	ND	ND	84.50
P. griseofulvum CBS 110.416	27.00	15.40	ND	24.60	46.96
P. griseofulvum CECT 2605	28.10	30.50	ND	9.90	34.43
P. griseofulvum CECT 2919	70.00	ND	33.70	15.10	81.13
P. griseofulvum IBT 14319	81.12	ND	35.20	18.90	29.23
P. dipodomyicola IBT 26223	90.30	ND	38.00	10.50	26.06
P. commune IBT 22298	15.50	12.40	ND	ND	53.14
P. commune CBS 169.44	62.90	28.60	ND	ND	46.23
P. commune CBS 311.48	21.30	18.00	ND	ND	28.63
P. commune CBS 341.59	ND	16.30	ND	ND	30.16
P. commune Pc7	12.40	35.60	ND	ND	41.00
P. commune Pc58	ND	ND	ND	3.00	36.13
P. commune CBS 247.32	26.30	ND	28.00	ND	50.40
P. commune CBS 282.36	ND	57.60	ND	25.80	55.60
P. commune Pc10	ND	20.80	ND	ND	45.30
P. commune Pc 4	29.50	30.50	12.00	30.00	36.63
P. commune Pc 20	ND	11.50	6.00	17.50	77.76
P. commune Pc 21	ND	31.20	ND	9.40	63.10
P. commune Pc 27	11.50	43.10	ND	19.00	63.20
P. commune Pc 31	ND	33.00	3.00	ND	77.96
P. commune Pc 36	ND	14.20	ND	33.60	48.56
P. commune Pc 54	15.00	12.50	ND	ND	26.76

[a] ND, not detected.

M1 A and B), 68.8% (Fig. 1, M2 A and B), and 31.2% (Fig. 1, M3 A and B), respectively, of the mold strains tested. From the above methods, method 2, which includes TES buffer, SDS, and proteinase K after liquid nitrogen freezing and mortar and pestle grinding, showed the best yield, but DNA was not extracted from 10 of the 32 mold strains tested. Methods with previous digestion with lyticase (method 4) or proteinase K and lyticase (method 5) improved DNA extraction, with method 5 being the only protocol able to yield a DNA band for all of the mold strains tested (Fig. 1, M5 A and B). Table 2 displays the concentrations of DNA extracted with the five extraction methods assayed. Methods 1 and 3 did not yield DNA from *Aspergillus flavus* or for some strains of *P. camemberti, P. griseofulvum,* and *P. commune.* Methods 2 and 4 extracted DNA from most of the strains tested and in higher amounts than methods 1 and 3, but all four methods failed to yield DNA from some of the strains of *A. flavus, P. camemberti, P. griseofulvum,* and *P. commune.* Method 5 yielded DNA from all the strains tested, at concentrations ranging from 17 to 89 ng/μl in the 150 μl of the final DNA extract. Since the amount of mycelium used was 50 mg, the DNA yield per gram of mycelium varied from 51 to 267 μg.

DNA extracted with method 5 was directly used for RAPD-PCR with primer $(GACA)_4$. Figure 2 shows RAPD profiles obtained with 1, 2, and 4 μl of the DNA extract of mold strains with different DNA yields (from 20 to 75 ng/μl). There were several differences in RAPD patterns obtained with the above DNA extracts amounts, but the best yields for the different molds tested were obtained with 2 μl of the DNA extract. Thus, this amount was selected for use in RAPD-PCR.

The quality of DNA extracted using method 5 was confirmed by successful amplification of the primer $(GACA)_4$ (Fig. 3). Different RAPD profiles between CPA producer and nonproducer mold strains in each species were observed. Figure 3 shows dendrograms obtained from RAPD profiles of CPA producer and nonproducer mold strains. In each species the higher percentage of similarity was found between CPA strains producers or between nonproducer strains, while producer and nonproducer strains always showed similarity of <55%. All strains were grouped in five clusters designated A to E (Fig. 3). Cluster A includes only CPA producer strains from *A. flavus* and *P. griseofulvum.* However, CPA nonproducer strains of *P. griseofulvum* were grouped in cluster E with 100% similarity. In this cluster, all strains grouped were CPA nonproducers. Cluster D includes nonproducer strains of *P. commune* showing similarity of >70%. CPA producer strains of *P. commune* were grouped in cluster B with similarity of >60%. In this cluster were included two nonproducer strains of this species, but the similarity with producer strains was low (around 50%). Cluster B also includes a producer strain of *Aspergillus tamarii* but with low similarity (<40%) with strains of *P. commune.* Cluster C includes CPA producer and nonproducer strains, but these are clearly separated. Thus, producer strains of *P. camemberti* showed a similarity of 100% but showed similarities of <55% with the two nonproducer strains of *P. commune* included in this cluster. Furthermore, a producer strain of *P. palitans* showed a similarity of <55% with the two nonproducer strains of *P. commune.*

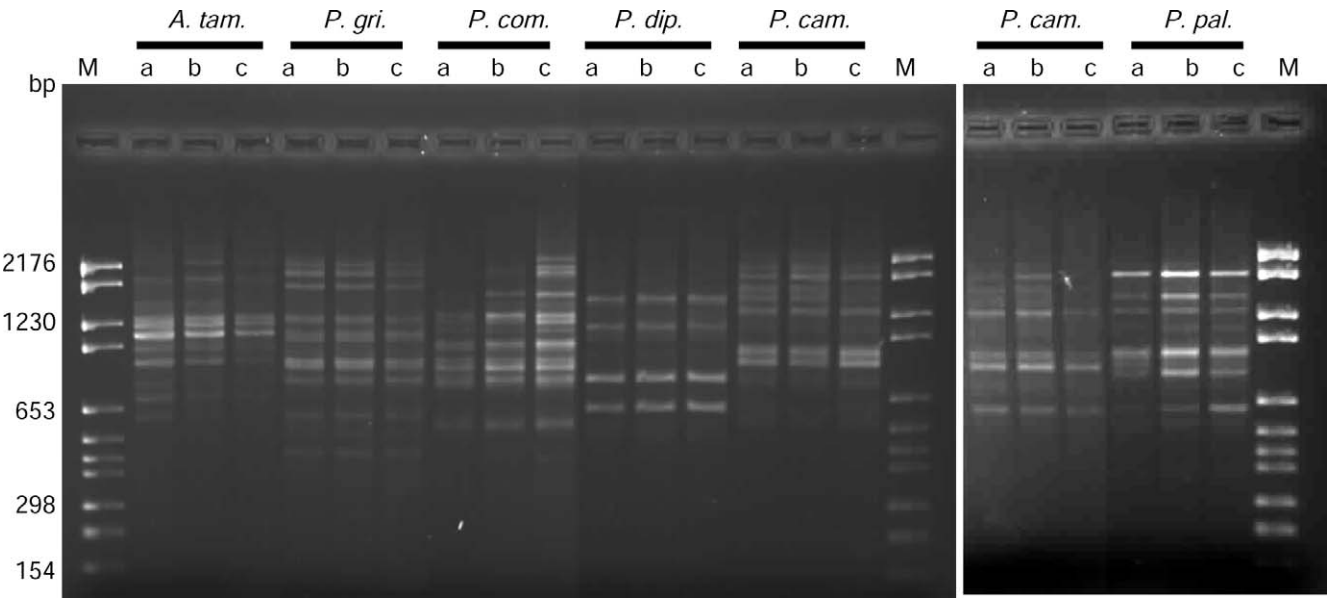

FIGURE 2. *Agarose gel of RAPD-PCR products with primer $(GACA)_4$ using different amounts (1, 2, and 4 μl) of DNA extracts obtained with method 5 (proteinase K and lyticase digestion, liquid nitrogen freezing, and mortar and pestle grinding). Lanes: M, 0.15- to 2.1-kbp DNA molecular weight marker VI (Roche Diagnostics, S.L.); a, 1-μl DNA extract; b, 2-μl DNA extract; c, 4-μl DNA extract. Organisms:* A. tamarii *IBT 26855;* P. griseofulvum *CECT 2919;* P. commune *Pc 4;* P. dipodomyicola *IBT 26223;* P. camemberti *CBS 273.97;* P. camemberti *CECT 2267;* P. palitans *IBT 15975.*

DISCUSSION

The analysis of CPA production in the producer and nonproducer strains used in the present study showed that HPLC-MS sensitivity was higher than that of MECE, since for five producer strains, CPA production was confirmed only by the former method. This difference in sensitivity can be explained by the detection systems used in the two techniques, i.e., mass spectrometry in HPLC-MS versus diode array in MECE. The five producer strains *P. palitans* IBT 15975, *P. camemberti* CECT 2267, *P. griseofulvum* CBS 485.84, *P. commune* CBS 169.44, and *P. commune* CBS 341.59 were considered for the present work to be CPA producers, since production of this mycotoxin was confirmed by HPLC-MS.

From the five DNA extraction methods tested, those including digestion prior to using liquid nitrogen freezing showed yields in DNA extraction similar to, or higher than, those methods without previous digestion. Thus, the method assayed with prior lyticase digestion showed a DNA yield similar to the best result obtained with methods without previous digestion (method 2, which includes TES buffer and proteinase K). In addition, when previous digestion was done, first with proteinase K and then with lyticase, the DNA yield was the highest, since this was the only method able to extract DNA from all mold strains tested. Proteinase or lyticase digestion before use of a mortar and pestle and a commercial kit was reported by Karakousis et al. *(15)* as essential to improve the efficiency of fungal DNA extraction. However, from these results, digestion with lyticase alone yielded no DNA in any of the strains and species tested. The action of lyticase is more effective if previously mycelium has been treated with proteinase K. In addition, liquid nitrogen freezing and mortar and pestle grinding after the described digestion with both proteinase K and lyticase contribute to an efficient DNA yield. Liquid nitrogen freezing and mortar and pestle grinding were previously reported by Karakousis et al. *(15)* as the most efficient physical disruption method for lysing fungal hyphae and conidia, especially when it was used after mycelium digestion.

The quantity of extracted DNA was similar to those reported by different authors for molds of clinical or plant origin *(9, 11, 15, 16, 23).* We reported here an efficient DNA extraction method for mold from food origin such as the *Penicillium* species *P. commune, P. camemberti,* and *P. griseofulvum,* none of which were reported in former studies.

Time of extraction is about 14 h, which includes overnight incubation. Thus, the real time of hands-on work is much lower (about 3 h). Furthermore, the use of a semiautomatic system operated by a vacuum pump to purify the DNA avoids use of purification reactives such as phenol-chloroform. This allowed a single person to extract 40 to 50 fungal samples per day.

The high quality of DNA extracted using method 5 was confirmed by the successful reproducible amplification of the RAPD-PCR with primer $(GACA)_4$, using different amounts of DNA extract for the different mold strains tested, which allowed selection of the amount of 2 μl as the most appropriate for use in further RAPD analysis. To ensure the quality of DNA, different strategies of amplification have been applied, such as RAPD-PCR *(11),* quantitative PCR *(9),* or real-time PCR *(8).* In most of the former works, the fungal DNA extracts yielded amplification. However, Guo et al. *(11)* did not find RAPD fingerprints in some fungal species of *Mucor, Cercospora, Phytophthora,* and *Trichoderma,* which the authors attributed to PCR inhibitors not being thoroughly eliminated in the DNA extracts. Since reproducible RAPD fingerprints were obtained

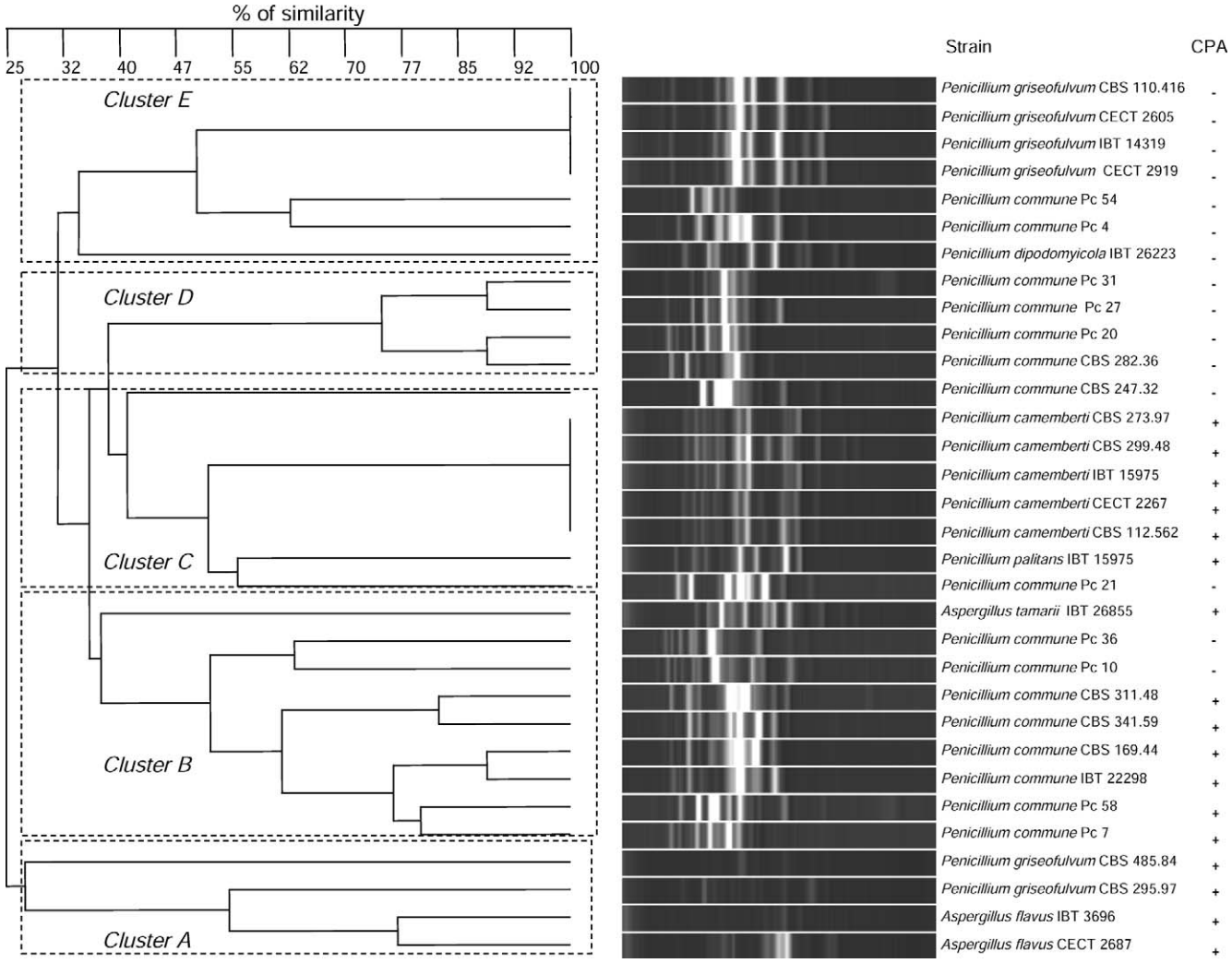

FIGURE 3. *RAPD profiles and dendrogram based on the PCR fingerprints obtained with primer $(GACA)_4$ of the different strains of molds tested. The dendrogram is based on the Dice similarity coefficient and cluster analysis by the unweighted pair group method with arithmetic mean. The percent similarity scale indicates the coefficients of similarity between strains. The production of CPA of the strains is shown to the right of the strain designation: +, producer; −, nonproducer.*

for all the strains tested in the present work, the method developed here yielded DNA extract without PCR inhibitors.

A further proof of the effectiveness of the method developed here to obtain DNA of quality was the ability of the reproducible RAPD fingerprints to differentiate CPA producer strains at the species level, since high similarity was observed between producer and nonproducer strains. In fact, in some cases RAPD pattern analysis showed higher similarity for the parameter production of CPA than for the parameter belonging to the species. Thus, strains of *P. griseofulvum* CPA nonproducers were grouped into cluster E, while those of CPA producers were grouped into cluster A with producer strains of *A. flavus.* CPA producer strains of *P. commune* were grouped into cluster B, while nonproducer strains were included in cluster D or in cluster C with a very low similarity with producer strains. In addition, the similarity of the RAPD fingerprints between producer and nonproducer strains was very low. Although no differentiation between CPA and CPA nonproducer mold strains has been previously reported, correlation between RAPD grouping and some secondary metabolites (CPA, rugulovasine, and cyclopaldic acid) produced by *P. commune* has been observed *(18).* In addition, for other mycotoxins such as mycophenolic acid, a high correlation between RAPD patterns and producing strains of *P. roqueforti* has been reported *(10).* Martín et al. *(20)* found a high correlation between RAPD analysis of toxigenic molds and profiles of secondary metabolites obtained by MECE. From our results, it is evident that there is a relationship between RAPD patterns and production of CPA. This relationship does not allow for the separation of all the strains tested in clusters, differentiated by CPA production. However, the differences in RAPD patterns observed between producer and nonproducer strains could be very useful to differentiate CPA producer from nonproducer strains by a simple RAPD analysis, if information about RAPD patterns associated to CPA production has been previously reported, as has been presented in this work. Thus, RAPD analysis reveals the DNA patterns of CPA producer strains without making it necessary to detect CPA production by methods such as growth of mold strains in culture medium, extraction with organic sol-

vents, and detection by a sensitive method such as HPLC-MS. Use of RAPD analysis as a routine technique may avoid false negatives associated with analysis of CPA production due to misproduction of CPA when culture conditions are not appropriate for a particular mold strain or if the detection method does not have sufficient sensitivity to detect produced CPA. RAPD analysis may be facilitated if the previous fungal DNA extraction method yields DNA extracts of good quality that could be used directly for RAPD-PCR without previous purification and quantification of DNA.

In summary, in this work an efficient fungal DNA extraction method that includes digestion with proteinase K and lyticase prior to using mortar and pestle grinding and a semiautomatic system operated by a vacuum pump was developed. This protocol is reproducible and generates good yields of DNA extracts that could be used directly without previous purification and quantification for RAPD analysis, which could be very useful in differentiating CPA producer from nonproducer mold strains. This combined analysis could be adaptable to other toxigenic fungal species to differentiate toxigenic from non toxigenic molds and may prove to be of great interest in food safety.

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