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Contamination of meat stored in home refrigerators in Qena (Egypt)

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Eighty samples were collected from different parts of home-refrigerators and meat stored herein, in the province of Qena, Egypt. Quantitative and qualitative estimations of moulds were carried out by conventional methods and the identified *Aspergillus* spp. were confirmed by the RAPD-PCR technique in the Institute of Applied Microbiology (IAM), University of Agricultural Sciences, Vienna, Austria. The obtained results revealed that the highest mould count was 3.9×10^4 CFU/cm^2 in the chest of the refrigerators, followed by 3.2×10^4, 2.6×10^3 and 2.5×10^3 CFU/cm^2 in samples of air and freezer of refrigerators and stored meat, respectively. Eleven mould genera could be identified, the most common of which were *Aspergillus, Penicillium* and *Cladosporium*. The counts and relative frequencies for these genera were 31 (25.4 %), 17 (13.9 %) and 16 (13.1 %), respectively. Five *Aspergillus* species were identified; mainly *A. flavus* 13 (42.0 %), *A. niger* 5 (16.1 %) and *A. nidulans* 5 (16.1 %). The isolated *Aspergillus* species were subjected to further identification by random amplified polymorphic DNA (RAPD) by using type strains from IAM. RAPD-analysis indicated that the *Aspergillus* strains isolated during this study were completely identical with the corresponding type strains from IAM. Public health hazard and significance of mould contamination in home-refrigerators, as well as hygienic measures and recommendations are fully discussed to prevent or minimise such contamination.

Key words: microscopic fungi, stored meat, refrigerators, *Aspergillus*, RAPD-PCR.

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Z různých míst chladniček používaných v domácnostech a z jídla tam uchovávaného bylo odebráno 80 vzorků (provincie Qena, Egypt). Kvalitativní a kvantitativní stanovení mikromycetů bylo provedeno běžnými metodami a určení druhů rodu *Aspergillus* bylo ověřováno molekulárními technikami (RAPD-PCR) v Institutu aplikované mikrobiologie (IAM) na Zemědělské univerzitě ve Vídni. Nejvyšší zjištěné množství mikromycetů bylo 3.9×10^4 CFU/cm^2 ve vnitřním prostoru chladniček, dále pak 3.2×10^4, 2.6×10^3 and 2.5×10^3 CFU/cm^2 ve vzorcích vzduchu, mrazicího boxu chladniček a v uchovávaném jídle. Bylo zjištěno 11 rodů mikromycetů, z nichž nejběžnější byli zástupci z rodů *Aspergillus, Penicillium* a *Cladosporium*. Absolutní a relativní frekvence pro tyto rody byla 31 (25.4 %), 17 (13.9 %) a 16 (13.1 %). Bylo určeno 5 druhů rodu *Aspergillus*, nejčastěji *A. flavus*: 13 (42.0 %), *A. niger*: 5 (16.1 %) a *A. nidulans*: 5 (16.1 %). Určení druhů z rodu *Aspergillus* bylo dále ověřováno metodou amplifikace náhodně zmnožených fragmentů DNA (RAPD) za použití ověřených kmenů z IAM. Tato analýza ukázala, že kmeny druhů z rodu *Aspergillus* izolované během této studie byly zcela identické s ověřenými kmeny z IAM. Závěrem je diskutováno zdravotní riziko, význam kontaminace chladniček v domácnostech a také hygienické podmínky a doporučení směřující k minimalizace kontaminace.

Introduction

Fungi and their mycotoxins are considered a major potential threat to public health and continue to have an extensive impact on the welfare of human and animal populations by affecting not only the quality of meat, but also the availability of clean products. Thus, mycotoxin residues have had a major impact on food industries of the world, which is reflected in the controlled movement of some foodstuffs across international borders. The ultimate concern is that some mycotoxins are highly carcinogenic, mutagenic and teratogenic for humans and animals (Thomas and Lawrence 1977, Bullerman et al. 1986).

Meat begins to be contaminated with moulds in slaughter-houses, mainly with the intestinal contents of slaughter animals, air and dust. The main isolated genera are *Aspergillus, Penicillium* and *Mucor* (Abdel-Rahman et al. 1985, El-Dally et al. 1988, Farghaly 1993). They found that *Aspergillus* species were the most frequent fungi in meat contamination and toxin production, especially *A. flavus* and *A. parasiticus,* which could produce aflatoxins (Ellis et al. 1991, Hamdy et al. 1993).

Some moulds can grow at temperatures in refrigerators typically used in houses and deep freezing has no significant destructive effect upon moulds (Kotinek et al. 1996). Therefore, several mould genera could be isolated from chilled meat, mainly *Aspergillus, Alternaria, Cladosporium, Fusarium, Mucor* and *Penicillium.* These fungi are responsible for spoilage and constitute a real risk to public health due to the production of mycotoxins (Refai and Loot 1969, Mislivec and Tuite 1970, Giradin 1997).

Detection of mould contamination in different parts of refrigerators and meat stored herein is essential to ensure safe and high quality of food. Direct microscopic observation of fungi or cultural methods are frequently used to assess such type of contamination. But as these conventional methods of detection have suffered from several drawbacks, new methods of identification based on PCR techniques have been developed (Messner et al. 1994, Giradin 1997).

Random PCR approaches are being increasingly used to generate molecular markers which are useful for taxonomy and for characterising fungal populations. RAPD-PCR assays have been used extensively to define fungal populations at species, intraspecific, race and strain levels. In general, most studies have been concentrated on intraspecific grouping, although other have been directed at the species level. RAPD-PCR has also been applied in the identification of individual strains within a particular population, some examples being toxin-producing strains of *Aspergillus flavus* (Bayman and Cotty 1993).

Therefore, this study was undertaken to assess mould contamination in home-refrigerators in Upper Egypt and to confirm the isolated strains of *Aspergillus* by using the RAPD-PCR method.

Materials and methods

The conventional techniques

Preparation and cultivation

The materials collected from the province of Qena (10 households) in Egypt were composed of 80 samples: 40 surface swabs taken from chest and freezer of the home-refrigerators, 20 samples of the meat stored (less than 4 weeks) herein, and 20 samples from air inside home-refrigerators. Air samples were obtained by exposing the plates to air for 15 min. while the surface area used for sample collection was 10 cm^2. Specimens were sent to the laboratory, without delay, for preparation of ten-fold dilution up to 10^6 by using sterile peptone water (1 %). One ml of each dilution was poured in duplicated sterile Petri-dishes (12 cm diam.) and one of each covered evenly with sterile melted Malt-extract agar and the other one with Czapek's-Dox agar (pH 4.5). Petri-dishes were then incubated at 25 °C for one week, and examined daily for detection of star-like mould colonies.

Isolation and identification

Detected colonies were picked up with mycological needles and inoculated into sterile slope Malt-extract agar (pH 7) and then incubated at 25 °C for 5 days. The sum of inoculated Malt-extract slope multiplied by the corresponding dilutions represented the total mould count (CFU/cm^2). Then, the isolated cultures in slope agar were further inoculated into Malt-extract agar and Czapek's-Dox agar (pH 7.5) by using 3-point techniques. The identification of mould genera and their species based on taxonomic information permits a general view of most known fungi. This depends mainly on the morphology of the colony, pigmentation of the reverse surface and fungus structures according to Raper and Fennel (1965), Hesseltine and Ellis (1973), Samson et al. (1976) and Rippon (1982).

The molecular technique (PCR)

All *Aspergillus* strains isolated during this study were subjected to further identification by using RAPD-PCR techniques as follows:

DNA extraction

Aspergillus strains were cultured in 100 ml Erlenmeyer-flasks containing 20 ml Mandles Andreoti Medium (per litre, 10 g glucose, 2 g peptone (Difco), 2.8 g ammonium sulphate, 4 g KH_2PO_4, 10 g Na_2HPO_4, 10 ml of a simplified Czapek concentrated (7 g $MgSO_4$, 0.05 g $CuSO_4$ $5H_2O$, 0.1 g $FeSO_4$ $7H_2O$, 0.1 g $ZnSO_4$ $7H_2O$, the final pH was adjusted to 5.0) for five days using a rotary shaker (30 °C, 150 rpm).

The mycelium was collected by filtration and ground to fine powder in liquid nitrogen. Fifty mg of the ground was transferred to a 1.5 ml Eppendorf tube and mixed with 700 μl 2 × CTAB buffer. Eppendorf tubes were incubated at 65 °C for 30 min., then 700 μl of chloroform was added and the mixture was vortexed briefly. The resulting mixture was centrifuged at a maximum speed of 5000 rpm for 30 min. and the clear supernatant was mixed with 600 μl isopropanol chilled to –20 °C. The mixture was centrifuged at a maximum speed for 5 min and the resulting pellet was washed twice with 1 ml of 70 % ethanol. The pellet was dried under vacuum and dissolved in 100 μl TE (10 mM Tris, 1 mM EDTA, pH 7.5) buffer. The DNA concentrations were evaluated by agarose gel electrophoresis.

RAPD – analysis

PCR conditions and separation of RAPD-PCR fragments were made according to Messner et al. (1994). The primers V1 (5' dACGGTCTTGG), V5 (5' dTGC-CGAGCTG) and V6 (5' dTGCAGCGTGG) were used. Synthesis of primers was performed with Codon Genetic systems (Vienna, Austria) using a model 392 DNA synthesizer (Applied Biosystems, Foster City, CA, USA). The temperature profile for primers was as follows: denaturation at 98 °C for 15 s; annealing at 40 °C for 90 s and extention at 72 °C for 100 s for a total of 40 cycles. Amplification products were electrophoresed in 1.4 % agarose gels with 10 × Trisborate-EDTA buffer and were stained with ethidium bromide.

Table 1. Mould counts of 20 contaminated samples from different places in refrigerators and stored meat.

Sources	Chest (CFU/cm^2)	Freezer (CFU/cm^2)	Air (CFU/cm^3)	Meat (CFU/cm^2)
Minimum	33000	220	2400	2000
Maximum	45000	280	4000	3200
Mean	39000	250	3200	2600

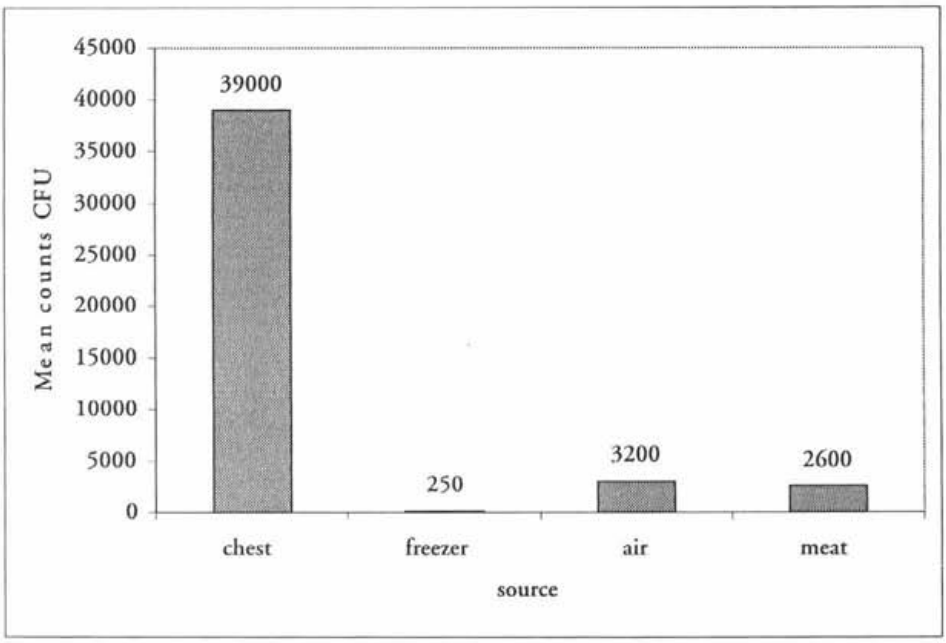

Fig. 1. Mean mould counts at different places in refrigerators and stored meat.

Table 2. Isolated mould genera from different places in refrigerators and stored meat.

Samples	Chest		Freezer		Air		Meat		Total	
	No.	F%	No.	F%	No.	F%	No.	F%	No.	F%
Mould genera										
Alternaria	3	2.5	2	1.6	4	3.3	2	1.6	11	9.0
Aspergillus	10	8.2	6	4.9	8	6.6	7	5.7	31	25.4
Botrytis	3	2.5	2	1.6	0	0.0	2	1.6	7	5.7
Cladosporium	4	3.3	5	4.1	4	3.3	3	2.5	16	13.1
Fusarium	2	1.6	1	0.8	0	0.0	0	0.0	3	2.5
Mucor	4	3.3	0	0.0	3	2.5	1	0.8	8	6.6
Paecilomyces	2	1.6	3	2.0	2	1.6	2	1.6	9	7.4
Penicillium	6	4.9	3	2.5	6	4.9	2	1.6	17	13.9
Rhizopus	0	0.0	2	1.6	2	1.6	2	1.6	6	4.9
Thamnidium	2	1.6	0	0.0	3	2.5	4	3.3	9	7.4
Trichoderma	3	2.5	1	0.8	0	0.0	1	0.8	5	4.1
Total	39	32.0	25	20.5	32	26.2	26	21.3	122	100

No. = number of cases of isolation out of 20 samples.
F% = Frequencies of occurrence in the total fungal isolates.

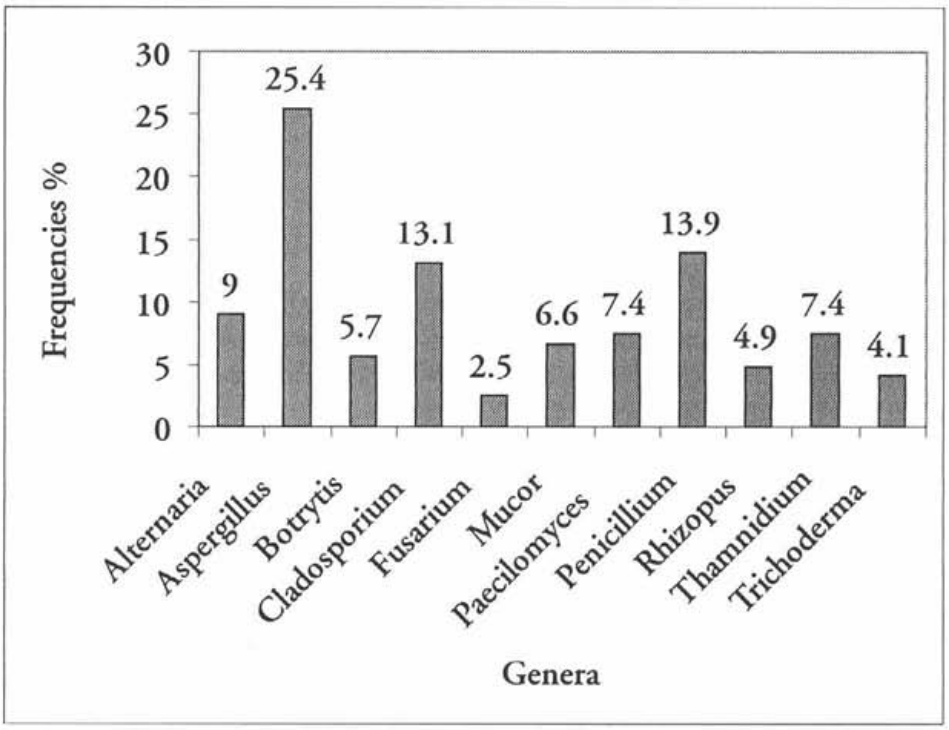

Fig. 2. Isolated mould genera from different places in refrigerators and stored meat.

RESULTS AND DISCUSSION

The obtained results in Table 1 and Fig. 1 show that the averages of total mould counts in the chest, freezer and air of the refrigerators and meat stored herein, were 3.9×10^4, 2.6×10^3, 3.2×10^4 and 2.5×10^2 CFU/cm^2, respectively. These results are more or less similar to those reported by Hamdy et al. (1993), but lower than those detected by Roushdy et al. (1996) and Saad et al. (1998).

Table 2 and Fig. 2 show that eleven mould genera were isolated from different parts of home refrigerators and meat stored herein, the main of which were *Aspergillus, Penicillium*, *Cladosporium* and *Alternaria*. The counts and relative frequencies for these genera were 31 (25.4 %), 17 (13.9 %), 16 (13.1 %) and 11 (9.0 %), respectively. *Paecilomyces, Thamnidium, Mucor*, *Botrytis, Rhizopus, Trichoderma* and *Fusarium* were isolated in lower percentages. For the genera of *Aspergillus* and *Penicillium*, similar results were tabulated by Gill and Lowry (1982) and Abdel-Rahman et al. (1985). Also, Mizakova el al. (2002) studied the occurrence of moulds in fermented raw meat products and reported

Table 3. Isolated *Aspergillus* species from different places in refrigerators and stored meat.

Sources	Chest		Freezer		Air		Meat		Total	
	No.	F%	No.	F%	No.	F%	No.	F%	No.	F%
Aspergillus spp.										
A. flavus	5	16.1	3	9.6	2	9.6	3	9.6	13	42.0
A. fumigatus	1	3.2	1	3.2	1	3.2	1	3.2	4	12.9
A. nidulans	2	6.4	1	3.2	1	3.2	1	3.2	5	16.1
A. niger	2	6.4	1	3.2	2	6.4	0	0.0	5	16.1
A. vitis	0	0.0	0	0.0	2	6.4	2	6.4	4	12.9
Total	10	32.2	6	19.4	8	25.8	7	22.6	31	100

No. = number of cases of isolation out of 20 samples.
F% = Frequencies of occurrence in the total fungal isolates.

that *Penicillium, Acremonium, Mucor, Cladosporium* and *Aspergillus* were the most frequently isolated genera of moulds. The genus *Cladosporium* responsible for black spots in chilled meat were previously detected by Gill et al. (1981) and Roushdy et al. (1996), but with higher frequency. The family *Mucoraceae,* which includes genera *Thamnidium*, *Mucor* and *Rhizopus,* was reported by Hadlok and Schipper (1974) and Refai el al. (1993). Other genera was isolated by Mislivec et al. (1970), Booth (1971) and Mansour (1986).

Table 3 and Fig. 3 indicate that *Aspergillus flavus* was the most predominant species followed by *A. niger, A. nidulans* (= *Emericella nidulans*), *A. fumigatus* and *A. amstelodami* (= *A. vitis*). These results are similar to those obtained by Mislivec et al. (1970) and Mansour (1986), but slightly different from those reported by Hamdy et al. (1993). Hamdy et al. (1993) recorded mean mould counts of surface swab samples obtained from 50 samples fresh and 25 cold stored meat collected from butcher's shops in Giza City of 9.4×10^2 and 8.9×10^3 CFU/cm^2, respectively. The isolated mould genera from the surface of cold stored meat were *Aspergillus, Alternaria, Cladosporium, Mucor, Penicillium* and *Rhizopus* with frequencies of 45.3, 1.9, 13.2, 5.7, 30.2 and 4.3 %, respectively.

Fig. 4 indicate that *Aspergillus* species (*A. flavus, A. fumigatus, A. niger, A. nidulans* and *A. amstelodami)* isolated from home-refrigerators in Qena, Egypt showed RAPD patterns identical with their corresponding type strains from IAM. These results indicate clearly that the results obtained by classical methods for identification of these strains were confirmed with the molecular method.

The results obtained in the present study indicate that moulds are capable of growing in home-refrigerators where they can tolerate lower temperatures. This contributes to various sources of mould contamination such as bad hygiene in abattoirs, meat transportation and personal hygiene in homes. The predominance

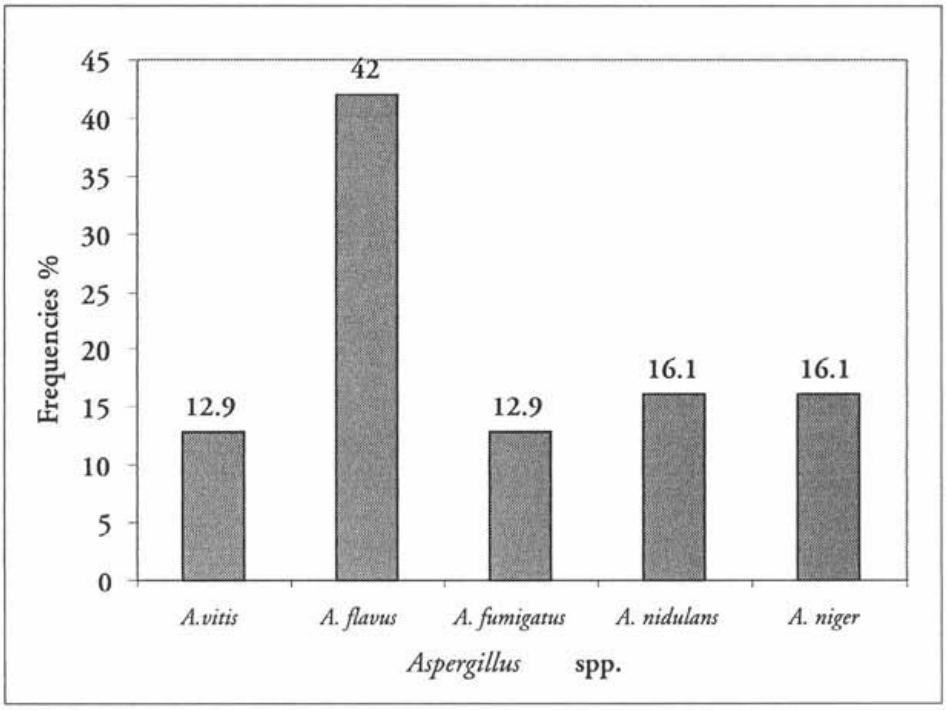

Fig. 3. Isolated *Aspergillus* species from different places in refrigerators and stored meat.

of *Aspergillus* and *Penicillium* species constitutes a real risk to human health due to their ability of mycotoxin production. Van Walbeek et al. (1969) reported that a strain of *Aspergillus flavus* produced aflatoxin at 7.5 °C and 10 °C in 4 weeks. Also, Kiermeier and Behringer (1977) noticed aflatoxin formation in moistened milk powder at temperatures between 1 and 5 °C and at 10 °C. Park and Bullerman (1983) reported that trace levels of aflatoxin (10 to 60 ppb) were detected on summer sausages produced by *Aspergillus flavus* at 5 °C. Hamdy et al. (1993) reported that different *Aspergillus* strains as well as aflatoxins B1, B2, G1 and G2 produced by *A. flavus* could be obtained in variable amounts from fresh and cold meat samples. Also, according to Ostrý (2001), mycotoxins in meat and meat products are produced at temperatures between 4 °C and 40 °C. There are several ways to minimise mould growth in refrigerators. Clean the inside of the refrigerator every month with one table spoon of backing soda dissolved in a quarter of a litre of water. Rinse with clean water, then dry. Mouldy food should be put in plastic bags for disposal in a covered trash can, so that animals and children cannot get into it. To remove mould odour, use 20 ml vinegar / l of water or 40 ml/ l chlorine bleach of water. Rinse with clean water and dry.

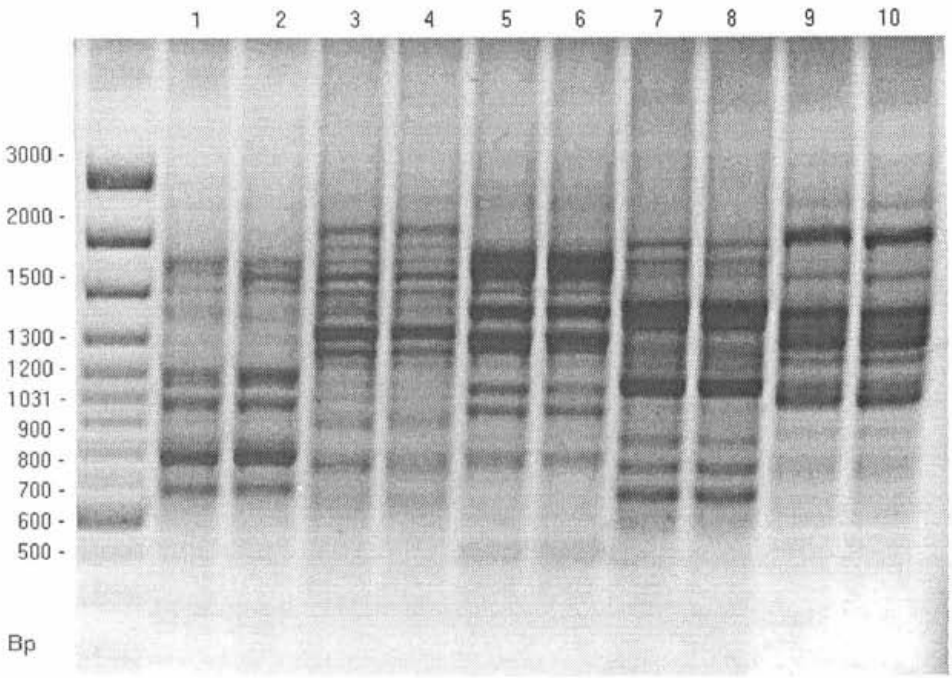

Fig. 4. Pattern of fragments from RAPD analysis of different *Aspergillus* species, printed by M13 oligonucleotide (GAGGGTGGCGGTTCT).

Lane 1 *Aspergillus flavus* from our study
Lane 2 *Aspergillus flavus* MA 86 from IAM (Institute of Applied Microbiology, Vienna, Austria)
Lane 3 *Aspergillus fumigatus* from our study
Lane 4 *Aspergillus fumigatus* MA 148 from IAM
Lane 5 *Aspergillus niger* from our study
Lane 6 *Aspergillus niger* MA 1922 from IAM
Lane 7 *Aspergillus nidulans* from our study
Lane 8 *Aspergillus nidulans* MA 337 from IAM
Lane 9 *Aspergillus vitis* from our study
Lane 10 *Aspergillus vitis* MA 1068 from IAM

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