Pectic zymogram variation and morphological identification of *Aspergillus* species

تنوع زیموگرام و شناسایی مورفولوژیکی گونه‌های آسپرژیلوس

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Abstract

One hundred and three *Aspergillus* isolates belonging to 12 species including: *A. alliaceus*, *A. candidus*, *A. carneus*, *A. flavus*, *A. fumigatus*, *A. niger* var. *niger*, *A. niger* var. *awamori*, *A. niveus*, *A. ochraceous*, *A. sydowii*, *A. terreus*, *A. ustus*, and *A. versicolor* based on morphological characters were obtained from various sources. The isolates were subjected to pectic zymogram electrophoresis. Based on the similarity of isozyme electrophoretic patterns, 45 zymogram patterns were identified. Analyses of the electrophoretic patterns revealed 26 isozyme loci corresponding to the polygalacturonase and pectin estrase. Although the examined species had a considerable intraspecific variation they were finely distinguished using pectic zymogram electrophoresis and no common zymogram pattern was observed among the species. The results imply that pectic zymogram electrophoresis may be used as a helpful approach for delimitation of *Aspergillus* species.

Keywords: Zymography, pectic enzymes, inter-specific variation

چکیده

تعداد ۱۰۳ جدایه قارچ متعلق به جنس *Aspergillus* از منابع مختلف جدا و خالص‌سازی گردید. این تعداد جدایه براساس مشخصات مورفولوژیکی و با مراجعه به کلیدهای معتبر شناسایی تحت ۱۲ گونه از جنس آسپرژیلوس شامل: *A. alliaceus*, *A. candidus*, *A. carenus* *A. flavus*, *A. fumigatus*, *A. niger* var. *niger*, *A. niger* var. *awamori*, *A. niveus*, *A. ochraceus*, *A. sydowii*, *A. terreus*, *A. ustus* و *A. versicolor* شناسایی شدند. این جدایه‌ها با استفاده از روش الکتروفورز آنزیمی پکتیک زیموگرام مورد بررسی قرار گرفتند. براساس تشابه الگوهای آنزیمی ۴۵ الگو به دست آمد. در بررسی این الگوها، تعداد ۲۶ جایگاه آنزیمی متعلق به پلی‌گالاکتورونازو پکتین استراز مشاهده شد. اگرچه تنوع درون گونه‌ای در بین گونه‌های جنس آسپرژیلوس براساس جایگاه‌های آنزیمی قابل توجه بود ولی با این حال، زیموگرافی توانست گونه‌های این جنس را از یکدیگر تفکیک کند و در بین جدایه‌های مورد مطالعه، الگوی مشترک آنزیمی بین گونه‌های مختلف دیده نشد. بنابراین، به نظر می‌رسد روش زیموگرافی از پتانسیل کافی برای تفکیک گونه‌های مختلف جنس آسپرژیلوس برخوردار است.

واژه‌های کلیدی: زیموگرافی، آنزیم‌های پکتین، تنوع بین-گونه‌ای

Introduction

The genus *Aspergillus* with 150 recognized species and many varieties has world-wide distribution. The genus with numerous species is recognized in some respects as beneficial, and in other respects as destructive (Klich & Pitt 1988). Some of the species infect agricultural crops, reducing their quality, produce mycotoxins and are toxic to animals and humans (Montesano 2001). Identification of the *Aspergillus* species is usually based on morphological characteristics, including morphology and measurements of vesicles, conidia, conidiophores, phialides and metulae (Raper & Fennell 1973). *Aspergillus* species are subjected to morphological changes due to their environmental conditions. Moreover, the proposed morphological characteristics for their discrimination are rather similar with extensive overlaps (Klich & Pitt 1988). The delimitation among the species requires controlled conditions and different standardized culture media which are time-consuming and lack high accuracy. That is why, since the beginning of its identification, numerous identification keys have been proposed, all with some ambiguity (Raper & Fennell 1973). The above mentioned factors and difficulties led researchers to exploit more accurate methods including biochemical techniques. Many fungi including *Aspergillus* species are able to produce extra cellular enzymes for the disintegration of plant cell wall pectin (Vries & Visser 2001). Some *Aspergillus* species produce pectinase and can be phytopathogen (Mullen & Jacobi 2002). The effect of different carbon sources on the pectinase production of *Aspergillus japonicus* Saito (1906) was studied by Teixeira *et al.* (2000) who stated that the presence of carbonic sources such as sucrose and glucose impede the process of pectinase enzyme production. According to Zhi-Gong *et al.* (1993) the production of pectinase enzymes by *A. flavus* is higher in the mycotoxin producing races. In spite of the extensive studies regarding the enzymes produced by *Aspergillus*, there is no report of application of this technique for studying inter and intraspecific variations. Pectic zymograms have been widely used for identification at species level of a number of fungi, *e.g. Sclerotinia* (Cruickshank 1983, Errampalli & Kohn 1995), *Gremmeniella* (Lecours *et al.* 1993), *Penicillium* (Cruickshank & Pitt 1987) and some genera of the family *Ceratobasidiaceae* (Sweetingham *et al.* 1986). Due to the fact that the majority of *Aspergillus* species have the ability to produce pectinase enzyme (Vries & Visser 2001), beside the morphology study, we also used zymogram analysis of extracellular pectic enzymes for species identification.

Materials and Methods

- Sampling and isolation of *Aspergillus* species

A total of 103 *Aspergillus* isolates were obtained from food materials, soil and different parts of diseased plants in agricultural fields of Isfahan using potato dextrose agar (PDA). Samples were transferred to PDA without surface sterilization.

- Morphological studies

Single-conidium cultures were subjected to macroscopic and microscopic studies. In order to identify *Aspergillus* species, isolates were grown on CYA, CYA20s, and MEA media. The identification keys presented by Klich & Pitt (1988) and Raper & Fennell (1973) were used to study macroscopic and microscopic features. Macroscopic features, including growth rate, colony diameter and colour of the colony were recorded. For microscopic studies, the fungi were placed on water agar for 3–10 days, depending on the species, size and ornamentation of conidia, length and width of the conidiophores, size of the vesicles, and length and width of phialides and metulae were studied under light microscope.

- Preparation of pectinase enzyme

From the margin of growing colonies, a 5 mm block was transferred to 2 ml of sterile liquid medium in Bijoax bottles containing 1% citrus pectin as a sole carbon source and the pH was adjusted at 5.5 (Sweetingham *et al.* 1986). Cultures were kept in the

dark, without shaking at 25º C for 11–17 days depending on the species. Mycelia were removed by filtration and 100 µl of each culture filtrate was mixed with 10 mg of Sephadex G-200 to make slurry and kept at room temperature for 30 min prior to electrophoresis (Sweetingham & MacNish 1994).

- Electrophoresis

The method of Cruickshank & Wade (1980) as modified by Cruickshank (1983) was used. Horizontal pectin-acrylamide gels were prepared as recommended by Sweetingham *et al*. (1986). A volume of 10 µl of each culture filtrate/sephadex slurry was loaded into the wells and 3 µl of 0.05% bromophenol blue was applied to each of the first and last wells as tracking dye. Cheese cloth was used as a wick at the cathodic and anodic ends of the gel. Power was supplied using a constant 16 mA per gel and electrophoresis was stopped when the tracking dye had migrated 5 cm toward the anodic end. An aqueous solution of boric acid (7.2 g/l) and sodium tetraborate dehydrate (15.75 g/l) was used as buffer and cold water (4–5º C) was circulated beneath the gel supporter plate during electrophoresis. The gel was rinsed briefly with distilled water and incubated for 1 h in 0.1 M malic acid at 25º C (Cruickshank & Wade 1980) before staining overnight at 5º C in 0.02% Ruthenium red to visualize the bands. Stained gels were washed with three changes of distilled water and incubated in 0.05% ammonium persulphate for 20 min at 25º C to increase the contrast for photography. The gels were rinsed briefly with distilled water and contact printed on high contrast photographic paper.

- Data analysis

The electrophoretic phenotypes were interpreted in terms of loci coding for polygalacturonase (PG, black zones in the print) and pectin esterase (PE, white zones in the print). Presence or absence of a band (allele) was determined by calculating the R*f* value, the ratio of the distance travelled by an enzyme to that travelled by the bromophenol blue. The phenotypes frequency, based on the PG and PE loci, were calculated. Genetic similarity among the electrophoretic phenotypes was calculated using the simple similarity statistic (S), $S_{xy} = 2\ N_{xy\ /}\ (N_x + N_y)$, where N_x and N_y are the number of bands in individual x and y, respectively, and N_{xy} is the number of shared bands by both (Lynch 1990). Pairwise comparisons were made between all isolates and a similarity matrix was generated. Cluster analysis was performed based on similarity values using group average analysis (UPGMA) method (Sneath & Sokal 1973). The NTSYS-pc ver. 2.2 software was used for data analysis.

Results

Using different sources, 103 *Aspergillus* isolates were obtained from food material, soil, dried medicinal, herbarium and diseased plants. According to Raper & Fennell (1973) and Klich & Pitt (1988) and using macroscopic and microscopic characters (Table 1), 12 species: *A. alliaceus*, *A. candidus*, *A. carneus*, *A. flavus*, *A. fumigates*, *A. niger* var. *awamori*, *A. niger* var. *niger*, *A. niveus*, *A. ochraceus*, *A. sydowii*, *A. terreus*, *A. ustus*, and *A. versicolor* were identified. *Aspergillus niger* (with two varieties: *awamori* and *niger*) was the predominant species followed by *A. flavus*.

- Pectic zymogram study

All 103 obtained isolates were subjected to pectic zymogram electrophoresis. Based on the similarity of isozyme patterns, 45 zymogram patterns designated as ZP1 to ZP45 were identified (Figs 1–3). The polygalacturonase isozymes (PG) and pectin esterase (PE) were observed separately or together in different species with polygalacturonase being dominant. Twenty-six loci (bands) corresponding to PG (16 bands) and PE (10 bands) were recognized based on R*f* value (Table 2). The comparison of 45 ZPs with morphology of isolates showed that ZP1, ZP2 and ZP3 corresponded to *A. candidus*, *A. carneus* and *A. alliaceus* respectively (Fig. 1) and ZP4 to ZP11 patterns to *A. flavus* (Fig. 1). ZP12 corresponded to *A. fumigatus* and ZP16, ZP17, ZP19, ZP21, ZP23 and ZP25 to *A. niger* var.

Table 1. Macroscopic and microscopic characters of *Aspergillus* species

***Aspergillus* species**	**Macroscopic characters**									**Microscopic characters**				
	CYA Medium (after 7 days)			**CY20S Medium (after 7 days)**			**MEA Medium (after 7 days)**			**Vesicle Diameter (µm)**	**Metulae (µm)**	**Phialides (µm)**	**Conidia**	
	Diam-eter (mm)	**Colour**		**Diam-eter (mm)**	**Colour**		**Diam-eter (mm)**	**Colour**					**Diameter (µm)**	**Surface**
		Above	**Reverse**		**Above**	**Reverse**		**Above**	**Reverse**					
A. alliaceus	60	Light yellow	Tan	65	Light yellow	Tan	65	Yellow, orange	Pale yellow	Biseriate 15–40	10 × 5	7 × 2	3–3.5	Smooth
A. candidus	23	Yellow	Light yellow	25	Yellow	Light yellow	20	Yellow or orange	Light yellow (Brown)	Biseriate 14–30	6 × 12	7 × 2	3–4	Smooth
A. carneus	33	Yellow	Yellow orange,brow n	35	Yellow	Yellow orange or brown	18	Yellow to yellow orange	Colourless	Biseriate 12	6 × 2	7 × 2	2.6	Smooth
A. flavus	65	Olive, olive yellow	Colourless or Brown	65	Olive, olive yellow	Colourless or brown	45	Olive or dark green	Colourless	Biseriate 25–28	8 × 5	10 × 4	3.9	Smooth
A. fumigatus	60	Dark green	Green or colourless	55	Dark green	Green or colourless	45	Grey green	White, pale yellow	Uniseriate 25	---	5 × 3	2.6	Smooth
A. niger var. *awamorii*	65	Black	Dull yellow, brown	70	Balck	Dull yellow, brown	60	Black	Dull yellow, Brown	Biseriate 35	10 × 5	8 × 3	3.9–4.2	Mostly smooth
A. niger var. *niger*	65	Black	Yellow, colourless	70	Black	Yellow, colourless	60	Black	Yellow, colourless	Biseriate 50	12 × 16	7 × 3	3.5–3.9	Mostly rough
A. niveus	25	White to pink	Yellow to browen	35	White to pink	Yellow to brown	20	Pale brown	Pale yellow	Biseriate 12	7 × 3	7 × 2	2.6	Smooth
A. ochraceus	50	White yellow	Brown	60	Wheat yellow	Brown	45	Yellow	Light brown	Biseriate 30	9 × 3	7 × 2	2.8	Smooth
A. sydowii	22	Dull (grey) turqoise or dull green	Red brown, Maroon	27	Dull (grey) turqoise or dull green	Red brown, maroon	20	Green or grey turqoise	Pale Brown	Biseriate 9.5	3.5 × 4	5 × 2.5	3	Very rough
A. terreus	45	Tan to brownish camel	Dull yellow	65	Tan to brownish camel	Dull yellow	35	Yellow to pale orange	Yellow	Biseriate 13	7 × 3	5 × 2	2.3	Smooth
A. ustus	50	Brown	Tan	30	Brown	Tan	45	Olive (gery) brown	yellow to grey	Biseriate 12	6 × 3	5 × 3	3.9	Rough
A. versicolor	25	Grey green, dull green	Red brown, Maroon	20	Grey green, dull green	Red brown, maroon	18	Light (grey) green (turqoise)	Pale yellow brown	Biseriate 13	7 × 3	5 × 2	2.6	Smooth

Table 2. The frequency and R*f* values of polygalacturonase (PG) and pectin estrase (PE) loci in zymogram patterns obtained for *Aspergillus* isolates

R*f* value	Locus	ZP1	ZP2	ZP3	ZP4	ZP5	ZP6	ZP7	ZP8	ZP9	ZP10	ZP11	ZP12	ZP13	ZP14	ZP15	ZP16	ZP17	ZP18	ZP19	ZP20	ZP21	ZP22	Band
0.14	PG																							a
0.21	PG																							b
0.25	PG			+																				c
0.3	PG	+																						d
0.32	PG						+					+	+	+										e
0.35	PG							+				+												f
0.4	PG					+		+																g
0.44	PG																						+	h
0.5	PG										+													i
0.55	PG																							j
0.6	PG				+																			k
0.65	PG																							l
0.7	PG			+		+	+	+																m
0.76	PG																							n
0.8	PG												+	+										o
0.84	PG												+											p
0.3	PE		+																					q
0.5	PE			+																				r
0.55	PE									+	+												+	s
0.6	PE															+					+			t
0.65	PE								+			+		+					+		+		+	u
0.68	PE													+		+	+			+	+	+		v
0.76	PE														+			+	+	+		+		w
0.8	PE																+	+						x
0.84	PE														+				+	+		+		y
0.9	PE																					+		z

Table 2. (countd)

R*f* value	**Locus**	**ZP23**	**ZP24**	**ZP25**	**ZP26**	**ZP27**	**ZP28**	**ZP29**	**ZP30**	**ZP31**	**ZP32**	**ZP33**	**ZP34**	**ZP35**	**ZP36**	**ZP37**	**ZP38**	**ZP39**	**ZP40**	**ZP41**	**ZP42**	**ZP43**	**ZP44**	**ZP45**	**Band**
0.14	PG																						+		A
0.21	PG													+			+	+							B
0.25	PG								+							+				+				+	C
0.3	PG	+		+	+	+				+					+						+				D
0.32	PG		+			+																			E
0.35	PG																								F
0.4	PG					+					+														G
0.44	PG						+																		H
0.5	PG					+																			I
0.55	PG			+				+																	J
0.6	PG								+																K
0.65	PG														+		+								L
0.7	PG									+						+	+	+					+		M
0.76	PG												+					+	+				+	+	N
0.8	PG					+						+	+					+	+	+				+	O
0.84	PG				+			+	+			+	+							+	+	+		+	P
0.3	PE																								Q
0.5	PE																								R
0.55	PE																								S
0.6	PE	+				+	+																		T
0.65	PE		+				+	+																	U
0.68	PE	+		+	+	+		+	+																V
0.76	PE		+	+	+		+		+																W
0.8	PE																								X
0.84	PE		+																						Y
0.9	PE																								Z

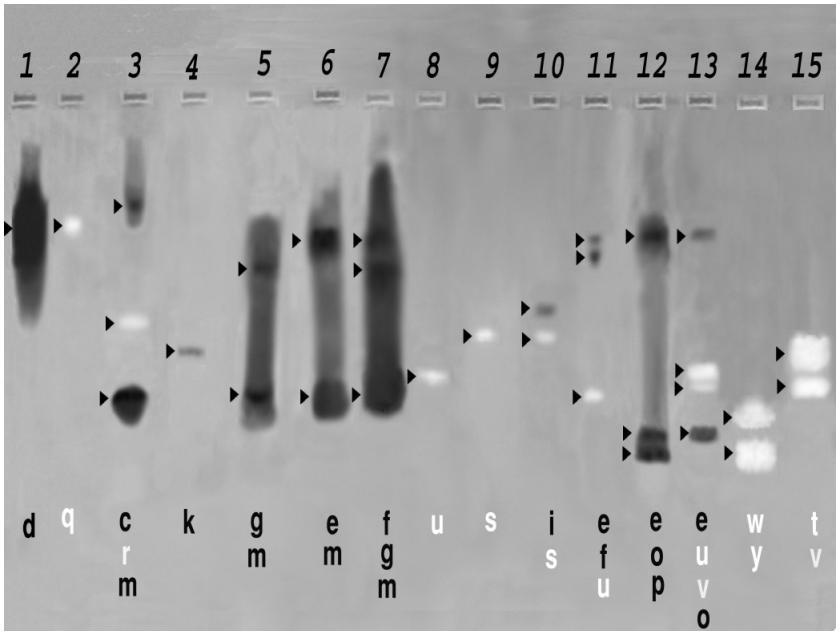

Fig. 1. Pectic zymogram patterns of the isolates of *Aspergillus* species: Lane 1. *A. candidus*, lane 2. *A. carneus*, lane 3. *A. alliaceous*, lanes 4–11. *A. flavus*, lane 12. *A. fumigates*, lane 13. *A. niger* var. *niger*, lanes 14–15. *A. niger* var. *awamori*. White and black letters stand for the pectin sterase and polygalacturonase loci, respectively.

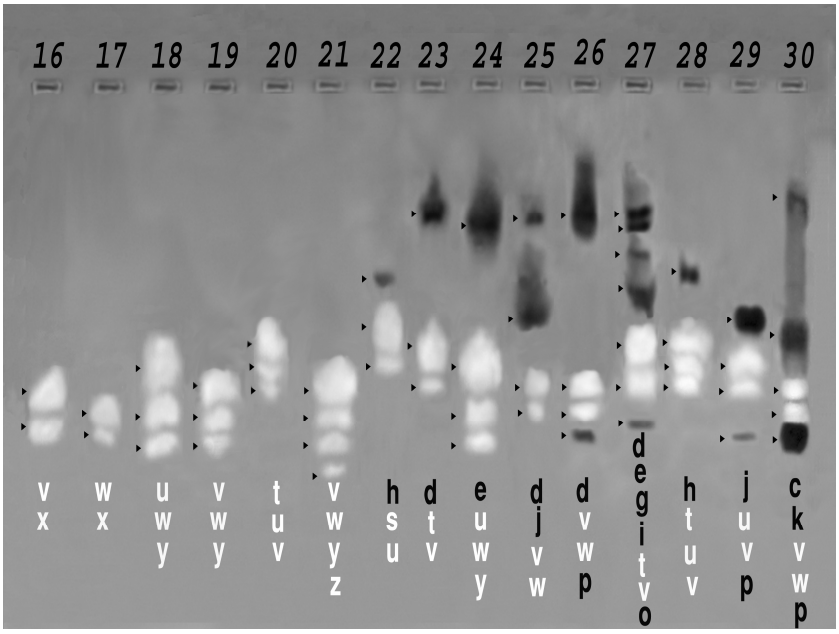

Fig. 2. Pectic zymogram patterns of the isolates of *Aspergillus* species: Lanes 16–17. *A. niger* var. *awamori*, lane 18. *A. niger* var. *niger*, lane 19. *A. niger* var. *awamori*, lane 20. *A. niger* var. *niger* and *awamori*, lane 21. *A. niger* var. *awamori*, lane 22. *A. niger* var *niger*, lane 23. *A. niger* var. *awamori*, lanes 24–30. *A. niger* var. *niger*. White and black letters stand for pectin esterase and polygalacturonase loci, respectively.

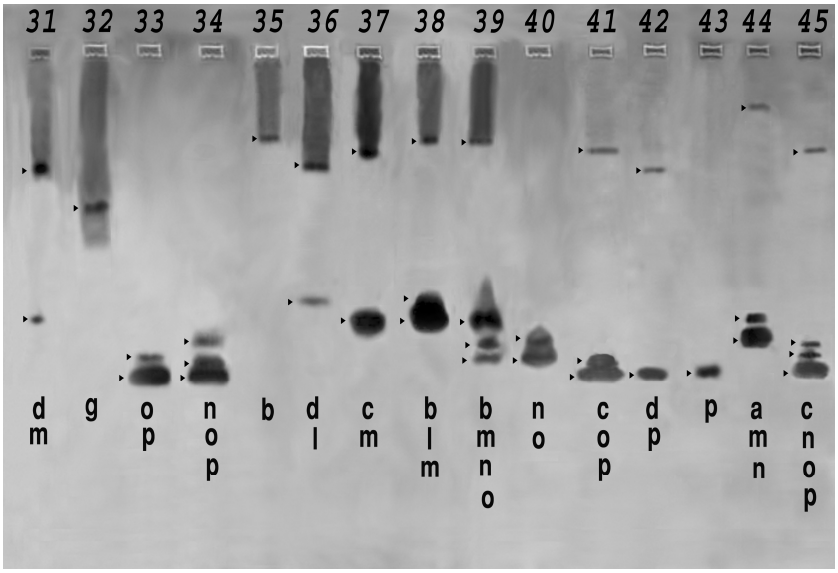

Fig. 3. Pectic zymogram patterns of the isolats of *Aspergillus* species: Lane 31. *A. niveus*, lane 32. *A. ochraceus*, lanes 33–34. *A. sydowii*, lanes 35–39. *A. terreus*, lane 40. *A. ustus*, lanes 41–45. *A. versicolor*. White and black letters stand for the pectin estrase and polygalacturonase loci, respectively.

awamori (Fig. 1); whereas ZP13, ZP18, ZP22, ZP24, ZP26, ZP27, ZP28, ZP29 and ZP30 corresponded to *A. niger* var. *niger* (Fig. 2). ZP14, ZP15 (Fig 1), and ZP20 (Fig. 2) were found in common with both of the above species. ZP31 and ZP32 belonged to *A. niveus* and *A. ochraceus*, respectively (Fig. 3). *Aspergillus sydowii* showed two patterns, ZP33 and ZP34 (Fig. 3). *Aspergillus terreus* showed five patterns of ZP35 to ZP39 (Fig. 3) and ZP40 belonged to *A. ustus* (Fig. 3). Patterns of ZP41 to ZP45 belonged to *A. versicolor* (Fig. 3).

- Cluster analysis

Cluster analysis of the similarity values assigned the 45 zymogram patterns to three primary groups which in turn were divided into a number of sub-clusters (Fig. 4). Group I was divided into two subclusters. However, different species such as *A. candidus*, *A. niveus*, *A. flavus*, *A. terreus*, *A. alliaceus*, *A. versicolor* and *A. ochraceus* clustered together but the majority of zymogram patterns in this clade belonged to *A. versicolor* and *A. terreus*. In group II, *A. fumigatus*, *A. sydowii*, *A. versicolor* and *A. ustus* clustered together, however, *A. versicolor* appeared with higher frequency. Isolates of *A. niger* including varieties *niger* and *awamori* were clustered in group III. There were only five zymogram patterns belonging to other species such as *A. flavus* and *A. carneus* (Fig. 4).

Discussion

Identification of *Aspergillus* species involves some complications from the beginning. The large number of species can be counted as one of the factors contributing to this complication. So far more than 150 species have been identified (Klich & Pitt 1988). The most important factor that complicates the identification, is the extent of morphological similarity of and their overlaps (Klich & Pitt 1988). Morphological studies of 12 species of *Aspergillus* identified in this study showed that the existing species in a given section have many macroscopic and microscopic similarities. For example, in section *Nigri*, *A. niger*, with two varieties *niger* and *awamori* which are similar with respect to microscopic characteristics except for the ornamentation on the surfaces of mature conidia. In this section, there are also other black *Aspergillus* species such as *A. foetidus* and *A. carbonarius*, with little morphological differences can be discriminated only based on one or two microscopic features. In section *Flavi*, besides *A. flavus*, there are many species close to it such as *A. parasiticus*, *A. soja* and *A. tamari*, with close affinity to *A. flavus* as Kurtzman *et al.* (1986) have suggested that these species

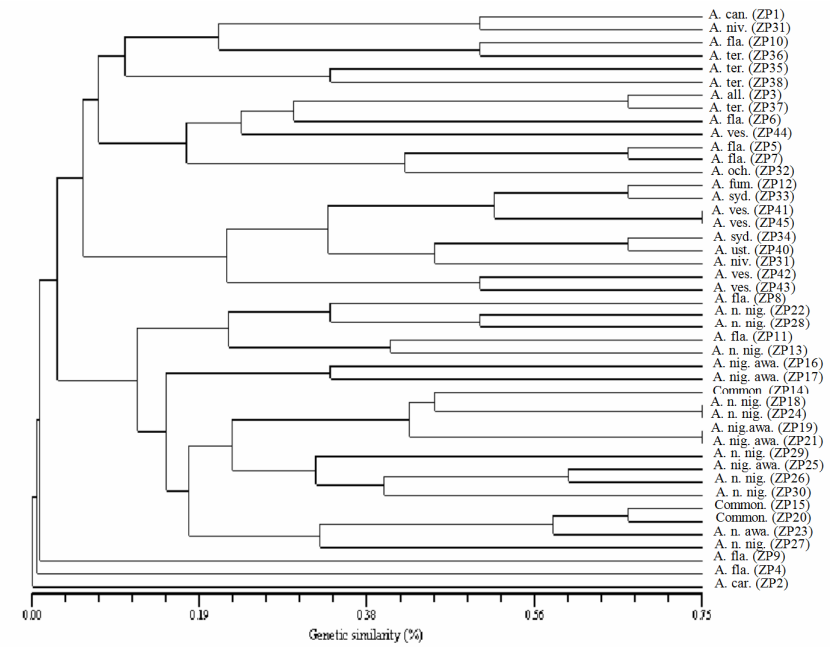

Fig. 4. Dendrogram showing relationships between zymogram patterns obtained for *Aspergillus* species, generated by group average analysis of similarity value.

species be considered as sub-species or varieties of *A. flavus*. However, further studies based on DNA restriction fragments length polymorphisms showed that they are distinct from *A. flavus* (Kurtzman *et al.* 1986). The above mentioned problems cause difficulties in morphological studies. In spite of the extensive molecular studies on *Aspergillus* species, especially on those with economical, industrial and medical importance, these studies were of less taxonomic nature and were more involved with physiological aspects of a few species (Anthony *et al.* 2003, Romero *et al.* 2003). In this study, besides morphological characteristics, a simple and rapid biochemical method was used, so that along with the interspecific variations, the intraspecific variations were also demonstrated. All examined *Aspergillus* isolates in this study had the ability of secreting pectinase enzyme. Based on this technique, obvious dissimilarity of patterns was observed among the species. Regarding the variation observed within the species, more isolates are needed to evaluate this variation more precisely. Different species with similar morphology showed completely different zymogram patterns. For example *A. carneus* and *A. niveus* which have extensive overlaps with respect to microscopic features (Klich & Pitt 1988), showed obvious differences in zymogram patterns. In *A. niveus*, two PG bands can be seen, whereas, in *A. carneus*, there was only one pectin esterase band (ZP3, Fig. 1, ZP31, Fig. 3). *Aspergillus ochraceus* (based on macroscopic features such as colony colour and growth rate) resembles *A. alliaceus*; however, they are completely different with respect to zymogram patterns. In *A. ochraceus* there was a PG band of the type 'g', whereas there was a PG band of the type 'd' in *A. alliaceus* (ZP1, Fig. 1, ZP35, Fig. 2). Another difference between these two patterns, i.e. larger and wider band in *A. alliaceus*, is probably due to the higher ability of this species in secreting polygalacturonase, compared with *A. ochraceus*, so that the growth rate of *A. alliaceus* is more than that of *A. ochraceus* in liquid culture of citrus pectin. It should be noted that in spite of the fact that samples of *A. ochraceus* species were isolated from different sources, their zymogram patterns were the same, which indicates the lack of intraspecific variations among the isolates studied. *Aspergillus terreus* showed noticeable intraspecific variations, so that six different zymogram patterns, ZP30 to ZP35 were observed for 12 isolates of this species. In all of these six patterns, there was one PG band. The variation in the zymogram patterns in these five groups was related to the

secretion of PG enzyme and no PE was observed in this species. The presence of a constant PG band in all the samples of this species points to the fact that probably the sequence related to this kind of PG is in a state of conservation and the gene responsible for its production is active in all studied isolates. In this study, no correlation was observed between the obtained zymogram patterns from *A. terreus* and the hosts from which they were isolated. In zymogram studies, differences were observed between the species *A. versicolor* and *A. sydowii*. The difference in zymogram patterns of these two species was corresponded only to the PG enzyme and no PE band was observed in any of them. Variation in the zymogram pattern was more in *A. versicolor* than *A. sydowii* and intraspecific variation was also more in *A. versicolor* than *A. sydowii*. It should be noted that in both species a PG 'p' band appeared in common. Moreover, the 'm', 'n', and 'o' PG bands were scattered in different isolates of the two species (Fig. 3). The main difference between these two species was the frequency of PG bands with different R*f*'. No correlation was observed between the zymogram patterns obtained for these two species with the hosts from which they were isolated. Another point worth noting is that there was more variation in patterns of *A. versicolor* compared with *A. sydowii*, and probably the degree of conservation of gene sequence responsible for the secretion of PG is much higher in *A. sydowii* than *A. versicolor*. According to Al-Musallam (1980), *A. niger* has two varieties: *awamori* and *niger*. These two varieties are similar based on macroscopic characteristics. Their microscopic characteristics, except for the conditions of the mature conidia are also very similar with extensive overlaps. The zymogram patterns of these two varieties revealed the presence of two consistent PE bands with a fixed distance from each other in all isolates. Probably gene sequence responsible for the production of this enzyme is highly conserved. Based on the patterns obtained in *A. niger* with the exception of three cases: ZP14, ZP15 (Fig. 1) and ZP20 (Fig. 2), which were in common between both varieties, var. *niger* with nine patterns showed higher variation than var. *awamori* with six patterns. The ability of producing PG was higher in *niger* isolates than *awamori*. The variation of the patterns of PE was higher in *A. niger* than in other species. Therefore, *A. niger* has likely been more successful than other species of *Aspergillus* regarding the secretion of pectinase isozymes and using plant tissues. Moreover, most of the pectinase studies regarding *Aspergillus* have been carried out on *A. niger* (Pashova *et al.* 1999, Angelova *et al.* 2000, Kittur *et al.* 2003). Among *Aspergillus* species used in this study, *A. flavus* ranks second to *A. niger*, regarding the variation in zymogram groups and the ability of using plant tissues and secretion of plant tissue disintegrating enzymes. This species showed the least consistency of the zymogram patterns, so that in this species, only the zymogram patterns containing PG were seen rather than those having only PE. Also the two patterns, ZP10 and ZP11 (Fig. 1) showed both isozymes of PG and PE. Langer *et al.* (2000) studied a number of *Aspergillus* species, stated that among the species, *A. flavus* has no host specificity. Probably the extreme lack of uniformity observed among isolates of this species, based on pectic zymogram method and the production of PE, is due to non-specific nature of the hosts for this species. Surve-Lyer (1995) stated that the species identification is primarily based on the morphological differences, whereas species and populations may be indistinguishable morphologically but different genetically. The biochemical and genetic methods, like isozymes could be used to study inter- and intraspecific variations. These methods could also be used to estimate the extent of variation among fungal populations isolated from different hosts. In this study, all isolates of a species, except for *A. flavus*, produced similar zymogram patterns. This similarity includes morphology, isozyme bands and R*f* values. The bands with different R*f*s in different patterns of one species indicate intraspecific variation. Similar studies were carried out on *Fusarium* by Szecsi (1990) and on *Sclerotinia* by Cruickshank (1983). The presence of specific zymogram patterns for each species shows that pectic zymogram can be considered as a rapid method for the identification of

Aspergillus species. In this method numerous isolates were prepared simultaneously and their electrophoresis patterns were obtained. Due to the fact that the extent of overlaps of zymogram patterns were minimal and easily recognizable for the species studied, it is possible to identify the species of the fungus under study through the determination of the type of the pattern and macroscopic characteristics. Cruickshank & Pitt (1987) studied species of *Penicillium* using zymogram technique. They found that identification of *Penicillium* species through morphology, growth characteristics or secondary metabolites is difficult. They concluded that this technique is a valuable tool for this purpose. MacNish *et al.* (1994) also found this method suitable for *Rhizoctonia* cultures. Based on the results obtained it seems that zymogram analysis could be employed as a practical and rapid method for distinguishing *Aspergillus* species. To increase the accuracy of the analysis of the zymogram patterns, especially in relation to intraspecific variation, other techniques such as DNA fingerprinting can be used simultaneously.

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