USE OF RAPD-PCR TO CHARACTERISE *EUROTIUM* STRAINS ISOLATED FROM DATE FRUITS

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ABSTRACT

Twenty- nine species and 15 fungal genera were collected from 30 samples of Egyptian date fruits on 50 % sucrose – Czapek's agar medium incubated at 27°C. *Eurotium* was the most frequent genus and it occurred in 83.3 % of the samples contributing 22.6 % of total fungi.

For further genotypic characterisation, the genus *Eurotium* represented by 5 species was compared with some type strains from culture collection of Institute of Applied Microbiology, Agricultural Sciences University, Austria. Random amplified polymorphic DNA (RAPD) markers were used for the estimation of genetic variability with this genus. Using three primers for the analysis, distinct fragments were obtained. The derived dendrogram clustered the strains according to the species.

INTRODUCTION

Dates are fruits of the palm, *Phoenix dactylifera* L., which has been cultivated in the Middle East and North Africa for thousands of years. For many Arab peoples the date is the staple carbohydrate food. Present world production is about 2.5 million tonnes, of which approximately one tenth enters international trade, chiefly from Iraq, Saudi Arabia, Pakistan, Tunisia and Iran (Snowdon, 1990). The percentage of date palm in Egypt represented 7% of total counts of the palm all over The World, and the total increase in the number of cultivated palm during 1990-1994 was 13.14% (Options Mediterranean, 1996). According to FAO (1998) Egypt achieved 536$ per Ton during 1990, while at 1996 it was 356 $ per Ton.

Susceptibility to physiological darkening, mould damage and mite infestation is determined by post harvest handling practices and, in particular, by the moisture content of the Dates of over 24% moisture in a warm moist atmosphere are an easy target for, microbial attack, especially yeast, but also moulds (Barreveld, 1993). Djerbi (1983) reported that the most common fungi causing fruit spoilage are the calyx-end rot by *Aspergillus niger* and the side spot decay caused by *Alternaria* sp.

Eurotium Link is a well circumscribed genus of Ascomycetes characterised by yellow or rarely white cleitothecia with glaborous, pseudoparenchymatous walls and *Aspergillus* anamorphs in which stipes bear phialides only. All species are xerophilic (Pitt, 1985: Abdel-Hafez *et al*., 1990). The genus was typified by Malloch and Cain (1972), with *E. herbariorum* Link as neotype. *Eurotium* was monographed by Basler (1975), but the taxonomy of Raper and Fennell (1965), where it is described under the name *Aspergillus glaucus* group, is still widely used. Samson (1979) has provided a very useful compilation and critique of species described since 1965.

Several phenotypic and genotypic approaches have been applied recently to clarify the taxonomic relationships within *Aspergillus* genus (Peterson, 1995; Geysers *et al*., 1998a,b; Mugnier, 1998; Sugiyama, 1998; Varga *et al*., 1999,2000a,b). Random amplified polymorphic DNA analysis has been used in phylogenetic studies of closely related species (buren *et al*., 1994 and Castagenone-Serone *et al*., 1994). RAPD-PCR techniques could be applied for genotyping isolates of *Aspergillus flavus* (Tran Dinh *et al*., 1998), *A. parasiticus* (Yuan *et al*., 1995; Carter *et al*., 1998) and *A. ochraceus* (Varga *et al*., 1999)

The objective of this investigation is to isolate osmophilic and osmotolerant fungi contaminated date fruits and characterisation of *Eurotium* strains by RAPD-PCR to show genetic variability within and between these strains.

MATERIALS AND METHODS

A- Determination of date fruit fungi

Thirty replicated samples of date fruits were collected from different localities in the City of Qena. Pieces of tissues from each sample (1 cm X 1 cm) were dipped momentarily into a 0.5 % (m/v) calcium hypochlorite solution and transferred without rinsing to 50 % Czapek's - agar plates. Four pieces were placed on the surface of the agar medium in each plate. For every sample, three plates were used. The plates were incubated at 28°C for 7 days, and the developing fungi were counted, identified (Pitt, 1979; Klick and Pitt, 1992; Domsch *et al*., 1993; Robert *et al*., 1996, Pitt and Hocking, 1997) and calculated per 360 pieces of date fruit (4 pieces from each sample X 3 plates from each sample X number of samples). The relative importance values (RIV) were calculated for each fungal species (Shearer and Webster, 1985; Ali - Shtayeh *et al*., 1988) as follows:

1 - Species frequency in a sample (A - values) = (number of pieces that yielded the species / total number of pieces transferred) * 100.

2 - Mean frequency of the species in all samples (B - value) = (A - value) of the species in all samples / total number of samples.

3 - Relative mean frequency of the species in the samples (C - values) = (B - value) of the species / (B - values) of all species isolated from the samples.

4 - Overall frequency of the species (D - values) = Number of samples from which the species were isolated / total number of samples.

5 - Relative importance value (RIV - value) = (C - value + D - value) *100.

B - Characterisation of *Erotium* strains by RAPD-PCR

B.1. Growth of *Eurotium* cultures and DNA extraction

Fungal cultures were maintained on slopes of 50% sucrose-Czapek's (Abdel-Hafez *et al*., 1990) and were subcultured onto 100 ml Erlenmeyer-flasks containing 25 ml. (per litter: 1 g K_2HPO_4; Czapek concentrate, 10 ml; yeast extract, 5 g and sucrose, 200 g) for ten days using a rotator shaker (27°C at 150 rpm) .The mycelia were collected by filtration and ground to fine powder in a liquid N2. Fifty mg of the powder transferred to 1.5 ml Eppendorf tube and mixed with 700µ / 2 x CTAB buffer .The tubes incubated at 65°C for 30 min., then 700µ of chloroform were added and the mixture vortexed briefly. The resulting mixture centrifuged at a maximum speed of 5000 rpm for 30 min. and the cleared supernatant was mixed with 600µ of isopropanol chilled to -20°C. The mixture was centrifuged at the maximum speed for 5 min and the resulting pellet washed twice with 1 ml of 70% ethanol the pellet was dried under vacuum and dissolved in 100µ TE (10 mM Tris, 1 mM EDTA, pH 7.5) buffer. The DNA concentrations were evaluated by agarose gel electrophoresis.

B.2. RAPD-amplification

PCR conditions and separation of RAPD-PCR fragments were carried out according to Messner *et al*. (1994). Using the primers of V5 (5´ dTGCCGAGCTG; Caetano – Anolles *et al*., 1992), V6 (5'dTGCAGCGTGG; Lopandic *et al*., 1996) and M13 (5' dGAGGGTGGCGGTTCT K.O'Donnell *et al*., 1999). Synthesis of primers performed by (Codon Genetical Systems, Vienna, Austria),

using a model 392 DNA synthesizer (Applied Biosystems, Foster city, CA, USA). The temperature profile of primers was subjected for denaturation at 98°C for 15 sec.; annealing at 40°C for 90 sec. and extension at 72°C for 100 sec. to a total of 40 cycles.

B.3- Analysis of RAPD profiles

RAPD profiles were scored by visually comparing RAPD amplification profiles and scoring the presence or absence of each band in each profile according to Halmschlager *et al* (1994). Basically, the formation obtained from agarose gel electrophoresis was digitalized by hand to a two - discrete - character - matrix (0 and 1 for absence and presence of RAPD - markers). For running cluster analysis, the two discrete characters of 0 and 1 had to be Guanine and Thymine in the RAPD data matrix. Complete alignment of data was performed with CLUSTALX software, then cluster analysis will be ready buy using Treecon program (van der Peer, 1994).

RESULTS AND DISCUSSION

Twenty-nine species belonging to 25 genera were collected from 30 samples of date fruit on 50 % sucrose –Czapek's agar medium incubated at 27°C (Table 1).

Eurotium was the most common genus and was recovered from 83.3 % of the samples matching 22.6% of total fungi and had RIV of 105.9. It was represented by *E. amstelodami* (50% of the samples, 6.3 % of total fungi and RIV of 56.3), *E. chevalieri* (66.7 %, 12.3 % and 78.9 %), *E. herbabriorum* (13.3%, 1.6 % and 14.9 %), *E. repens* (1.2 %, 10 % and 11.2 %) and *E. rubrum* (1.2 %, 10 % and 11.2). Nassar (1986) isolated *Aspergillus* represented by three species, *A. niger* and 2 species from *Aspergillus glaucus* group namely *A. ruber* (= *Eurotium rubrum*) and *A. amstelodami* (= *E. amstelodami*) from dates in Aswan, Egypt. From the point of view of food spoilage and loss, Abellana *et al.* (1999) reported that *Eurotium* species are probably the most destructive of all other fungi isolated from sponge cake analogue.

Aspergillus ranked second in the number of cases of isolations; occurring in 70% of the samples comprising 18.8% of total fungi and had RIV of 88.8. Of the genus 4 species were isolated of which *A. niger* was the most frequent; emerging in 56.7 % of the samples matching 11.3 % of total fungi and had RIV of 68.0. *Aspergillus flavus, A. sydowii* and *A. terreus* were less common (Table 1).

Alternaria (*A. alternata*), *Mycosphaerella* (*M. tassiana*), *Penicillium* (*P. brevicompactum, P. chrysogenum, P. digitatum, P. expansum, P. funiculosum, P. italicum* and *P. variable*) and *Rhizopus* (*R. stolonifer*) were also isolated in high frequencies of occurrence. They emerged in 50.0 – 53.3% of the samples comprising 6.3 – 10.7% of total fungi and possessed RIVs of 56.3 – 63.4.

Cladosporium (*C. cladospoioides*) and *Fusarium* (*F. avenaceum* and *F. culmorum*) were isolated in moderate frequencies of occurrence. They were isolated from 40 % and 26.7% of the samples representing 5.5% and 6.3% of total fungi and had RIVs 45.5 and 33.0, respectively. The remaining genera and species were recovered, but with different numbers and frequencies and these were *Acremonium* sp., *Charaposis thevi, Cochliobolus spicifer, Mucor racemosus, Nectria haematococca, Neosartory fischeri* and *Ulocladium chartarum* (Table 1).

Abu Zinada and Ali (1977) in KSA reported that, *Aspergillus niger*, *A. flavus*, *Rhizopus stolonifer*, *Penicillium* spp., *Fusarium* sp and *Stemphylium verruculosum* were the most common fungi associated with dates. Alavi and Sonblokar (1998) studied the mycoflora of date on PDA medium from Iran. They isolated *Penicillium* sp., *Alternaria* sp., *Cladosporium* sp., *Aspergillus* sp., *Rhizopus* sp. And predominant to all was *Fusarium* sp. Elarosi *et al*. (1983) reported that, fungi belonging to the genera *Alternaria*, *Aspergillus*, *Aureobasidium*, *Botryodiploida*, *Cladosporium, Fusarium, Nigrospora*, *Paecilomyces* and *Penicillium* were frequently isolated from date fruits showing signs of preharvest infections.

Eurotium species collected during this study were subjected for further characterisation by using RAPD-PCR techniques. Firstly, the strains of each species compared together by using three different primers to determine percentage of similarities between those strains. Then with the same way each species compared with it's type strain to confirm the identification of these strains with DNA fingerprinting method. All *Eurotium* strains showed highly similar RAPD patterns with the comparison to the correspondence type strain. The percentage of similarities between the strains of each species were 95-100 % (Fig.1). Then, comparison between representative strains from each species was carried out to examine the relationship between these strains (Fig.2). Farghaly *et al*. (2000) reported that *Aspergillus amstelodami* (= *E. amstelodami*) showed identical patterns with it 's type strain.

From the RAPD-PCR results of three different primers, dendrogram was constructed (Fig. 3). The derived dendrogram clustered

the strains according to the species and each species clustered with its type strain in distinct group. These results gave indication that each of *E. herbabriorum, E. repens* and *E. rubrum* is distinct species. Fig. 3 showed that each of *E repens* and *E. herbabriorum* clustered together in large group and this group clustered to the group of *E. rubrum.* These results indicated that those species are closely related. Blaser (1975) placed *E. rubrum* in synonymy with *E. herbariorum*. Domsch *et al*. (1980) disposed of both *E. rubrum* and *E. repens* in a similar fashion but Pitt (1985) depending on morphological features could easily be distinguished between those three species of *Eurotium*. RAPD-PCR dendrogram for *Eurotium* species fits mostly well with the dendrogram constructed from gene bank for *Eurotium* strains derived from partial sequence of 28S ribosomal RNA and using CLUSTALX and TREECON programs (Fig.4). In this study RAPD markers proved to be a reliable, fast and easy tool for the differentiation of *Eurotium* species.

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Table 1: Total isolates (TI , calculated per 360 pieces of date), number of cases of isolation (NCI out of 30 samples), occurrence remarks (OR), percentage frequency (F%) and relative importance values (RIV) of various fungal genera and species isolated from date fruits on 50 % sucrose – Czapek's agar medium incubated at 27°C.

Genera & species	TI	NCI&OR	F%	RIV
***Acremonium* sp.**	**8**	**2 R**	**6.7**	**8.3**
Alternaria alternata	**32**	**15 H**	**50**	**56.3**
Aspergillus	**95**	**21 H**	**70**	**88.8**
A. flavus	6	3 R	10	11.2
A .niger	57	17 H	56.7	68.0
A. sydowii	16	4 L	13.3	16.5
A. terreus	16	6 L	20	23.2
Charaposis thevi	**11**	**5 L**	**16.7**	**18.8**
Cladosprium cladosporioides	**28**	**12 M**	**40**	**45.5**
Cochliobolus spicifer	**6**	**3 R**	**10**	**12.4**
Eurotium	**114**	**25 H**	**83.3**	**105.9**
E. amstelodami	32	15 H	50	56.3
E. chevalieri	62	20 H	66.7	78.9
E. herbabriorum	8	4 L	13.3	14.9
E. repens	6	3 R	10	11.2
E. rubrum	6	3 R	10	11.2
Fusarium	**32**	**8 M**	**26.7**	**33.0**
F. avenaceum	14	4 L	13.3	16.1
F. culmorum	18	6 L	20	23.6
Mucor racemosus	**6**	**4 L**	**13.3**	**14.5**
Mycosphaerella tassiana	**51**	**16 H**	**53.3**	**63.4**
Nectria haematococca	**10**	**4 L**	**13.3**	**15.3**
Neosartory fischeri	**4**	**2 R**	**6.7**	**7.5**
Penicillium	**50**	**16 H**	**53.3**	**63.2**
P. brevicompactum	8	5 L	16.7	18.3
P. chrysogenum	16	10 M	33.3	36.5

Table 1 : (Cont'd)

Genera & species	TI	NCI&OR	F%	RIV
P. digitatum	4	1 R	3.3	4.1
P. expansum	8	3 R	10	11.6
P. funiculosum	6	2 R	6.7	7.9
P. italicum	4	1 R	3.3	4.1
P. variable	4	1 R	3.3	4.1
Rhizopus stolonifer	**54**	**15 H**	**50**	**60.7**
Ulocladium chartarum	**4**	**1 R**	**3.3**	**4.1**
Total isolates	505			
Number of genera	15			
Number of species	29			

Occurrence remarks: H = high occurrence, from 15-30 cases (out of 30): M = moderate occurrence, from 8-14 cases; L = low occurrence, from 4-7 cases; R = rare occurrence, from 1-3 cases.

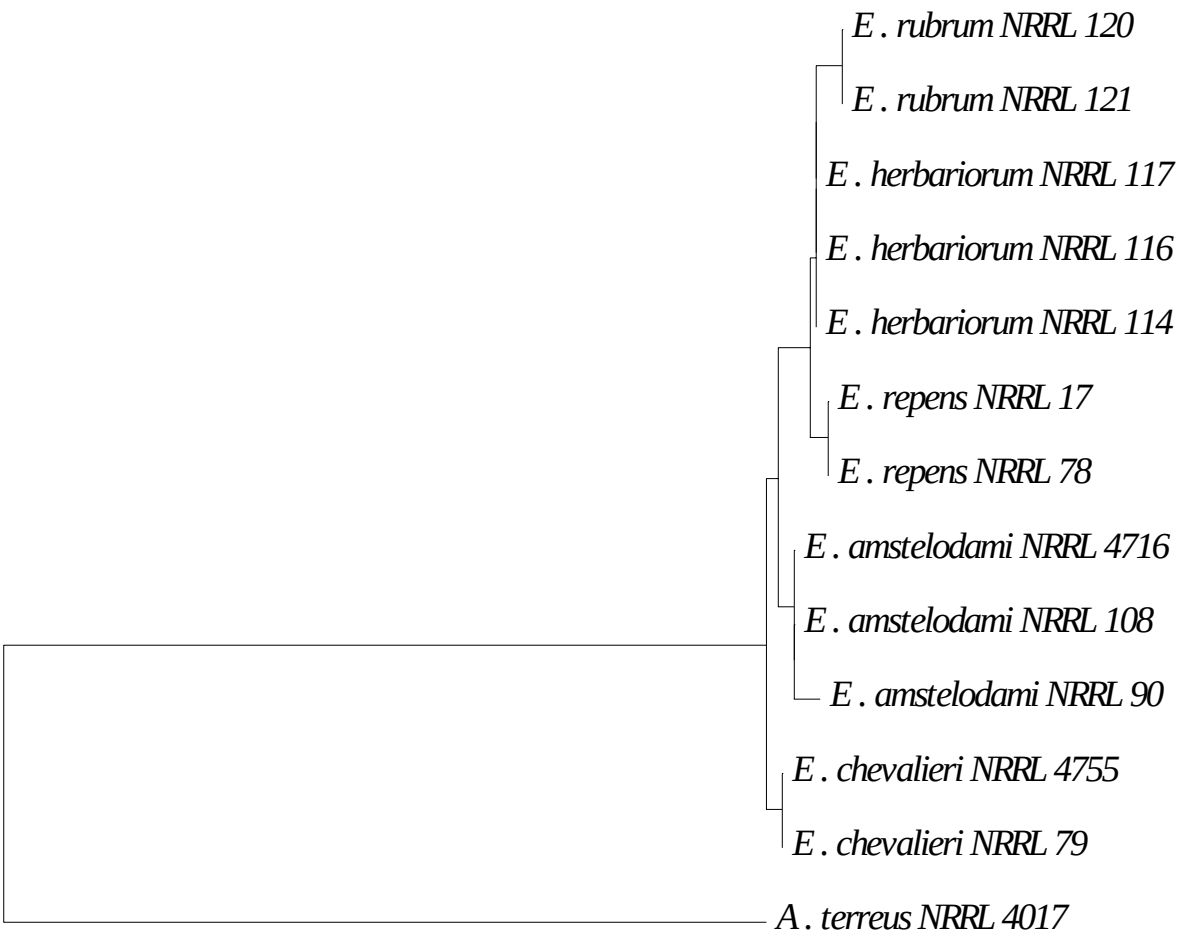

Fig.4 Dendrogram of some *Eurotium* strains (from gene bank) derived from partial sequence of 28S ribosomal RNA and using CLUSTALX and TREECON programs.

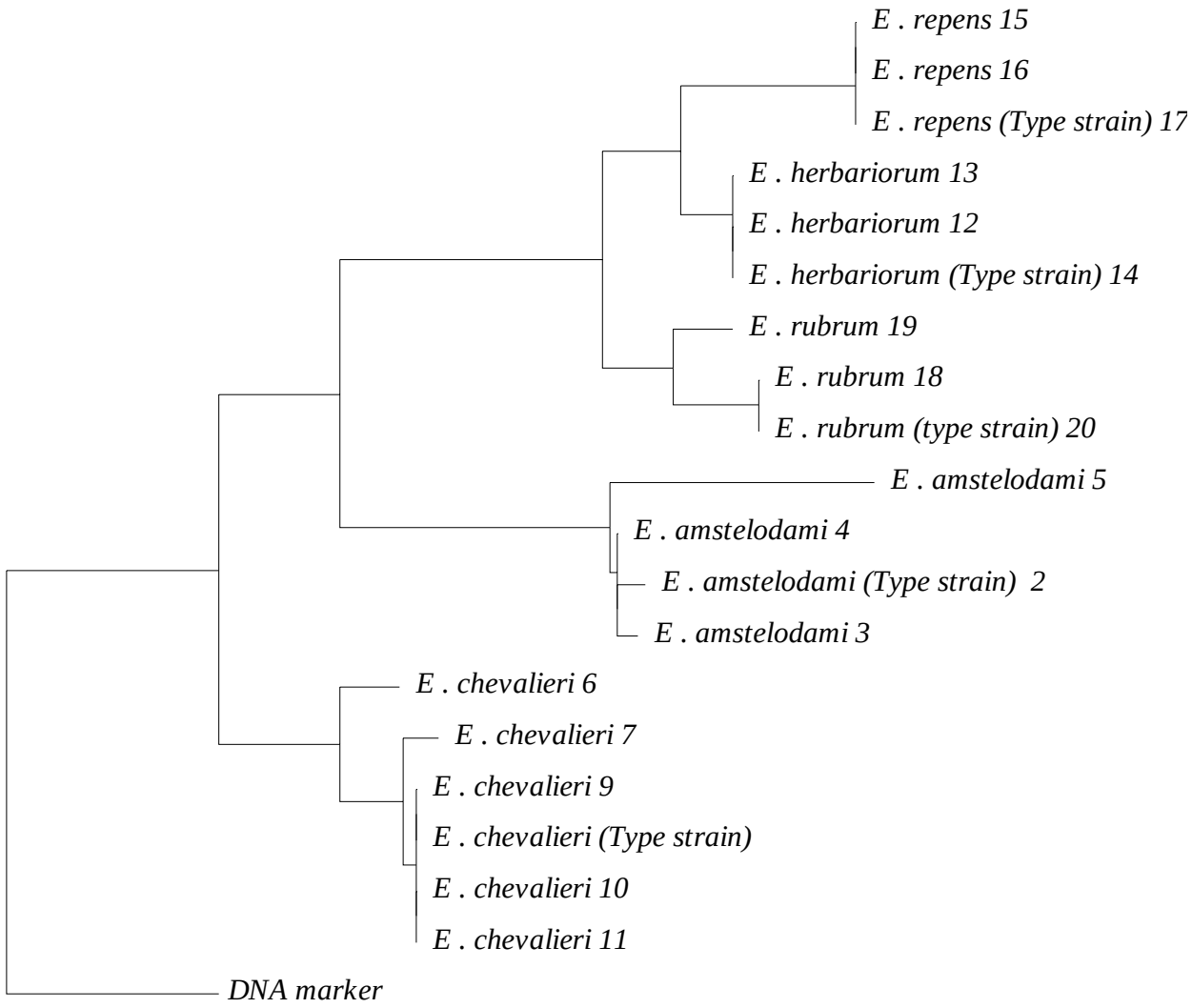

Fig.3 Dendrogram of 19 isolates of different *Eurotium* species (compared with their type strains) based on RAPD data of three different primers (Numbers shows the position of species in the gel).