Research Journal of Agriculture and Biological Sciences, 5(5): 709-718, 2009

Parameters Governing Sexuality in Two *Aspergillus* Species

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Abstract: Random amplification of polymorphic DNA (RAPD), Fatty acid and secondary metabolite profiles were performed for four *Aspergillus* isolates; two cleistothecia producing isolates (*Emercilla nidulans* and *Eurotium chevalieri*) and two non-cleistothecia producing ones (*Aspergillus nidulans* and *Aspergillus chevalieri*). Changes in fatty acid, secondary metabolite and RAPD profiles of sexual and their corresponding asexual isolates were observed. Oleic acid was of lower concentrations in asexual cultures than in sexual ones while the opposite was observed for linoleic and linolenic acids. RAPD bands of molecular weights of 559 and 790 bp were the only different ones between *A. chevalieri* and *E. chevalieri* using primer 5 while those of molecular weights of 1239 and 1757 bp using primer 3 as well as that of 1209 bp using primer 5 represented the only different bands between *A. nidulans* and *E. nidulans*. Some intra- and extracellular secondary metabolites were undetected in the imperfect isolates while were detected in the corresponding perfect ones. Deep relationship among cleistothecia formation, fatty acid and secondary metabolite biosynthesis has been shown. Results reflected the possible presence of mutation and/or repression in the non-cleistothecia producing isolates.

Key words: Fatty acids, secondary metabolites, RAPD-PCR, cleistothecia.

INTRODUCTION

Fungi are ubiquitous eukaryotes that are estimated to comprise a quarter of the entire biomass on earth and consist of nearly 1.5 million species, with only 5% identified thus far [18]. They are the primary degraders of cellulose and lignin and devastating pathogens of plants and animals. Their success is attributed to their multilateral reproductive strategies, which are uniquely represented by the development of specialized reproductive cells, the meiospore and mitospore. These two spores provide the sexual and asexual modes of fungal reproduction that occur in distinct reproductive organs [2]. The genus *Aspergillus* comprises a diverse group of species with many members capable of producing only mitospores, a few that produce only meiospores, and several that can produce both spores. The homothallic genetic model *Aspergillus nidulans* is a classic example of the latter, producing both meiospores (e.g., ascospores) and mitospores (e.g., conidia) [26,4].

Ascospore formation in *A. nidulans* requires the GATA-type transcription factor NsdD, necessary for cleistothecia (sexual fruiting bodies bearing the ascospores) and Hülle cell production. Conidia formation in *A. nidulans* requires the function of BrlA, a zinc finger transcription factor essential for conidiophore development. Deletion of either gene blocks formation of the respective meiotic or mitotic fruiting body, resulting in a strict asexual morph (Δ*nsdD*) or a strict sexual morph (Δ*brlA*). In contrast to NsdD and BrlA, which are solely involved in the regulation of the sexual or the asexual cycle, respectively, physiological studies of Champe and el-Zayat [10] led to the identification of secreted lipogenic signal molecules, collectively named “psi factor” (for precocious sexual inducer), that govern the timing and balance of meiotic to mitotic spore development. Biochemical analysis showed that *A. nidulans* psi factor is an endogenous mixture of hormone-like oxylipins composed of hydroxylated oleic (18:1), linoleic (18:2), and linolenic (18:3) acid molecules termed psiβ, psiα, and psig, respectively. The position and number of hydroxylations of the fatty acid backbone further identifies the psi compounds as psiB, psiC, and psiA [7,9,32].

It has been noted since the earliest days of fungal manipulation that many species of filamentous fungi readily synthesize complex compounds named secondary metabolites that are putatively helpful but not necessary for survival and whose production is presumably costly to maintain. Furthermore, production is often linked to fungal development. Some compounds might function as virulence factors, or their presence could give a competitive edge to the producing organism or enhance the survivability of spores. Some secondary metabolites stimulate sporulation and therefore influence the development of

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the producing organism and neighboring members of the same species, perhaps enhancing the fitness of a community of related species. Natural products are often produced late in fungal development, and their biosynthesis is complex. This complexity is due to a number of factors that affect secondary metabolite production. These include (i) the influence of a number of external and internal factors on natural product biosynthesis, (ii) the involvement of many sequential enzymatic reactions required for converting primary building blocks into natural products, (iii) tight regulation of natural product enzymatic gene expression by one or more transcriptional activators, (iv) close association of natural product biosynthesis with primary metabolism, and (v) close association of natural products with later stages of fungal development, particularly sporulation. Furthermore, the genes required for biosynthesis of some natural products are clustered, perhaps as a consequence of these factors. Gene clusters contain all or most of the genes required for natural product biosynthesis, and logic suggests that their maintenance could only be selected for if the final natural product conferred some advantage to the producing organism. Moreover, natural product biosynthetic gene clusters can be conserved between organisms, for example the sterigmatocystin-aflatoxin biosynthetic gene cluster in several *Aspergillus* spp. [28,6].

Lipids have been shown to regulate virulence and development, including both spore and secondary metabolite production in fungi [5]. Shared intracellular signaling pathways for sporulation and secondary metabolite production suggest a common trigger(s) for both these processes. Lipid signals also affect secondary metabolites and sporulation [5]. Among lipid signals, of particular note are the previously mentioned oxylipins.

Brodhagen and Keller [5] additionally reported that the processes of sporulation and secondary metabolite production have been demonstrated to share common regulatory elements, for instance, in *Colletotrichum lagenarium*, deletion of a mitogen-activated kinase (MAPK) gene lowers both production of conidia and expression of melanin genes [30].

A. nidulans generates both sexual (ascospores) and asexual (conidia) spores; ascospores can arise from either homothallic or heterothallic crosses. From studies of *A. nidulans* strains deleted of genes (ppoA, ppoB and ppoC) encoding oxylipin-generating dioxygenases [32,33,34], it is inferred that oxylipins generated by Ppo gene products regulate the expression of transcription factors required for meiotic (NsdD)[16] and mitotic (BrlA) [17] sporulation processes. In fact, there is evidence that oxylipins are physically associated with spores: PpoA, which catalyses oxylipin synthesis, is localized to lipid bodies in sexual and asexual fruiting structures [33]. Oxylipins are physically associated with reproductive structures in other fungi as well, suggesting a conserved role in development [29,19].

In the current study, fatty acid and secondary metabolite profiles as well as RAPD patterns of two *Aspergillus* cultures uncapable of producing sexual spores (*Aspergillus nidulans* and *Aspergillus chevalieri*) and two other perfect ones (*Emercilla nidulans* and *Eurotium chevalieri*) were investigated in an attempt to figure out reasons for cleistothecia unproductivity by the imperfect cultures taking into account these three interconnected parameters.

MATERIALS AND METHODS

All experimental work except for fatty acid analysis was carried out at the Regional Center for Mycology and Biotechnology, Al-Azhar Univ., Cairo, Egypt.

***-Aspergillus* isolates:** *Aspergillus chevalieri*, *Eurotium chevalieri*, *Aspergillus nidulans* and *Emercilla nidulans* were obtained from the culture collection of The Regional Center for Mycology and Biotechnology.

-DNA - Based Techniques:

a- Fungal DNA Extraction Using Qiagen kit: The mycelial growth from 5–7 day old cultures on Malt Extract Agar (MEA) slopes were scraped by using 2 ml of sterile distilled water. The two ml of spore suspension were used to inoculate a 100 ml of Yeast Extract Sucrose (YES) medium in a universal 250ml flask and incubated with gentle shaking (180 rpm at 22˚C for 48hrs). The mycelia from the flasks were harvested by filtration under aseptic conditions using a microcloth, washed with sterile distilled water and stored at -20 overnight in a sterile Petri dishes. The mycelia were lyophilized in a Heto lyophilizer system model Maxi Dry Plus. The freeze-dried mycelia were ground in a mortar using a sterile pestle, and the powdery samples were placed in eppendorf tubes (1.5 ml). DNA extraction was conducted using DNeasy kit (Qiagen-Germany).

b- RAPD-PCR: Ready to go PCR beads kit (purchased from Amershambioscience) was used to amplify DNA genomic fragments using a thermal cycler machine (gradient Robocycler 96 Stratagene, USA) by combining the lyophilized bead, 25 pmole of each primers, 100 ng DNA as a template in 25ml of total reaction volume. The mixture was then placed to the thermal cycler machine directly to start the appropriate PCR program including a universal denaturation cycle (5 min at 94°C), 45 cycles of annealing/extension

reactions (1 min at 94°C, 1 min at an optimum annealing temperature 36°C for each used universal primer and 2 min at 72°C) and cycle of final extension step (5 min at 72°C) was followed by soaking at 4°C.

The primers used in this study were supplied with the Ready to go kit and are of the following sequences: Primer3: 5'-d {GTAGACCCGT}; Primer5: 5'-d {AACGCGCAAC}; Primer6: 5'-d {CCCGTCAGCA}

e- Agarose Gel Electrophoresis: The desired amount of agarose 2% was added to 100 ml (1X) of electrophoresis buffer (10X TBE, tris-base 108g/l; boric acid, 55g/l; 40 ml of 0.5M EDTA (pH8)). The gel was boiled and ethidium bromide solution (10 mg/ml) was added at 55°C, then poured into sealed gel tray and the appropriate comb was inserted. All the molecular biology techniques were carried out according to Sambrook *et al*. [25].

Secondary Metabolite Analyses: 7-day old fungal isolates were grown on YES medium for the determination of extracellular secondary metabolites while CYA medium was used for the determination of intracellular secondary metabolites using agar plug technique of Paterson and Bridge [20]. Extraction, analysis and identification of intracellular and extracellular metabolites were carried out according Paterson and Bridge [20], using the automatic HPTLC system (CAMAG, model scanner 3- Switzerland).

Fatty Acid Analysis: Intracellular fatty acids were extracted according Peter and Michael [22]. Gas chromatography analysis was achieved using Dani GL\C-FID 1000 at the Central Laboratory of Ain Shams University. The fatty acid standard was manufactured by Supelco [tm] containing mixture of 37 fatty acid methyl esters (C_4-C_{24}).

RESULTS AND DISCUSSION

Fatty Acid Profiles: Sixteen fatty acids (eleven saturated and five unsaturated) were detected in the cell free extract of *A. chevalieri*, *A. nidulans* and their corresponding isolates capable of cleistothecia production; *E. chevalieri* and *E. nidulans*, respectively (Table I).

A. chevalieri represented the isolate with the maximum number of fatty acids (thirteen out of the sixteen detected); only oleic, tridecanoic and stearic acids were undetected, followed by *E. chevalieri* (twelve detected fatty acids) and then *E. nidulans* and *A. nidulans* (each possessing ten fatty acids) (Table I).

The detected unsaturated fatty acids were palmitoleic, oleic, elaidic, linoleic and linolenic. Palmitoleic was only detected in the cultures producing asexual conidia (*A. chevalieri*, 3.7 µg/ml and *A. nidulans*, 6.2 µg/ml) while oleic acid was only detected in the cultures producing sexual cleistothecia (*E. chevalieri*, 0.9 µg/ml and *E. nidulans*, 3.73 µg/ml). Linoleic acid was detected in the four investigated isolates with lower concentrations in the sexual cultures (*E. chevalieri*, 0.1 µg/ml and *E. nidulans*, 0.2 µg/ml) than in the asexual ones (*A. chevalieri*, 1.9 µg/ml and *A. nidulans*, 2.3 µg/ml). Elaidic acid was also present in the four investigated cultures with the highest concentration being detected in *E. chevalieri* (22.9 µg/ml) while with much lower concentrations in the rest of the investigated isolates (*A. chevalieri*, 1µg/ml; *A. nidulans*, 3 µg/ml; *E. nidulans*, 0.5 µg/ml).

Intracellular Secondary Metabolite Profiles: Eight intracellular secondary metabolites were detected with *E. chevalieri* being the richest in secondary metabolites for detecting seven of the eight secondary metabolites in its cell free extract (xanthocillin, ochratoxin A, carlosic acid, 2-carboxy-3,5-dihydroxyphenylacetylcarbinol, schizopaltic acid, genestic acid and psoromic acid). While, *A. nidulans* represented the poorest isolate for possessing only three metabolites (ochratoxin A, carlosic acid and 2-carboxy-3,5-dihydroxyphenylacetylcarbinol). *E. nidulans* exceeded its anamorph by Viridicatum toxin. While, *E. chevalieri* exceeded its anamorph by schizopaltic, genestic and psoromic acids (Table II).

Extracellular Secondary Metabolite Profiles: Six extracellular secondary metabolites were detected in the culture filtrate of the investigated aspergilla (Table III). *E. chevalieri* was the richest for possessing five of the six detected metabolites (α-collatolic acid, chaetoglobosin A, chaetochromin A, 2-pyruvoylaminobenzamide and rosepurpurine) followed by *E. nidulans* (α-collatolic acid, chaetoglobosin A and 2-pyruvoylaminobenzamide). Both Asexual cultures produced poor extracellular secondary metabolites (2-pyruvoylaminobenzamide and rosepurpurine for *A. chevalieri* while alectronic and α-collatolic acids for *A. nidulans*).

RAPD-PCR: Three primers (primers 3, 5 and 6) were used to evaluate the genomic profile of each of *A. chevalieri* and its perfect culture (*Eurotium chevalieri*) as well as *A. nidulans* and *Emercilla nidulans*.

Exactly the same RAPD pattern was obtained for both *A. chevalieri* and *E. chevalieri* using primer 3 (Fig.1 and Table IV). For primer 5, only two bands (559 bp and 790 bp) were different; the band of molecular weight of 559 bp was present in *A. chevalieri* while absent in *E. chevalieri*, however the

opposite was observed for the band of molecular weight of 790 bp (Fig. 2 and Table V). Primer 6, like primer 3, resulted in exactly the same RAPD pattern (Fig. 3 and Table VI).

In case of *A. nidulans* and *E. nidulans*, primer 3 resulted in differences in two bands of molecular weights of 1239 bp (present in *A. nidulans* while absent in *E. nidulans*) and 1757 bp (present in *E. nidulans* while absent in *A. nidulans*) (Fig. 4 and Table VII). Amplification of their DNA using primer 5 resulted in differences in only one band of molecular weight of 1209 bp which was present in *A. nidulans* while absent in *E. nidulans* (Fig. 5 and Table VIII). Exactly the same RAPD pattern was developed using primer six (Fig. 6 and Table IX).

Discussion: The genus *Aspergillus* includes fungi of importance in the food and biotechnology industries, as well as pathogens. It would therefore be of major economic and medical advantage to be able to study their biochemistry as well as the inheritance of genes of interest and to bring together desirable genetic traits in the aspergilli. Unfortunately, such genetic efforts have been impeded because most *Aspergillus* species are only known to reproduce asexually, thus the sexual cycle cannot be used for strain improvement and inheritance studies. However, the 'genomics revolution' is now beginning to reveal the sexual secrets of *Aspergillus*, thereby offering the prospect of understanding reasons for sexuality and asexuality, and the basis of homothallic (selfing) or heterothallic (obligate outcrossing) modes of sexual reproduction in this group of fungi [23].

The current work studies biochemical and genomic differences between an asexual culture of *A. nidulans* and another capable of reproducing sexually (*E. nidulans*) as well as between *A. chevalieri* (asexual culture) and *E. chevalieri* (sexual culture) in an attempt to investigate genomic and metabolic differences between the sexual and the interestingly asexual culture through studying the three interconnected chosen parameters; fatty acids, intracellular and extracellular secondary metabolites representing the biochemical investigations, as well as RAPD analysis using three universal primers (primer 3, 5 and 6) representing the molecular investigations.

In the current study, oleic acid was undetected in the imperfect cultures (*A. nidulans* and *A. chevalieri*) while was of concentrations of 0.9 ppm in *E. chevalieri* and 3.7 ppm in *E. nidulans* suggesting its increased requirement in conidia formation (asexual reproduction). While, in case of linoleic acid, it was of lower concentrations in perfect cultures (0.1 ppm for *E. chevalieri* and 0.2 ppm for *E. nidulans*) than in imperfect ones (1.9 ppm for *A. chevalieri* and 1.1 ppm for *A. nidulans*) suggesting its increased demand in sexual spore production. The latter was also observed for linolenic acid which was undetected in the perfect cultures while was of concentrations of 3.05 and 1.2 ppm in *A. chevalieri* and *A. nidulans* respectively.

This agrees with the results of Tsitsigiannis *et al.* [32, 34] who reported that in *A. nidulans*, mutations in *ppoA* (encoding the dioxygenase PpoA contributing to the generation of 8-hydroxy linoleic acid, psiBα or 8-HODE) enhanced the ratio of asexual to sexual spore production, reflecting the role of linoleic acid in sexual spore production. However, Δ*ppoC* (encoding the dioxygenase PpoC necessary for optimal production of 8-hydroxy oleic acid, psiBβ or 8-HOE) mutant exhibited an increase in sexual spore production reflecting the role of oleic acid in asexual reproduction. These effects on sporulation are reflected in expression levels of the sporulation-specific transcriptional regulatory genes, *brlA* and *nsdD*.

It has been reported that *odeA* deletion (encoding a Δ-12 desaturase that converts oleic acid to linoleic acid) resulted in a strain depleted of polyunsaturated fatty acids (18:2 and 18:3) but increased oleic acid (18:1) and total percent fatty acid content. Linoleic acid-derived psi factors were absent in this strain but oleic acid-derived psi factors were increased relative to wild type**,** also a 2–3-fold increase in total fatty acids/weight of fungal biomass was observed. Moreover, the chemical makeup of the fatty acid profile was altered in these strains: palmitic acid content was decreased and both stearic and oleic acid content increased compared with wild type strains [7]. The increase in stearic and decrease in palmitic acids agree with the current results where in cases with increased oleic acid (0.9 and 3.37 ppm for *E. chevalieri* and *E. nidulans* respectively) production there were also increased stearic acid (3 and 9.69 ppm for *E. chevalieri* and *E. nidulans* respectively) but decreased palmitic acid production (0.5 and 0.2 ppm for *E. chevalieri* and *E. nidulans* respectively). It could also be observed that the fatty acid profile of the asexual culture of *A. nidulans* and *A. chevalieri* was different than that of their corresponding sexual ones (*E. nidulans* and *E. chevalieri*, respectively).

The ability or inability of the investigated fungal cultures to produce cleistothecia (which also resulted in different fatty acid profiles and concentrations) resulted in different intracellular and extracellular secondary metabolite profiles, where certain metabolites were detected in case of cleistothecia production which were ceased when the culture lost its ability to produce cleistothecia. It has been reported that reproduction in fungi is accompanied by developmental changes among which are changes in secondary metabolite profiles [5]. Fungal secondary metabolism and sporulation are associated both temporally and functionally [1,8].

Table I: Concentration of fatty acids (µg/ml) detected in the cell free extract of sexual and asexual states of the investigated aspergilla.

Fungal isolate Fatty acid	*Aspergillus chevalieri*	*Eurotium chevalieri*	*Aspergillus nidulans*	*Emercilla nidulans*
Butyric acid (4:0)	0.5	0.65	0.7	1.76
Caproic acid (6:0)	2.63	0.6	0.84	1.2
Caprylic acid (8:0)	0.7	1.2	0.5	0.8
Capric acid (10:0)	1.0	0.7		0.6
Lauric acid (12:0)	3.5	1.2		4
Tridecanoic acid (13:0)		9.28		
Pentadecanoic acid (15:0)	0.7			
Palmitic acid (16:0)	1.5	0.5	8.5	0.2
Margaric acid (17:0)	10.23	0.3	5.4	
Stearic acid (18:0)		3	1.9	9.69
Arachidic acid (20:0)	0.5			
Palmitoleic acid (16:1)	3.7		6.2	
Oleic acid (18:1 Δ^9 cis)		0.9		3.73
Elaidic acid(18: 1 Δ^9 trans)	1.0	22.9	3	0.5
Linoleic acid (18:2)	1.9	0.1	2.3	0.2
Linolenic acid (18:3)	3.05		1.2	

Table II: Intracellular secondary metabolites of sexual and asexual states of the investigated aspergilla.

Fungal isolate Secondary metabolite	*A. chevalieri*	*E. chevalieri*	*A. nidulans*	*E. nidulans*
Xanthocillin	0	0	-	-
Ochratoxin A	0	0	0	0
Carlosic acid	0	0	0	0
2-carboxy-3,5-dihydroxyphenylacetylcarbinol	0	0	0	0
Schizopaltic acid	-	0	-	-
Genestic acid	-	0	-	-
Psoromic acid	-	0	-	-
Viridicatum toxin	-	-	-	0

Table III: Extracellular secondary metabolites of sexual and asexual states of the investigated aspergilla.

Fungal isolate Secondary metabolite	*A. chevalieri*	*E. chevalieri*	*A. nidulans*	*E. nidulans*
Alectronic acid	-	-	0	-
α-collatolic acid	-	0	0	0
Chaetoglobosin A	-	0	-	0
Chaetochromin A	-	0	-	-
2-pyruvoylaminobenzamide	0	0	-	0
Rosepurpurine	0	0	-	-

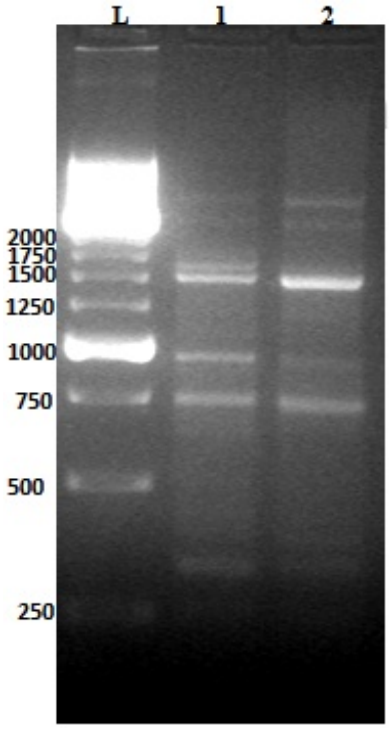

Fig. 1: RAPD patterns of *A. chevalieri* (1) and *E. chevalieri* (2) using primer 3. L, 250 bp ladder.

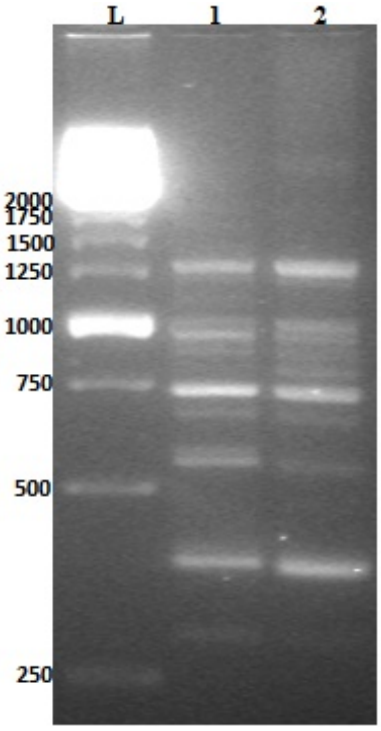

Fig. 2: RAPD patterns of *A. chevalieri* (1) and *E. chevalieri* (2) using primer 5. L, 250bp ladder.

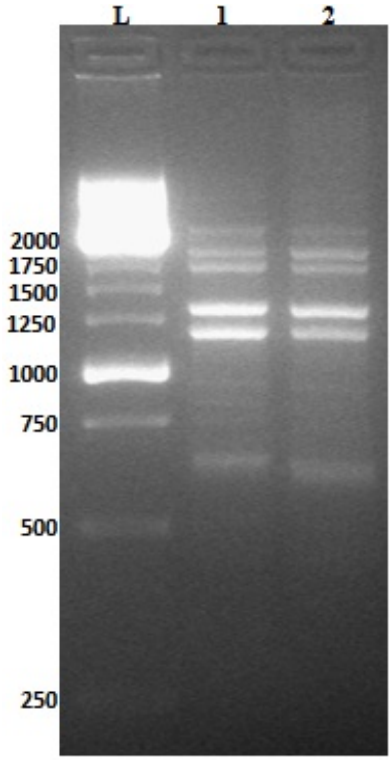

Fig. 3: RAPD patterns of *A. chevalieri* (1) and *E. chevalieri* (2) using primer 6. L, 250bp ladder.

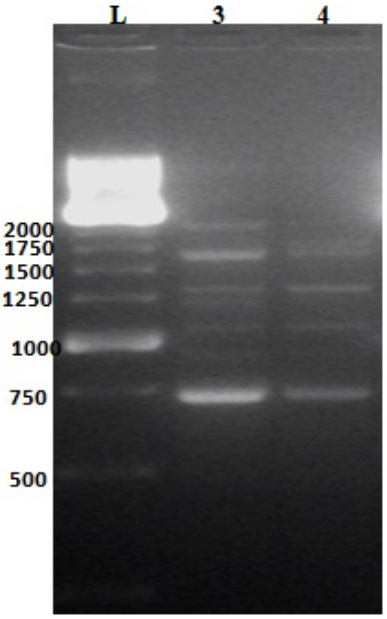

Fig. 4: RAPD patterns of *A. nidulans* (1) and *E. nidulans* (2) using primer 3. L, 250bp ladder.

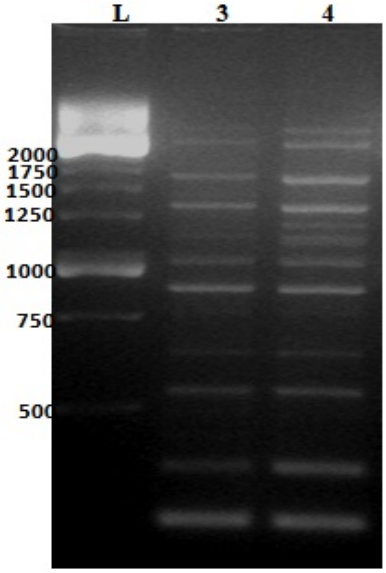

Fig. 5: RAPD patterns of *A. nidulans* (1) and *E. nidulans* using primer 5. L, 250bp ladder.

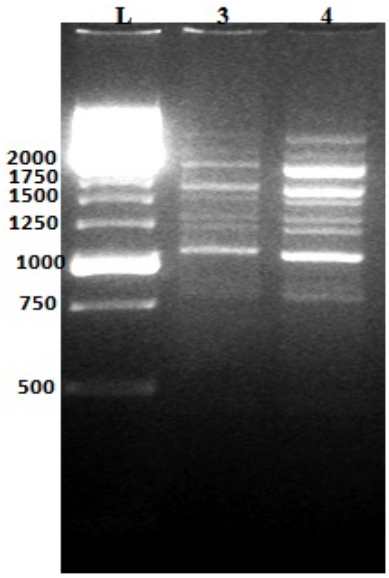

Fig. 6: RAPD patterns of *A. nidulans* (1) and *E. nidulans* (2) using primer 6. L, 250bp ladder.

Table IV: Molecular weights of fragments generated using primer 3 for *A. chevalieri* and *E. chevalieri*. +, band present; -, band absent.

Fungal isolate Band (bp)	*A. chevalieri*	*E. chevalieri*
300	+	+
713	+	+
1100	+	+
1500	+	+
1598	+	+
2019	+	+
2378	+	+

Table V: Molecular weights of fragments generated using primer 5 *A. chevalieri* and *E. chevalieri*. +, band present; -, band absent.

Fungal isolate Band (bp)	*A. chevalieri*	*E. chevalieri*
294	+	+
380	+	+
402	+	+
550	+	+
559	+	-
721	+	+
750	+	+
790	-	+
883	+	+
970	+	+
1014	+	+
1250	+	+
1280	+	+

Table VI: Molecular weights of fragments generated using primer 6 for *A. chevalieri* and *E. chevalieri*. +, band present; -, band absent.

Fungal isolate Band (bp)	*A. chevalieri*	*E. chevalieri*
630	+	+
1200	+	+
1379	+	+
1759	+	+
1890	+	+
2141	+	+

Table VII: Molecular weights of fragments generated using primer 3 for *A. nidulans* and *E. nidulans*. +, band present; -, band absent.

Fungal isolate Band (bp)	*A. nidulans*	*E. nidulans*
720	+	+
900	+	+
1087	+	+
1239	+	-
1300	+	+
1666	+	+
1757	-	+
2080	+	+

Table VIII: Molecular weights of fragments generated using primer 5 for *A. nidulans* and *E. nidulans*. +, band present; -, band absent.

Fungal isolate Band (bp)	*A. nidulans*	*E. nidulans*
200	+	+
300	+	+
382	+	+
515	+	+
670	+	+
945	+	+
1100	+	+
1209	-	+
1380	+	+
1740	+	+
2476	+	+
2750	+	+

Table IX: Molecular weights of fragments generated using primer 6 for *A. nidulans* and *E. nidulans*. +, band present; -, band absent.

Fungal isolate Band (bp)	*A. nidulans*	*E. nidulans*760
760	+	+
800	+	+
1040	+	+
1250	+	+
1306	+	+
1500	+	+
1740	+	+
2137	+	+
2224	+	+
2495	+	+

One remarkable property of secondary metabolites produced by fungi is that the genes involved in their biosynthesis and regulation are frequently clustered [38]. The significance of biosynthetic gene clusters in filamentous fungi has long been debated. One hypothesis is that they represent an extended form of selfish genes, facilitating simultaneous mobilization of a discrete biosynthetic function for horizontal transfer [35]. Another rationalization is that clustering is associated with co-regulation of genes within the cluster, reminiscent of operons and regulons in prokaryotes. However, eukaryotes effectively orchestrate the functioning of many biosynthetic pathways for which the various genes are dispersed throughout the genome [38].

Regarding the RAPD patterns of the four investigated fungal isolates, it could be observed that the maximum number of different bands was only two bands using primer 5 with *A. chevalieri* and *E. chevalieri* and primer 3 with *A. nidulans* and *E. nidulans*. Primer 5 resulted in only one band difference with *A. nidulans* and *E. nidulans*. The rest of the investigated primer-isolate combinations resulted in no band differences. This might suggest that the asexual isolates unable of cleistothecia production suffered sexual, fatty acid or secondary metabolite gene mutations as these three criteria are connected.

The accompanying changes in secondary metabolite and fatty acid profiles between the sexual and asexual cultures with RAPD profile differences agrees with the results of Tsitsigiannis and Keller [31] who reported that deletion of *ppo* genes (affecting sexuality) affected the production of at least three different secondary metabolites in *A. nidulans*, including sterigmatocystin, the antibiotic penicillin and an octaketide, shamixanthone, where for sterigmatocystin and penicillin, these effects were reflected and supported by levels of biosynthetic gene transcription.

Comparing the RAPD results with fatty acid results, it could be concluded that variations in the DNA are very low when compared to fatty acid variations, this reflects that it might be the expression of the genes that govern fungal sexuality which might be repressed or blocked, or the parts of the gene regulators are mutated so these isolates need DNA repair (differences in some of the bands with this great DNA similarity might reflect the possibility of some mutations).

Data from mutants in diverse genes involved in different aspects of fatty acid metabolism indicate that appropriate amounts and composition of fatty acids and their derivatives are essential for sexual development. *N. crassa* mutants of a fatty acid synthase subunit are sterile in homozygous crosses, and *A. nidulans* mutants of several desaturase genes show changes in the balance between sexual and asexual development [14,7,36]. In *A. nidulans*, psi factors are necessary for correct developmental decisions, and changes in fatty acid composition also influence psi factor composition, which could explain morphological defects in the mutants [24].

Analyses of other mutants point to a more general requirement for fatty acids, probably as energy source for fruiting body formation. A mutation example showing implications for fatty acid metabolism in fruiting-body development is *car1* of *Podospora anserina*. CAR1 is a peroxisomal protein necessary for peroxisome biogenesis, and interestingly, the *car1* mutant has defects in karyogamy, and therefore is sterile [3]. In peroxisomes, mobilization of fatty acids by β-oxidation takes place, and the phenotype of the *car1* mutant might be due to a disturbed fatty acid metabolism.

Hence mutations in fatty acid synthesis, secondary metabolite synthesis and/or sexuality genes as well as differences in the promoter sequence of either genes (or gene clusters) might be responsible for the ceasing of fungal sexual reproduction especially that it has been recently reported that sexual genes exist in the formerly thought asexual forms; *A. fumigatus* has long been considered to reproduce only by asexual means, however, it has been reported that *A. fumigatus* has the potential to reproduce by sexual means [12,37]. Additionally, genomic sequences of *A. fumigatus* and *A. oryzae*, considered to be asexual, have implications for the presence of mechanisms for sexual reproduction [13,27]. Mating type genes have also been identified from other "asexual" species including *A. niger* [21], *A. clavatus*, *A. sojae*, *A. flavus* and *A. parasiticus* [11]. Again in 2008, the presumably asexual pathogen *A. fumigatus* was reported to encode functional regulators of mating and sexual development [15].

Conclusively, minor DNA variations between the perfect and imperfect states reflects that both possess the sexuality genes however these genes need, in the asexual state, to be derepressed by removing the

repressors effect on them or retransform the repaired genes to the same anamorph with new promoter to activate the inactive pathway which might be connected to the biosynthesis of secondary metabolites and fatty acids. Or it is a mutation in fatty acid or secondary metabolite biosynthetic genes which consequently affects the rest of the investigated studied parameters.

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