The isoepoxydon dehydrogenase gene PCR profile is useful in fungal taxonomy

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Summary

This study evaluates the specificity of PCR isoepoxydon dehydrogenase (*idh)* primers on fungi associated with patulin production. The DNAs of 93 strains were extracted and analysed by PCR using primers of the *idh* gene of patulin biosynthesis. A single band at 620 bp was obtained on 17% of the analysed strains. Different molecular weight amplicons were observed in other strains. These were employed as binary characters for numerical analysis to obtain a dendrogram. Clusters were observed, which corresponded to morphological identifications in some cases. Amplicons at 400 and/or 500 bp were related to patulin non-detection for strains, whereas a 450 bp amplicon was associated with some *Aspergillus* and both of the *Byssochlamys nivea* strains tested. Hence, the *idh* primers are not specific for the gene and provide other amplicon products in other species. These results were useful providing (a) profiles of DNA to identify and classify fungi and (b) insights into patulin production. The DNA profiles in this study may be useful for determining patulin producing fungi. Obtaining multiple bands in culture-independent PCR of environmental samples by using the primers could indicate that more than one species is present.

Key words

Isoepoxydon dehydrogenase gene, Patulin, PCR, Numerical analysis, *Penicillium, Aspergillus, Byssochlamys*

Utilidad en taxonomía fúngica de los patrones obtenidos por PCR del gen de la isoepoxydon deshidrogenasa

Resumen

El interés del presente estudio fue investigar la especificidad de los cebadores para la PCR del gen de la isoepoxydon deshidrogenasa en hongos asociados a la producción de patulina. El DNA de 93 cepas fue extraído y analizado mediante PCR utilizando cebadores del gen *idh* implicado en la biosíntesis de la patulina. Se obtuvo una banda simple de 620 pb en un 17% de las cepas y bandas de peso molecular variable para el resto. Estos datos fueron utilizados como caracteres binarios en un análisis numérico. Se pudo comprobar que los diferentes clusters que aparecían en el correspondiente dendograma a veces se correspondían con determinados caracteres morfológicos de las cepas. Amplificados de de 400 y/o 500 pb se relacionaron con cepas no productoras de patulina y una banda de 450 pb se asoció con algunos *Aspergillus* y con las dos cepas de *Byssochlamys nivea* incluidas en el estudio. Ello demostraba que los cebadores no eran específicos para el gen y amplificaban otras regiones en algunas especies. Estos resultados demostraron ser útiles en para la identificación y clasificación de hongos y para un mejor conocimiento de la producción de patulina. Determinados patrones de DNA pueden ser útiles para detectar hongos potenciales productores de patulina. La obtención de múltiples bandas en una PCR de muestras ambientales no cultivadas puede indicar que más de una especie está presente.

Palabras clave

Gen de la isoepoxydon deshidrogenasa, Patulina, PCR, Análisis numérico, *Penicillium, Aspergillus, Byssochlamys*

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Introduction

The significance of the polyketide patulin as a mycotoxin has increased greatly from the introduction of new European Union regulations that decrease the acceptable limits in fruit products and especially those used in baby foods. Evidence of this fact was recently reported in two patulin reviews [6,7]. Furthermore, the possibility for controlling the mycotoxin in food commodities has improved with greater understanding of the patulin metabolic pathway [17] and was further enhanced when the sequences of crucial genes were determined [4]. In particular, the sequencing of the isoepoxydon dehydrogenase gene *(idh)* which produces the enzyme to transform isoepoxydon to phyllostine, has been of greatest use.

Paterson et al. [8] were the first to employed *idh* primers to screen fungi specifically in the context of patulin as a mycotoxin: the fungi that were analysed were all penicillia. In addition, primers were employed seminally to analyse environmental samples as a cultural independent PCR (CIP). The analyses were extended to many other species within penicillia using culture dependant PCR (CDP), indicating that strains could be classified as to whether they were positive for *idh* and patulin detection [10]. Varga et al. [18] indicated the presence of *idh* and patulin production within *Aspergillus* section *Clavati* and included an evolutionary perspective on fungi with, or without, the trait. Paterson [12] analysed species from *Penicillium*, *Aspergillus,* and *Byssochlamys* that were associated with patulin production: *B. nivea* was positive for *idh* and patulin detection. In addition, positive *idh* results were obtained for *Paecilomyces lilacinus* and a strain of *B. fulva* [11]. *Paecilomyces* is the anamorphic form of *Byssochlamys*. Finally, an applied scheme for the classification of penicillia was developed using *idh* and patulin production as characters [13] to compliment other schemes e.g. Samson and Frisvad [16].

DNA sequencing demonstrated that the *idh* of *P. griseofulvum* was different from *P. expansum* [2] and that maximum parsimony trees based on rDNA and *idh* sequences were congruent [3] in the cases of these two important terverticillate penicillia. Unrelated *B. nivea* had a high degree of homology (88%) with the two penicillia: The *B. nivea* strains were identical independent of geographical region of isolation [1]. Puel et al. [15] determined that *B. fulva* could not produce patulin because of the absence of the 6-methylsalicylic acid synthase gene and *idh*. However, one other strain was *idh* positive in Paterson [11] and the situation requires clarification. White et al. [19] determined that *idh* was up-regulated under patulin permissive culture conditions.

During the investigations involving the use of *idh* primers on fungi it was noticed by the present author that other amplicons, apart from the expected product, appeared on gels at 620 bp. An assessment is made in the current report of the complete profiles for the classification of various fungi associated with patulin production using *idh* primers as such novel characters may be useful for the classification and identification of these organisms.

Material and methods

Ninety-three strains were obtained from the CABI Bioscience, UK culture collection and were identified by expert taxonomists as associated with that organisation. Methods of growth were as in Paterson et al. [10]. Isolates were grown on slopes of potato dextrose agar or 2% (w/v) malt agar. The isolates were harvested and extracted. The PCR analysis used has been described thoroughly [10] where images of representative gels are available. The band patterns were recorded to the nearest 50bp and subjected to numerical analysis using the statistical programme, Statistical Package for the Social Sciences version 14.0 and a dendrogram was obtained (Figure 1). However, the *idh* product was recorded as 620bp, which is the typical size. Control taxa were a *Ganoderma* sp. (IMI 357185), a basidiomycete that may cause disease in oil palm; and *Aspergillus flavus* (IMI 380661), which produces aflatoxins that are polyketides, as is patulin. Neither of these fungi is known to produce patulin.

The PCR mixture consisted of 200 mM of dNTPs (Pharmacia, Herts), 1.25 units of Tth polymerase, Tth buffer (both from HT Biotechnology, Cambridge, UK), 0.8

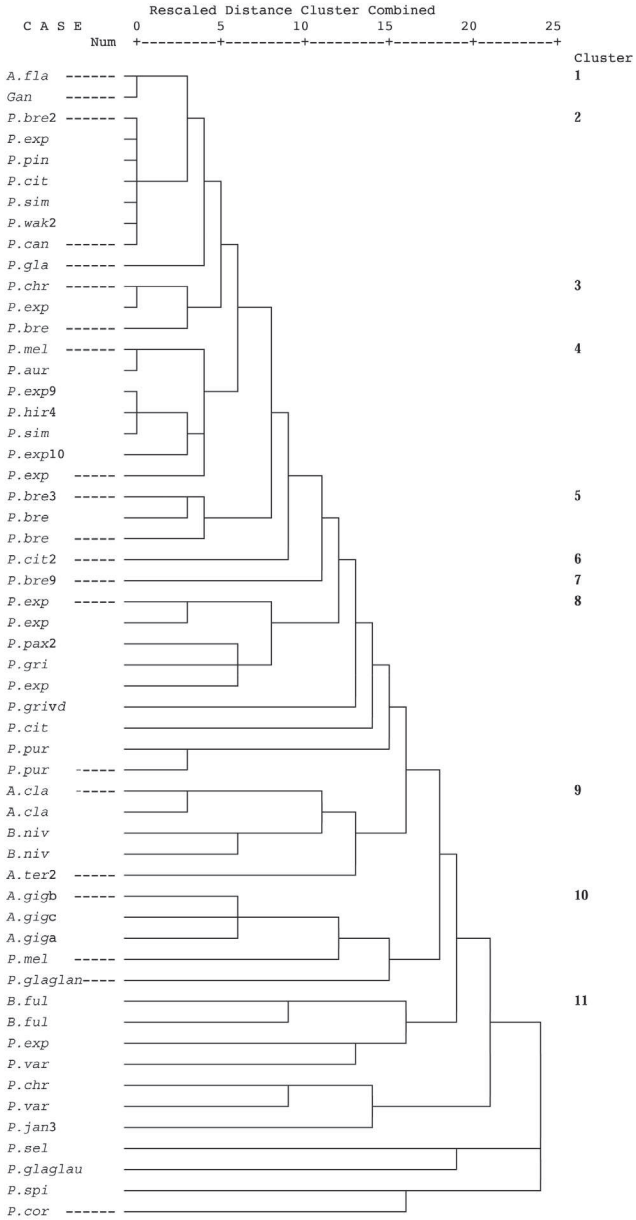

Figure 1. Dendrogram from a hierarchical cluster analysis by average linkage (between groups) of the bands resulting from the isoepoxydon dehydrogenase primers applied to total DNA from the fungal strains. The numbers after the abbreviated species names are the number of strains of the same species that were analysed. The broken horizontal lines indicate the limits of each cluster. The full names of the species can be determined from table 2.

Table 1. List of strains employed and PCR products from the isoepoxydon dehydrogenase gene primers. The six-figure number is the IMI culture collection identification code.

Fungus		PCR products (base pairs). *idh* is 620							
Aspergillus	Species/strain								
Subgen. *Aspergillus*									
Sect. *Flavi*	*A. flavus* 380661						800		
Sect. *Terrei*	*A. terreus* 135817, 016043	250	450		620				1100
Subgen. *Fumigati*									
Sect. *Clavati*	*A. clavatus* 015949v	300	450		620		800		1200
	232883	300	450		620		800		1100, 1200
	A. gigantus 343711	350		500	620	700, 750	850		1100
	016154	350		500	620	750	850		
	358435	350		500	620		850		1100
Byssochlamys nivea	361545		450		620		800	900	1050
	058423		450		620		800		
B. fulva	058422		400		620	750	850		1050, 1100
	040021	200	400	500	620	750			1100, 1050
Ganoderma sp.	357185						800		
Paecilomyces variotii	321342		400	500	620				1050, 1300
	204127	200	400			700	800		1050, 1300
Penicillium									
Subgen. *Aspergilloides*	*P. glabrum* 380331								1000
	P. spinulosum 380642	350			620	700		950	1050, 1200, 1500, 2000
Subgen. *Biverticillium*	*P. pinophilum* 380659	No bands							
	P. purpurogenum 380952			500		700			1100, 1400
	380948			500					1100, 1400
	P. waksmanii 381974, 381990		400		650	750			1200
Subgen. *Furcatum*	*P. canescens* 380320	No bands							
	P. citrinum 380961, 380969								1250, 1400
	380323			500			800		2000
	380342	No bands							
	P. corylophilum 380322	350			620				1500, 1550, 2000
	P. janczewskii 381948, 380968, 380633			550			800	900	1050, 1300
	P. melinii 304279	350	400		620		850		
	040216ii				620	700			
	P. paxilli 380639, 381272		400			750			
	P. selandiae 304284		400	500	620	700		950	1000, 1400
	P. simplicissimum 380333				620				
	380971	No bands							
Subgen. *Penicillium*	*P. aurantiogriseum* 265302				620	700			
	P. brevicompactum 381970, 380944, 380346, 380347,380348, 380329, 380341, 380656, 380321				620				1150, 1050
	380334				620				1500
	380330				620				
	380352							900	1100
	380349, 380643, 380648							900	
	380353		400					900	
	380634, 380645	No bands							
	P. chrysogenum 380958								1500
	380332					700			1050, 1300
	P. expansum 381952, 381969, 381268, 380345, 380350, 380665, 380657, 380662, 380653				620				
	299046				620	750			
	380324, 380326, 380327, 380325, 380344, 380337, 380336, 380335, 381265, 380970				620				1200
	232297	400		500	620	750			
	380960	400			620	750			1200
	380654				620	750			1200
	381266								1500
	380636	No bands							
	028619	200	400	500	620				1200
	P. griseofulvum 075832ii	300	400		620	750			
	P. griseofulvum var. *dipodomyicola* 296935	350			620	750		900	
	P. glandicoladicola var. *glandicoladicola* 321513	300		500	620		850		1150
	P. glandicoladicola var. *glaucovenetum* 321511	200, 300	400	500	620	700		900	
	P. hirsutum 381967, 381951, 382063, 382064				620				

mM of primer IDH1, and IDH2, and template DNA. The IDH gene was amplified at 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min for 30 cycles with a Hybaid, Omn-E thermal cycler. The initial denaturing step was 94 °C for 3 min and the programme was completed with an extension step of 72 °C for 5 min. The primers (GIBO BRL, Paisley) were: IDH1 sequence: 5k-CAATGTGTCGTACT GTGCCC-3k, and IDH2 sequence: 5k-ACCTTCAGTCGCTGTTCCTC-3k. A list of the 93 strains used is provided (Table 1) together with the DNA products observed.

Results

A single amplicon at 620 bp was obtained by 17% of the 93 strains tested wich were mainly strains of *P. simplicissimum*, *P. expansum, P. brevicompactum* and *P. hirsutum* (Table 1). The remainder strains showed 2 to 7 amplicons, with the exception of *P. spinulosum,* which showed 8 bands. The different banding patterns are shown in table 1 some of which are specific for species as revealed in the dendrogram (Figure 1). The strains representing each cluster are provided in table 2. From "top" to "bottom" of the dendrogram, the first cluster is formed by the *A. flavus* and the *Ganoderma* sp strains, (outgroup). The second cluster was made of strains, which bands were not apparent (Table 1). A single *P. glabrum* strain was loosely linked to this cluster. A cluster of three unrelated subgenus penicillia formed the fourth cluster. This was followed by a cluster containing most of the *P. expansum* strains and which were all *idh* positive. Approximately 50% of these strains produced also a band above 1000 bp. However, this expansum cluster included the *P. hirsutum* strains together with one *P. aurantiogriseum*. *P. melinii* and *P. simplicissimum* from subgenus Furcatum were also included in this cluster. A cluster of five *idh* negative *P. brevicompactum* strains was followed by a cluster of two *P. citrinum*. However, these were followed by most of the *P. brevicompactum* strains, which possessed the *idh* band at 620 bp and other higher molecular weight products at 1050 bp and 1150 bp. Those strains without the 620 amplicon showed a high molecular weight band at approximately 900 bp. A mixed collection of predominately single strains clustered below these strains, although the *P. paxilli* strains were identical and the *P. purpurogenum* were similar to each other in terms of the banding patterns. The next group was formed by *A. clavatus, A. terreus* and *B. nivea* strains, all of which were *idh* positive. However, *A. giganteus* clustered separately and were linked loosely to two penicillia. Strains with complex patterns containing an average of 5 bands were separate from the other strains and formed numerous "single strain clusters" towards the lower portion of the dendrogram. However, two strains of *B. fulva* clustered here and were *idh* positive: Three strains of *P. janczewskii* were *idh* negative and formed a loose group with two other *idh* negative strains of *Paecilomyces variotii* and *P. chrysogenum*. The other strains in this group were *idh* positive, the predominant bands being at 400 bp and 500 bp.

Table 2. Strains included in each cluster. The six-figure number is the IMI culture collection identification code.

Clusters	Fungi
1.	*A. flavus* 380661, *Ganoderma* sp. 357185.
2.	*P. pinophilum* 380659, *P. waksmanii* 381974, 381990. *P. citrinum* 380342, *P. simplicissimum* 380971, *P. canescens* 380320, *P. brevicompactum* 380634, 380645, *P. expansum* 380636.
Single strain	*P. glabrum* 380331.
3.	*P. brevicompactum* 380334, *P. chrysogenum* 380958, *P. expansum* 381266.
4.	*P. expansum* 299046, 381952, 381969, 381268, 380345, 380350, 380665, 380657, 380662, 380653, 380324, 380326, 380327, 380325, 380344, 380337, 380336, 380335, 381265, 380970. *P. hirsutum* 381967, 381951, 382063, 382064. *P. aurantiogriseum* 265302. *P. melinii* 040216ii, *P. simplicissimum* 380333.
5.	*P. brevicompactum* 380352, 380349, 380643, 380648, 380353.
6.	*P. citrinum* 380961, 380969.
7.	*P. brevicompactum* 381970, 380944, 380346, 380347, 380348, 380329, 380341, 380656, 380321.
8.	*P. expansum* 380960, 380654. *P. paxilli* 380639, 381272. *P. griseofulvum* 075832ii. *P. expansum* 232297 *P. griseofulvum* var. *dipodomyicola* 296935 *P. citrinum* 380323 *P. purpurogenum* 380952, 380948.
9.	*A. clavatus* 015949v, 232883. *B. nivea* 361545, 058423. *A. terreus* 135817, 016043.
10.	*A. giganteus* 343711, 016154, 358435. *P. melinii* 304279 *P. glandicoladicola* v. *glandicoladicola* 321513
11.	*B. fulva* 058422, 040021. *P. expansum* 028619 *Pa. variotii* 321342 *P. chrysogenum* 380958 *Pa. variotii* 204127 *P. janczewskii* 381948, 380968, 380633 *P. selandiae* 304284 *P. glandicoladicola* var. *glaucovenetum* 321511 *P. spinulosum* 380642 *P. corylophilum* 380322

Discussion

It is apparent that the *idh* primers were not specific for the expected 620 bp product. The bands produced did not allow for complete separation of taxa into individual clusters in all circumstances. Nevertheless, the banding patterns provided are useful taxonomic characters in addition to determining the presence of *idh*. A group of predominately *P. expansum strains* was observed, all of which produced detectable amounts of patulin and were *idh* positive. Some non-*P. expansum* species were observed in the same cluster. However, these tended to be negative for patulin production as reported in [8,10,12]. Differences within *P. expansum* require further investigation as the fungus is an important pathogen of economic food plants and a known mycotoxin producer.

The *P. brevicompactum* strains consist of at least two separate groups, one of which contains strains that were positive for *idh*. These may form the other members of the *Olsonii* series, as the morphological methods employed to identify the fungi would not necessarily separate the different species of the series (see [14]). The *Aspergillus* and *B. nivea* were separate from *Penicillium,* as would be anticipated from conventional taxonomic analysis. Related to this, the *A. clavatus, A. terreus* and *B. nivea* all provided a unique amplicon at 450 bp compared to the other species. More work is necessary to determine if this is a

genuine difference. The *B. nivea* were also separate from *B. fulva,* which is interesting as these species are difficult to differentiate by conventional methods. It is notable that the *B. fulva* strains were positive for *idh,* contradicting the hypothesis of Puel et al. [15] that *B. fulva* are not capable of producing patulin because the species does not possess *idh,* amongst other genes. It is necessary to consider the possibility of inhibition of PCR in the circumstances where CDP is employed [5,9]. The other banding patterns obtained provide additional characters for identification of taxa.

In conclusion, PCR using *idh* primers provides more information than a simple, single band at 620 bp. This needs to be appreciated when undertaking CIP analysis where many bands can be obtained [8] and, for example, multiple bands may not be indicative that numerous species could be present in environmental samples. The data provided additional taxonomic characters that may be useful in classification and identification of species, in addition to indicating whether strains possess the *idh* gene.

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References

1. Dombrink-Kurtzman MA, Engberg AE. *Byssochlamys nivea* with patulin-producing capability has an isoepoxydon dehydrogenase gene *(idh)* with sequence homology to *Penicillium expansum* and *P. griseofulvum.* Mycol Res 2006; 110: 1111-1118.
2. Dombrink-Kurtzman MA. The isoepoxydon dehydrogenase gene of the patulin metabolic pathway differs for *Penicillium griseofulvum* and *Penicillium expansum.* Anton van Leeuw 2006; 89: 1-8.
3. Dombrink-Kurtzman MA. The sequence of the isoepoxydon dehydrogenase gene of the patulin biosynthetic pathway in *Penicillium* species. Anton van Leeuw 2006; 91: 179-189.
4. Fedeshko RW. Polyketide enzymes and genes in *Penicillium urticae*. PhD Dissertation, University of Calgary, Alberta, Canada 1992.
5. Hoorfar J, Cook N, Malorny B, de Medici D, Abdulmawjood A, Fach P. Diagnostic PCR: making internal amplification control mandatory. J Appl Microbiol 2004; 96: 221-222.
6. Jackson L, Dombrink-Kurtzman MA. Patulin. In: Sapers GM, Gorny JR, Yousef AE (Eds.) Microbiology of Fruits and Vegetables. Boca Raton, FL, CRC Press, 2006: 281-311.
7. Moake MM, Padilla-Zakour OI, Worobo RW. Comprehensive Review of Patulin Control Methods in Foods. Comp Rev Food Sci Food Safety 2005; 1: 8-21.
8. Paterson RRM, Archer S, Kozakiewicz Z, Lea A, Locke T, O'Grady E. A gene probe for the patulin metabolic pathway with potential use in novel disease control. Biocon Sci Technol 2000; 10: 509-512.
9. Paterson RRM. Internal amplification controls have not been employed in diagnostic fungal PCR hence potential false negative results. J Appl Microbiol 2007; 102: 1-10.
10. Paterson RRM, Kozakiewicz Z, Locke T, Brayford D, Jones SCB. Novel use of the isoepoxydon dehydrogenase gene probe of the patulin metabolic pathway and chromatography to test penicillia isolated from apple production systems for the potential to contaminate apple juice with patulin. Food Microbiol 2003; 20: 359-364.
11. Paterson RRM. The isoepoxydon dehydrogenase gene of patulin biosynthesis in cultures and secondary metabolites as candidate PCR inhibitors. Mycol Res 2004; 108: 1431-1437.
12. Paterson RRM The isoepoxydon dehydrogenase gene of the patulin biosynthetic pathway, patulin detection, and the utility of species names in patulin-producing fungi. In: Lima N, Smith D (Eds.) Biological Resource Centres and the use of Microbes, European Culture Collections Organisation XXII. Braga, Micoteca da Universidade do Minho, 2003; 259-266.
13. Paterson RRM, Venâncio A, Lima, N. A novel identification system based on 318 penicillia strains using the isoepoxydon dehydrogenase gene and patulin production. Rev Iberoam Micol 2006; 23: 155-159.
14. Peterson SW. Multilocus DNA sequence analysis shows that *Penicillium biourgeianum* is a distinct species closely related to *P. brevicompactum* and *P. olsonii.* Mycol Res 2004; 108: 434-440.
15. Puel O, Tadrist S, Delaforge M, Oswald I, Lebrihi, A. The inability of *Byssochlamys fulva* to produce patulin is related to absence of 6-methylsalicylic acid synthase and isoepoxydon dehydrogenase genes. Inter J Food Microbiol 2006; 115: 131-139.
16. Samson RA, Frisvad JC, *Penicillium* subgenus *Penicillium*: New taxonomic schemes, mycotoxins and other extrolites. Stud Mycol 2004; 49: 1-258.
17. Sekiguchi J, Shimamoto T, Yamada Y, Gaucher GM. Patulin biosynthesis: enzymatic and nonenzymatic transformations of the mycotoxin (E)-ascladiol. Appl Environ Microbiol 1983; 45: 1939-1942.
18. Varga J, Rigó K, Molnár J, Tóth B, Szencz A, Téren J, Kozakiewicz Z. Mycotoxin production and evolutionary relationships among species of *Aspergillus* section *Clavati,* Anton *van* Leeuw 2003; 83: 191-200.
19. White S, O'Callaghan J, Dobson ADW. Cloning and molecular characterization of *Penicillium expansum* genes upregulated under conditions permissive for patulin biosynthesis. FEMS Microbiol Lett 2006; 255: 17-26.