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$(GTG)_5$ microsatellite regions in citrinin-producing *Penicillium*

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Summary

Morphological and cultural characteristics, as well as biochemical properties, are the main criteria used in fungal taxonomy and in the standard description of fungi species. Sometimes, however, this criterion is difficult to apply due to fungal phenotypic variations. This is particularly true in the genus *Penicillium*. The aims of this work were to determine $(GTG)_5$ microsatellite sequence in potentially citrinin-producing *Penicillium* strains and to investigate if this sequence could be useful to characterize such fungi.
Penicillium citrinum Thom and *Penicillium chrysogenum* Thom were isolated from different foods. The identification of the isolates at species level was carried out according to classical taxonomy. The production of citrinin was determined by thin layer chromatography.
This study proved that microsatellite regions exist as short repeated sequences in all tested strains. The patterns were very similar for all *P. citrinum* isolates and it was possible to group them in function of the quantity of citrinin produced. Yet, not similar clusters were obtained when *P. chrysogenum* isolates were analyzed.

Key words *Penicillium,* $(GTG)_5$ microsatellite, Citrinin - producing *Penicillium*

Regiones microsatélites $(GTG)_5$ en *Penicillium* productores de citrinina

Resumen

Los principales criterios que se utilizan en la taxonomía fúngica, así como en la descripción de las especies, son sus caracteres morfológicos y de cultivo y sus propiedades bioquímicas. Sin embargo, a veces, resulta muy difícil clasificarlos debido a su variabilidad fenotípica, lo que es particularmente cierto con el género *Penicillium*.
Los objetivos de este trabajo fueron determinar regiones microsatellites $(GTG)_5$ en *Penicillium* potenciales productores de citrinina e investigar la utilidad de las mismas para caracterizarlos.
Penicillium citrinum Thom y *Penicillium chrysogenum* Thom fueron aislados a partir de diferentes alimentos. La identificación de las especies se llevó a cabo aplicando las claves taxonómicas clásicas y la producción de citrinina se determinó mediante cromatografía en capa delgada.
Se demostró la existencia de los microsatélites, como secuencias cortas y reiteradas, en todas las especies estudiadas. Los patrones obtenidos en todas las cepas de *P. citrinum* fueron muy similares y permitieron agruparlas según la cantidad de toxina producida. Los aislamientos de *P. chrysogenum* no pudieron ser agrupados de la misma manera.

Palabras clave *Penicillium,* Regiones microsatélites $(GTG)_5$, Citrinina

Penicillium spp. are ubiquitous filamentous fungi that play an important role under natural conditions in the aerobic decomposition of organic materials. Besides, they are natural contaminants of nearly every food or feed commodities. Many of the food-borne *Penicillium*, and other filamentous fungi are capable of producing mycotoxins as well as antibiotics [3].

The identification of *Penicillium* isolates to species level is not easy and it must be carried out under carefully standardized conditions of media, incubation time, and temperature. Gross physiological features, colony diameters, colours of conidia, colony pigments, etc. as well as microscopic morphology, have been used to distinguish species.

Penicillium citrinum Thom and *Penicillium chrysogenum* Thom have been isolated from nearly every kind of food surveyed for fungi, and toxin production is likely to be a common occurrence. Both of them are known as citrinin-producing fungi, being *P. citrinum* the major producer of this mycotoxin. Even though the effect of citrinin on humans remains undocumented, kidney damage appears to

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be a likely result of prolonged ingestion. Yet, citrinin is a significant renal toxin to animals [9].

In the last few years, several molecular typing methods, including restriction fraction length polymorphism (RFLP), ribotyping, randomly amplified polymorphic DNA pattern (RAPD pattern) analysis and amplification of microsatellite regions, have been successfully used for the identification and epidemiological characterization of different organisms [1,11].

Microsatellite loci are regions on a chromosome that contain a short DNA sequence (1-10 bp), repeated in tandem up to 100 times [7]. PCR techniques using primers based on microsatellite sequences are used to detect DNA polymorphisms. This is a reproducible and easily performed method which only needs very small amounts of DNA for typing related microorganisms [17,19] and allows to compare results among different laboratories.

There are not enough reports about microsatellite regions in fungi even though this method has been applied to opportunistic fungi such as *Aspergillus fumigatus* and *Penicillium marneffei* [5].

The aims of this work were to determine $(GTG)_5$ microsatellite regions in potentially citrinin-producing *Penicillium* strains and to investigate if this sequence could be useful to characterize such fungi.

Material and methods

All *Penicillium* isolates that were studied are listed in table 1. These isolates were maintained on malt extract slants and stored at 4 °C for daily work and lyophilized for longer periods of time.

Morphological identification and citrinin detection in Penicillium *isolates*. The identification of isolates at species level was carried out according to classical taxonomy, based on their micro and macro-morphological and physiological characteristics in culture [12-15]. Citrinin production was detected and analyzed by thin layer chromatography (TLC) on Merck Kiesegel 60 by means of Cole & Cox's [2] and Hald & Krogh's method [4] respectively. Spots were also observed by UV light and those of interest were sprayed with a commercial boron triflouride (Voltaix, Inc.) solution (10-15% in methanol). Blue-green fluorescence under 365 nm light confirmed the presence of citrinin [4]. Rf values and semiquantitative analysis of citrinin were performed in comparison to citrinin results with an authentic commercial toxin standard (SIGMA, lot 76H404) (0.4% in acetone:water solution, by 80:20 vol.) [8].

DNA extraction. DNA extraction was performed as described in Lee & Taylor [6] with a slight modification. In brief, each *Penicillium* was subcultured onto a malt extract agar slant, and the slant was incubated at 25 °C for 4 days. Conidia suspension in water (10^5-10^6 UFC/ml) was inoculated into 100 ml of *Colletotrichum* broth (MLC) [10]. Broth was incubated at 28 °C for 24 h at 180 rev/min. Cultures were harvested by filtration, washed with sterile water, blotted dry and finally powdered. Mycelium (0.06 g) was treated with 600 µl lysis buffer [Tris-HCl (pH 7.2) 0.05 M, EDTA 0.05 M, SDS 3%, 2-mercaptoetanol 1%] and 60 µl sarkosyl (10%). The suspension was incubated at 65 °C for 1 h and 200 µl of 5 M potassium acetate and 100 µl 4 M sodium chloride were added. After a gentle inversion ten times, the suspension was allowed to rest on ice for 10 min. Following a centrifugation at 15,000 *g* for 15 min, the upper phase was carefully transferred into a new tube and an equal volume of phenol-chloroform-isoamilic alcohol solution (25:24:1) was added. The suspension was gently mixed by inversion, pelleted by centrifugation for 5 min at 15,000 *g* and the aqueous phase was then carefully removed. Phenol-chloroform-isoamilic alcohol extraction was performed twice. An equal volume of chloroform-isoamilic alcohol solution (24:1, v/v) was added and emulsified by inversion 5 times, followed by centrifugation for 5 min.

The aqueous phase was recovered and DNA was precipitated by adding 0.7 volume of isopropanol. The tube was kept at room temperature for 20-30 min and the DNA was collected by centrifugation.

Pellet was washed with 500 µl of 70% ethanol (previously cooled at -20 °C) and completely dried at 55 °C. Finally, DNA was solubilized in 100 µl of milliQ water, treated with 1 µl RNAsa (Promega, 10 mg/ml) and stored at -20 °C.

DNA concentrations were determined by measuring the A_{260} with a spectrophotometer and by gel electrophoresis [16].

Microsatellite PCR-assay. Microsatellite PCR-assay proposed by Longato et al. was followed [7]. Briefly, primer (5'-GTGGTGGTGGTGGTG-3') obtained from FAGOS/Ruralex (Argentina) was used. The reaction mixture (final volume: 50 µl) consisted in approximately 20 ng of DNA and 5 µl adequate buffer (INBIOWAY) with 1.5 mM $MgCl_2$, 1 mM each of dNTPs (INBIOHIGHWAY), 100 pmol of primer and 1 U of *Taq*DNA Polymerase

Table 1. Sources of *Penicillium* isolates and citrinin production.

Isolates	Isolate number	Sources	TLC results[1]
Penicillium citrinum[2]	–	CECT 2274	Citrinin (> 0.4 mg/100 ml)
P. citrinum[2]	–	CECT 2269T	Citrinin (< 0.4 mg/100 ml)
P.steckii[2]	–	CECT 2268	Non-producer
P. chrysogenum[2]	–	CECT 2784	Non-producer
P. citrinum	74	Dairy products (cheese)	Citrinin (>0.4 mg/100 ml)
P. citrinum	99	Bakery products (bread)	Citrinin (< 0.4 mg/100 ml)
P. chrysogenum[3]	85	Cereals (oat)	Citrinin(> 0.4 mg/100 ml)
P. chrysogenum	112	Peanuts	Citrinin (> 0.4 mg/100 ml)
P. chrysogenum[3]	122	Cereals (maize)	Citrinin (> 0.4 mg/100 ml)
P. chrysogenum[3]	126	Cereals (oat)	Non-producer
P. chrysogenum	132	Peanuts	Citrinin (> 0.4 mg/100 ml)

[1] Thin layer chromatography (TLC) performed by means of Cole and Cox [2] and Hald and Krogh's [4].
[2] Strain kindly given by Cultures Type Spanish Collection (CECT).
[3] Originally identified as *P. notatum.*

(INBIOHIGHWAY) (5 U/µl). Mineral oil (15 µl) was added to the top of the reaction. Control experiments were performed without template DNA. The amplification reaction was performed in a MJ Research Thermal Cycler programmed as follows: starting at an annealing temperature of 70 °C, reductions by 2 °C each at two subsequent cycles to 55 °C and 25 extra cycles at 55 °C. Extension temperature was of 72 °C.

The amplification products were analyzed by electrophoresis in 1.3 % agarose gel, run in 1X TBE buffer [16] at 100 V and subsequently stained with ethidium bromide. Lambda DNA/*Eco*RI+*Hind* III and 100-bp DNA ladder (Promega) were used as molecular weight markers.

Each assay was performed at least four times.

Fingerprint evaluation. Banding patterns similarities were scored by the Dice coefficient (D) with "RAP-Distance 1.04" software and cluster analysis was performed by agglomerative technique using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method from Molecular Evolutionary Genetic Analyses program (MEGA). The relationships among the different isolates studied were portrayed graphically in the form of a dendogram.

Results

Sources of *Penicillium* isolates and citrinin production are shown in table 1.

The variations in the banding pattern obtained by DNA fingerprinting enabled all these species to be distinguished according to number, size and intensity of the polymorphic fragments. Amplification results were reproducible for each isolate.

Microsatellite primed PCR produced 16 amplification products (max: 9, min: 5) with sizes that ranged from 1500 to 200 bp for *P. citrinum* and *Penicillium steckii* Zaleskii CECT 2268 isolates, and 13 bands (max: 8, min: 1) lower than 611 bp for *P. chrysogenum* isolates.

The relationship of *P. citrinum* and *P. chrysogenum* isolates is shown in figures 1 and 2, respectively. Microsatellite patterns were very similar for all *P. citrinum* isolates and it was possible to group them in function of the quantity of citrinin production. However, non similar clusters were found when analyzing the microsatellites patterns obtained with $(GTG)_5$ primer for *P. chrysogenum* isolates, due to their high variability.

Discussion

So far, few reports about microsatellite sequences have been carried out in *Penicillium* spp. [5]. Since amplification products were obtained in all *Penicillium* isolates, $(GTG)_5$ microsatellite regions are present in the studied species.

In this study, microsatellite patterns were very similar for all *P. citrinum* isolates. Although a few isolates were studied, it was possible to group them according to their citinin-production capabilities (Figure 1), suggesting that $(GTG)_5$ microsatellite regions could be a possible tool for their classification.

Moreover, our results showed slight genotypic differences between *P. citrinum* isolates and *P. steckii* CECT 2268. Since the analyzed fungi had a high degree of similarity at the genetic level, this finding would reinforce the taxonomic scheme proposed by Pitt where *P. steckii* is regarded as a synonym of *P. citrinum* [12].

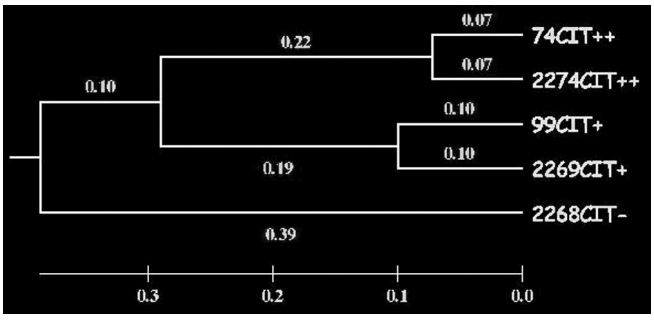

Figure 1. Dendogram showing the relationship among *Penicillium citrinum* according to the results obtained with $(GTG)_5$-primed PCR. 74: *P. citrinum* isolated from cheese; 2274: *P. citrinum* CECT 2274; 99: *P. citrinum* isolated from bread; 2269: *P. citrinum* CECT 2269T; 2268: *Penicillium steckii* CECT 2268; CIT++: > 0.4mg/100 ml Citrinin; CIT+: < 0.4 mg/100 ml Citrinin; CIT-:Non-producer.

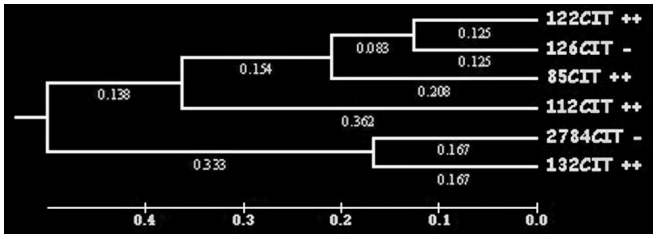

Figure 2. Dendogram showing the relationship between *Penicillium chrysogenum* according to the results obtained with microsatellite-primed PCR. 122: *P. chrysogenum* isolated from maize; 126: *P. chrysogenum* isolated from oat; 85: *P. chrysogenum* isolated from oat; 112: *P. chrysogenum* isolated from peanuts; 2784: *P. chrysogenum* CECT 2784; 132: *P. chrysogenum* isolated from peanuts. CIT++: > 0.4 mg/100 ml Citrinin; CIT-:Non-producer.

Several additional taxa have recently been described that appear to be related to *P. chrysogenum*. Neither the clonal nature nor the placement of these taxa relative to *P. chrysogenum* have been investigated by means of molecular phylogenetic methods [18].

The number and size of the DNA fragments obtained with $(GTG)_5$ primer demonstrated the lack of homogeneity among *P. chrysogenum* isolated from different foods. However, isolates number 85, 122 and 126 were closely grouped (Figure 2). They were originally identified as *Penicillium notatum* Westling according to Raper and Thom [15], until Pitt's classification [12] was applied.

Although it occurred with *P. citrinum*, $(GTG)_5$ primer was not useful for grouping *P. chrysogenum* isolates, according to the citrinin-production (Figure 2).

These experiments proved that $(GTG)_5$ microsatellites exist as short repeated sequences in all tested species. As different banding patterns were detected, this technically simple tool could be useful for assaying genetic variability in the *Penicillium* studied.

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