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Molecular epidemiology of isolates of the *Cryptococcus neoformans* species complex from Spain

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ABSTRACT

To study genetic diversity of *Cryptococcus neoformans* species complex in Spain, 97 isolates of the yeast recovered from human, animal and environmental samples have been analysed using three molecular epidemiological techniques. One of these, *URA5* gene fragment length polymorphism (RFLP) analysis, has been previously described as a molecular epidemiology tool. Thus, standard profiles and reference strains have been defined for it. In addition, 5S rDNA/IGS RFLP and [GACA]₄ microsatellite PCR fingerprinting were also used. Our results show five of the previously defined *URA5* genotypes with a high frequency (33%) of the VNI type, which is in concordance with other studies. The high presence of VNIII pattern (28.9%) among our strains is remarkable and could be a specific feature of the isolates from our country. 5S rDNA/IGS RFLP showed a low intra-species discriminative power. Three different molecular profiles (S1-3), which showed a good correlation with the different species, varieties and genotypes, were obtained. [GACA]₄ microsatellite PCR-fingerprinting analysis showed a high variability of patterns among the studied strains. Molecular profiles represented in a dendrogram clustered strains in four main groups related with the source of the yeast and also in concordance with some of the described genotypes (VNI-IV and VGI).

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Epidemiología molecular de aislamientos del complejo *Cryptococcus neoformans* en España

RESUMEN

Para ampliar el conocimiento de la diversidad genética que presenta el complejo *Cryptococcus neoformans* en España, se analizaron 97 aislamientos de esta levadura obtenidos a partir de muestras ambientales, veterinarias y clínicas, utilizando 3 técnicas epidemiológicas moleculares diferentes. Uno de estos marcadores moleculares, el análisis del *URA5* RFLP, se ha descrito previamente como herramienta epidemiológica, por lo que se dispone de perfiles estándar y cepas de referencia. Además, se utilizaron también el análisis del RFLP del 5S rDNA/IGS y la huella digital tras amplificación por PCR de la secuencia microsatélite [GACA]₄. Nuestros resultados muestran 5 de los genotipos *URA5* con una elevada frecuencia del tipo VNI (33%), lo que concuerda con otros estudios. Es de resaltar la elevada presencia del perfil VNIII entre nuestras cepas (28,9%), lo que podría ser el rasgo específico más destacable de los aislamientos de nuestro país. El marcador 5S rDNA/IGS mostró un bajo poder discriminativo intraespecie. Se obtuvieron 3 perfiles moleculares distintos (S1-3), que presentaron una buena correlación con las especies, variedades y genotipos. La obtención de perfiles de huella digital con [GACA]₄, presentó una gran variabilidad entre las cepas estudiadas. La representación en dendrograma agrupó las cepas en 4 agrupamientos principales relacionados con el origen de las levaduras y también con cierta concordancia con los genotipos descritos (VNI-IV y VGI).

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The “*Cryptococcus neoformans* species complex” is a denomination for the diverse yeasts that cause cryptococcosis, one of the most prevalent life-threatening mycoses^{10,28,33}. Since the first descriptions of *C. neoformans*, many intraspecific differences have been described and a complicated sub-classification has been established within the species^{17,23,25}. Further studies revealed several differences between the anamorphs of the two described varieties (*C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*) regarding ecology, epidemiology, pathogenicity, biochemistry and genetics^{5,6}. Thanks to molecular biology tools, the existence of two species (*Cryptococcus neoformans* and *Cryptococcus gattii*) has been well established²⁶. Each of them corresponds to two distinct set of serotypes. In the case of *C. neoformans* the serotypes are A and D, and two varieties are proposed for each of them (*C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*) but the existence of hybrid strains with an AD serotype is well known⁵. For *C. gattii* the serotypes are B and C. Nevertheless, some anomalous serotypes BD have been described. These strains could be considered as hybrids between the two species *C. neoformans* and *C. gattii*⁵.

Although serotyping was the first method used to study the epidemiology of cryptococcosis, the analysis of genomic patterns obtained after amplification of minisatellite/microsatellite sequences, intergenic spacer regions and/or the digestion of some specific genes improved the sensitivity and specificity of strain typing^{1,10,13,20,21,29,31}. These molecular tools provide a new possibility for the epidemiological survey of *Cryptococcus* infections and ecological assessment of the source of the disease.

Cryptococcosis by *C. neoformans* is cosmopolite mostly in immunocompromised patients. *C. gattii* has more aggressive behaviour and causes infections more frequently in immunocompetent patients²⁴. Until recently, this species has been considered to be restricted to warm areas (tropical and subtropical climates). These free-living yeasts can be isolated from soils, avian excreta and plant material^{1,6}. However, the outbreak of cryptococcosis by *C. gattii* in the temperate climate of Vancouver Island (British Columbia, Canada)²² indicated a shift in the ecological niche of this species.

In the last decade, a number of DNA typing techniques have been used to study the epidemiology of *C. neoformans*. PCR fingerprinting has been used as the major typing technique for molecular epidemiological surveys of *C. neoformans* species complex¹². In 1999 Meyer et al²¹ described eight major molecular types, VNI-IV for *C. neoformans* strains, and VGI-IV for *C. gattii* strains, which were proposed as epidemiological markers. The strains were typed using M13 PCR fingerprinting and orotidine monophosphate pyrophosphorylase (*URA5*) gene restriction fragment length polymorphism analysis (RFLP). In 2003 the same group carried out a study of more than 400 clinical and environmental isolates from Latin-American countries and Spain³⁰.

Our laboratory leads the coordination of the Spanish cryptococcosis survey – within the European Conference of Medical Mycology Societies (ECMM) Cryptococcosis Working Group – which allowed us to type a large number of Spanish strains from environmental, human and animal samples^{8,9}. Some of them were previously studied with pulsed field gel electrophoresis (PFGE) and their electrophoretic karyotypes showed a very high genomic variability¹⁹. At that time, there was no clear explanation for that genomic variability and no relationship with pathogenic aspects could be established. The possibility of studying the strains with newly proposed molecular markers encouraged the present work.

Three molecular techniques evaluating different molecular markers have been assayed for strain typing in this study. These were considered as low discriminative (5S rDNA RFLP analysis), moderately (*URA5* RFLP analysis) and highly discriminative methods ([GACA]₄ PCR fingerprinting)³⁵. Therefore, each could provide a different applicability in epidemiological studies, i.e. [GACA]₄ PCR fingerprinting is a good method for discrimination between highly

related strains from the same origin. On the other hand, 5S rDNA RFLP analysis is a good discriminative tool to differentiate between *C. neoformans* and *C. gattii* species and varieties. The results obtained by *URA5* RFLP analysis allowed classification of the strains in previously defined genotypes and comparison with other epidemiological studies.

Material and methods

Yeast isolates

A total of 97 strains of *C. neoformans* species complex were analysed in this study. All of them were isolated in Spain: 20 from pigeon droppings, 64 from human clinical samples and 13 from veterinary samples (goats)². In addition, 3 reference isolates provided by J.M. Torres (IMIM, Barcelona, Spain) and 8 from W. Meyer (University of Sydney, Australia), were used as controls for reproducibility of the techniques^{3,31}. Detailed information for the isolates is provided in Table 1. As indicated in Figure 2, four pairs of strains from clinical samples were isolated from the same patient at different times during their disease follow-up.

Phenotype characterization

All of the isolates were identified as belonging to the *C. neoformans* species complex by standard methods: yeast and colony morphology, ability to grow at 30 and 37 °C on Sabouraud dextrose agar (SDA; Oxoid Ltd., Basingstoke, Hampshire, England), urease production (Urease Christensen), presence of capsule by India ink negative stain, pigment production on Staib medium, colorimetric sugar assimilation, actidione sensitivity and phenoloxidase production (Auxacolor Bio-Rad, France). Isolates were grown on Canavanine Glycine Bromothimol Blue (CGB) medium to discriminate between *C. neoformans* and *C. gattii*³².

Serotyping

The strains were serotyped by latex agglutination with the Crypto Check commercial kit (Iatron Laboratories, Tokyo, Japan) according to the manufacturer's instructions.

Genotyping

High molecular weight genomic DNA was extracted from cryptococcal cells by a modified method¹⁶ based on the standardized protocol described by White et al³⁹. At least two independent DNA preparations were performed for each isolate. PCR reactions were carried out in a final volume of 50 µl. Each reaction contained 1 × PCR buffer (75 mM Tris-HCl pH 9; 3 mM MgCl₂; 50 mM KCl; 20 mM (NH₄)₂SO₄; 0.001% BSA; Biotools B&M Labs, S.A., Madrid, Spain), a final concentration of 200 µM of dNTPs, 1 U Taq DNA polymerase (Biotools B&M Labs, S.A., Madrid, Spain), 25 pmol of each indicated primer and 5 µl of extracted DNA. All PCR reactions were carried out in an Eppendorf Mastercycler gradient[®] (Eppendorf, Hamburg, Germany).

Amplification products from each PCR reaction were mixed with 20 µl of MilliQ water and separated by 2% (PCR fingerprinting) or 3% (*URA5* RFLP products) agarose gel electrophoresis at 60 V for 2 or 3 h (PCR fingerprinting and RFLP analysis, respectively). Patterns were assigned visually by comparison with the molecular standard (Gene Ruler 100 pb DNA ladder Plus, MBI Fermentas, Vilnius, Lithuania). Images were captured by DigiDoc-It System v.1.1.27 (UPV, Cambridge, UK).

– 5S rDNA RFLP analysis: PCR amplification was carried out with primers 5S1 (5' GATCTACTGAGGCTAAGCCC 3') and 5S2 (5' AGACAA-GCATATGACTACTG 3'). Conditions were adjusted as follows: 30 cycles; 95 °C 5 min initial denaturation, 1 min of denaturation at 95 °C, 2 min annealing at 52 °C, and 3 min extension at 72 °C, followed

Table 1
Species, serotypes and source of isolation of studied strains of *Cryptococcus neoformans* and *Cryptococcus gattii*

Species	<i>Cryptococcus</i>			Source of strains		Reference strains	
	Serotypes	Human	Veterinary (Cáceres)	Environmental (Alicante)	WM	RV	
<i>C. neoformans</i>	A	39	–	19	2	1	
	D	12	–	1	1	–	
	AD	10	–	–	1	–	
	Not determined	2	–	–	–	–	
<i>C. gattii</i>	B	1	13	–	3	1	
	C	–	–	–	1	1	
Total		64	13	20		11	
			97			106	

All the studied strains are from Spain. Reference strains for genotyping (WM strains) and serotyping (RV strains) were included.

by a final extension cycle of 10 min at 72 °C^{11,14}. Five microliters of PCR products were digested with 1 U of *CfoI* (ROCHE, Barcelona, Spain) for 2 h at 37 °C.

– URA5 RFLP analysis: PCR were performed with primers URA5 (5' ATGTCCTCCCAAGCCCTCGACTCCG 3') and SJ01 (5' TTAAGACC-TCTGAACACCGTACTC 3'). Conditions were adjusted as follows: 35 cycles at 94 °C for 2 min initial denaturation, 45 s of denaturation at 94 °C, 1 min annealing at 61 °C and 2 min extension at 72 °C, followed by a final extension cycle for 10 min at 72 °C³⁰. Fifteen microliters of PCR products were double digested with *Sau96I* (10 U/ml) and *HhaI* (20 U/ml) for 3 h at 37 °C.

– [GACA]₄ PCR fingerprinting: the microsatellite-specific primer [GACA]₄ (5' GACAGACAGACAGACA 3') was used as a single primer in the PCR reactions^{7,30}. Amplification conditions were 40 cycles at 94 °C for 5 min initial denaturation, 30 s of denaturation at 94 °C, 45 s annealing at 43 °C and 30 s extension at 72 °C, followed by a final extension cycle for 10 min at 72 °C.

Data analysis

The NTSYS-pc version 1.8 (Applied Biostatistics, Inc.) was used to express results in a quantitative form. DNA bands obtained with each molecular method were defined manually. Similarity coefficients were estimated using the Jaccard algorithm and cluster analysis was performed by the Unweighted Pair Method with Arithmetic mean (UPGMA)³⁴.

Reproducibility of the techniques

To assess intra-laboratory reproducibility of the methods of DNA extraction, PCR amplification and digestions were repeated three times with each strain. Inter-laboratory reproducibility of the methods was tested using reference strains. The eight molecular profiles described in the literature for URA5 RFLP analysis were obtained by including reference strains in the analysis. For [GACA]₄ PCR fingerprinting, previous works have reported the high variability of the patterns^{7,30} and subsequently the inter-laboratory instability. Only intra-laboratory reproducibility could be assessed by obtaining the DNA fingerprinting for each strain at least three times.

Results

Phenotypes and serotype diversity of the isolates

After performing the different tests described in Materials and methods, it was determined that 83 (84.7%) of the isolates corresponded to *C. neoformans* and 14 (15.3%) to *C. gattii*. All studied isolates showed positive results for urease and phenoloxidase production, and growth at 37 °C. Carbohydrate assimilation showed 10 different patterns. Lactose was always negative and inositol was positive for 98%. The most frequent phenotypes were Auxacolor

Table 2
Distribution of strains on the different serotypes and genotypes obtained with restriction fragment length polymorphism of IGS/5S and URA5 genes

Serotype	RFLP URA5					Total
	VNI	VNII	VNIII	VNIV	VGI	
5S RFLP						
S1						
A	27	8	9	1	45	48 + 1*
D				1	1	
AD		1	1		2	
S2						
A	4	2	7		13	33 + 1*
D			3	9	12	
AD			7	1	8	
S3						
B					14	14
Total	31 + 1*	11	27 + 1*	12	14	95

S4 pattern (for IGS/5S RFLP method) and VGII, VGIII and VGIV (for URA5 RFLP) were not found in this study.

*Not serotyped strains.

75774 and 75775, with almost the same frequency of isolates, 37 (38.1%) and 34 (35.1%), respectively. All other auxonotypes presented a low (5–10 strains) or very low rate (< 5 strains).

The serotyping results showed that 58 isolates were serotype A (59.8%), which was the most frequent; 13 isolates (13.4%) were serotype D and 10 (10.3%) belonged to the AD hybrid. All the 14 *C. gattii* strains (14.4%) were serotype B. Two strains could not be serotyped (3.1%). Serotype C was absent in our study but reference strains were used to obtain and compare other possible molecular patterns (Table 1).

Genotype analysis of the isolates

Molecular profiles obtained by RFLP of the amplified genes IGS-5S and URA5 showed a number of different molecular patterns, which could be analysed manually by visual comparison of profiles. Data obtained after PCR fingerprinting with the microsatellite [GACA]₄ showed a very high intra-species variability. For analyses, such high variability required the use of a specialized software that compared patterns and then clustered strains in dendrograms.

IGS RFLP (5S) analysis grouped the isolates in three major previously described genotypes (5S1–5S3). This molecular marker clearly differentiates the two species and varieties. For S1 and S2 types, 100% belonged to *C. neoformans*, and 100% of S3 types belonged to *C. gattii* isolates (VGI genotype). The S1 pattern was the most frequent among our isolates, which mainly corresponded to VNI/VNII *C. neoformans* var *gribii* strains (Table 2). The S4 pattern was not found in our study.

Concerning URA5 gene RFLP analysis, analyses of our isolates showed five of the eight patterns described previously^{30,31}. Figure 1 shows a typical result of this kind of analysis. The profiles VNI and

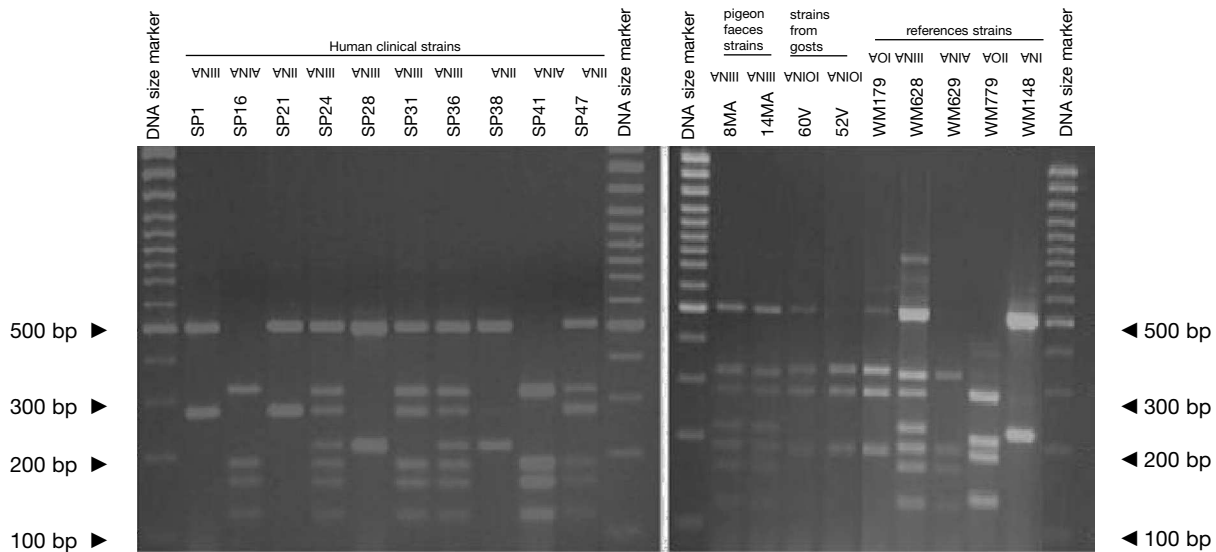


Figure 1. RFLP patterns obtained after double digestion of the *URA5* gene. SP strains: isolates from human clinical samples; MA strains: isolates from pigeon faeces; V strains: isolates from goat tissues; WM strains: reference strains used to reproduce the molecular patterns VNI, II, III, IV, VGI and VGII, according to the molecular standards previously described (see Material and methods).

VNIII showed the highest frequency (33% and 28.9%, respectively). Profiles VGI (14.4%), VNIV (12.4%) and VNII (11.3%) had similar frequencies. The virulent VGII genotype, which is related to some *C. gattii* B strains, was not found in this study²². VGIII and VGIV profiles were also absent among the studied isolates. All our serotype B strains belonged to the VGI molecular type (Table 2).

Reference strains included in the study showed identical profiles for *URA5* gene RFLP (Fig. 1) as previously published^{30,31}, providing us with appropriate tools for pattern identification and assessment of reproducibility.

PCR fingerprinting with the microsatellite $[GACA]_4$ showed 48 different profiles. Patterns were described taking into account each band that was constant for the same strain in successive DNA amplifications (repetitive analysis). This molecular marker showed the highest diversity. Results were analysed using the NTSYS software³⁴, which clustered the strains on the basis of their similarity. The dendrogram in Figure 2 summarizes the results that were obtained. Strains were grouped in four main clusters, which show a good correlation with the different origin of the isolates. Cluster 1 groups the veterinary isolates, clusters 2 and 3 harbour 95.3% of the clinical isolates and cluster 4 consists of 60% of the environmental strains. The four pairs of strains isolated from the same patient always appear in the same branch in identical yeasts. Isolates of VGI and VNII genotypes clustered together in the dendrogram. Nevertheless, other genotypes (VNI, VNIII and VNIV) are randomly distributed in the groups of *C. neoformans* isolates (Fig. 2).

Geographical distribution of the isolates

Geographical distribution of the strains in the map of Spain (Fig. 3) shows a random distribution of all the genotypes obtained from clinical samples. The *URA5*-VNIII profile, which would be considered especially important in our country, was detected in different parts of our geography. Regarding environmental strains, the high presence of this VNIII genotype among the isolates from pigeon droppings (50%) obtained in Alicante is remarkable. *C. gattii* was detected in two different regions of the country that were approximately 700 km apart. All the strains obtained from goats in Cáceres showed a high clonality and had the same genotype with all the molecular markers used. The unique isolate of this species obtained from a human patient in Alicante also showed the same genotype.

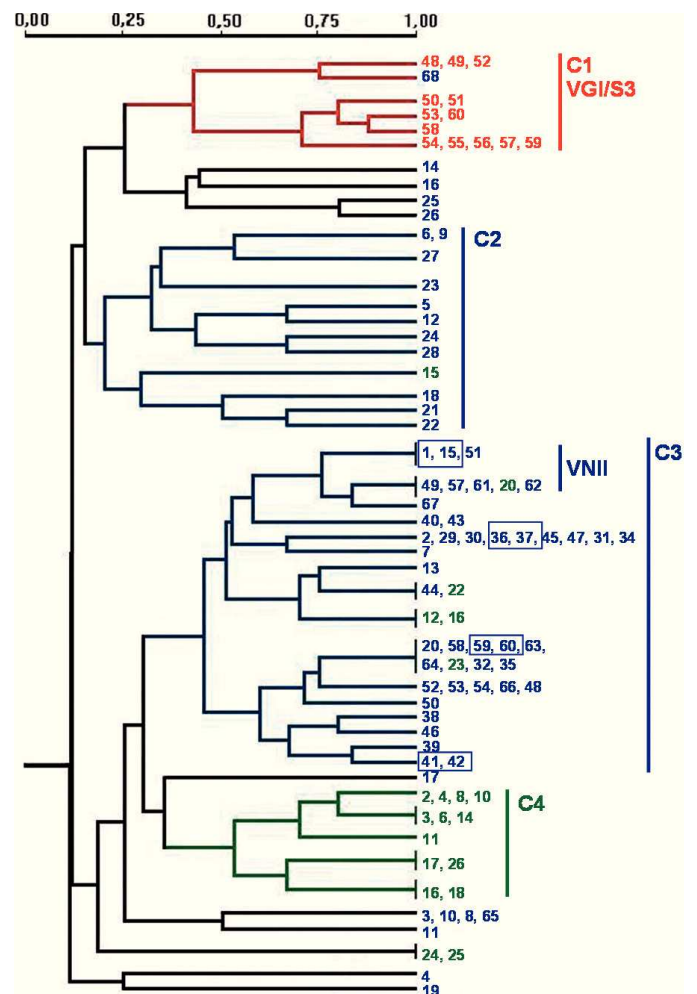


Figure 2. Dendrogram obtained with molecular profiles of all studied isolates after $[GACA]_4$ amplification. Blue numbers correspond to strains obtained from human clinical samples (SP1-SP68). Green numbers: environmental strains from pigeon droppings (MA1-MA45); red numbers: strains from goat tissues (48V-60V). Blue boxes indicate strains obtained from a same patient. Main clusters identified by numbers from C1 to C4.

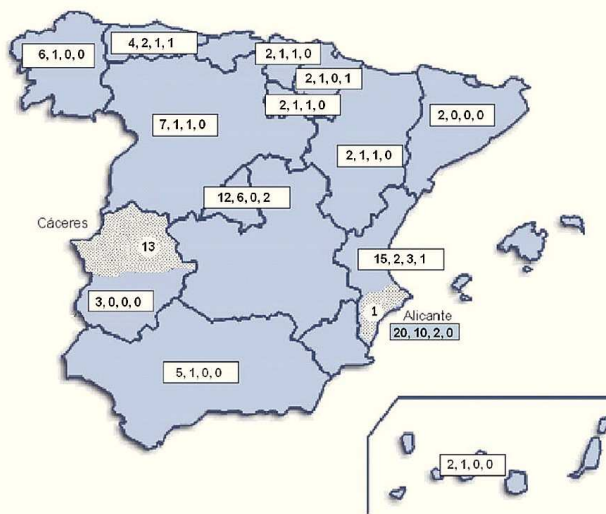


Figure 3. Geographic distribution of the isolates studied. Numbers in the boxes indicate correlatively the follows: first – total number of clinical isolates from that region of Spain; second – isolates showing URA5 VNI pattern; third – isolates showing URA5 VNIII pattern. Dotted areas correspond to the provinces of Alicante and Cáceres, where the environmental and veterinary strains were, respectively, isolated.

Discussion

During the last 10 years and from different parts of the world, a number of interesting reports have been published regarding the molecular typing of yeasts belonging to the *C. neoformans* species complex^{4,15,20,37,36}. Besides the geographical differences reported for *C. neoformans* and *C. gattii* species, most of the results published showed that there is a predominant genotype for each of these two pathogenic species: VNI is the most prevalent profile among *C. neoformans* isolates all over the world and VGI within the *C. gattii* strains. In the present study, we also found VNI and VGI to be the most prevalent genotypes for each species in Spain. Nevertheless, the main finding in our work is, undoubtedly, the high presence of VNIII genotype of *C. neoformans* in our country.

The RFLP study of *URA5* gene showed five of the eight previously described profiles (VNI-IV and VGI). The VGII, VGIII and VGIV profiles were absent among *C. gattii* serotype B strains and only VGI genotype was detected for this species. For *C. neoformans* isolates, VNI was the most prevalent type (33%) followed by VNIII (28.9%), which showed a very high prevalence when compared with other studies. These results agree with those obtained by Meyer et al³⁰, on a limited number of Spanish isolates. In Meyer's study of Iberoamerican isolates, they found 42% of VNIII among Spanish strains. This genotype is absent in a number of studies from different parts of the world^{30,36} or had a very low presence in some others like the 3.6% and 1.5% in Argentina and Mexico^{15,30}. Only in Chile, a significant presence of this profile had been described (15.8%).³⁰ In our study, VNIII pattern had the highest presence among the environmental strains (50%) obtained from pigeon droppings, and also showed a very important presence within clinical isolates (28%).

This similarity between Chile and Spain is not restricted to the VNIII pattern. *C. neoformans* genotype VNIV also had a remarkable presence in our study (12.4%), again similar to the rate found in Chile (26.3%) by Meyer et al³⁰. This prevalence is higher than in other Latin-American countries, where the VNIV pattern was almost absent. VNIII pattern is mainly related to hybrid *C. neoformans* yeasts (AD serotypes) and VNIV to *C. neoformans* var. *neoformans* (D serotype)³⁰. The high presence of D serotype in Europe is well assessed by some previous studies^{3,9,37,38}. So the origin of the high prevalence of hybrid genotypes could be explained for the co-

existence of the two haploid forms (A and D) in our geographical area. In this regard, it is important to remark that most of the VNIII strains in our study exhibited serotype A (59.3%), although 80% of the serotype AD isolates were also VNIII. This probably means that some hybrid forms of *C. neoformans* only show one serotype.

Amplification with microsatellite $[GACA]_4$ revealed a highly discriminative power. Among the studied isolates, 48 different profiles were obtained. As in other studies, strains from a same species clustered together^{20,27} and we also found a good correlation between the PCR fingerprinting and the origin of the isolates. The dendrogram obtained with this method grouped the strains in four main clusters that correlate with the source of the isolates and, in some cases, with the molecular types (Fig. 2). The first cluster – C1 – harbours all VGI/S3 genotypes, which also correspond to all the *C. gattii* isolates studied. Therefore, all strains from veterinary origin were grouped here with the unique *C. gattii* of human origin. The low number of strains and the common origin of the veterinary ones (from a cryptococcosis outbreak among goats) make it difficult to assess that the $[GACA]_4$ profile is specific to this origin, species or genotype. For *C. neoformans* strains, two big clusters grouped 95.3% of the clinical isolates and a third one grouped 60% of the environmental isolates (Fig. 2). It is also remarkable that 72.7% of the VNII isolates are grouped in the same branch inside the second clinical cluster – C3 – while other *C. neoformans* genotypes (VNI, VNIII and VNIV) appeared randomly distributed among the three clusters.

The specific case of the strains obtained from the same patient at different times during the follow-up of the disease showed that they were consistently grouped together in the same branch as identical strains. Other studies with $[GACA]_4$ and $[GTG]_5$ as molecular markers also proved that strains from the same patient, but from different parts of the body, always showed the same molecular type^{20,31}. From these results, we can conclude that the diverse patterns obtained with $[GACA]_4$ fingerprinting are stable (intra-laboratory) and thus, could be a useful method for either the follow-up of a strain or the differentiation between relapse and reinfection in patients who suffer from repetitive episodes of cryptococcosis.

Molecular patterns obtained by digestion of 5S-IGS amplified fragments showed three different profiles within the studied strains. These "S types" can be easily related with species, varieties and serotypes. In fact, serotype D and AD strains of *C. neoformans* belonged to the S2 profile at a significantly high rate (92.3% and 80%, respectively) and showed VNIII and VNIV genotypes (Table 2). Serotype A strains were distributed in S1 (77.6%) and S2 (22.4%) profiles. S1 type was the most prevalent and harboured most of *C. neoformans* VNI and VNII (*C. neoformans* var. *grubii*) isolates included in the study. Serotype B isolates (*C. gattii*, VGI) corresponded 100% to the S3 type. This method has been scarcely used by other authors^{11,14}. So the results are somehow independent and difficult to compare. Therefore, inter-laboratory reproducibility could not be evaluated.

Nowadays, the source of most *Cryptococcus* infections is not very clear, and some epidemiological events, like the Vancouver outbreak, ought to make us think about the significant lack of knowledge we have about their route from the environment to human or animal patients, the worldwide spread of the species or genotypes of the yeast, and the different virulences they could display. The excellent work carried out with the Vancouver outbreak strains is a good example to reinforce the study of *Cryptococcus* typing²². The study based on some genotyping methods like *URA5* RFLP, AFLP analysis and Multilocus Sequence Typing (MLST) showed a special situation in the Vancouver outbreak with a high spread of two subtypes of VGII molecular type¹⁸. From that moment on, a relationship between high virulence and genotype VGII could be established within *C. gattii* B serotype and some hypothesis of the probable spread from their origin was proposed.

The results shown here could indicate the importance of local environmental factors for strain distribution of this pathogen in a

limited geographical area. We can confirm the high frequency of the VNIII profile previously described by Meyer and his co-workers in their Iberoamerican's strain study. This fact, together with the still unexplained goat's cryptococcosis outbreak that occurred in Cáceres some years ago, makes our country different from other European and Mediterranean areas regarding the epidemiology and ecology of *Cryptococcus* species complex. The similarity between Spain and Chile in the prevalence of some *C. neoformans* var. *neoformans* and hybrid strains could be an interesting feature to explain the spread of this yeast around the world.

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