Population structure and diversity of the pathogenic fungus *Aspergillus fumigatus* isolated from different sources and geographic origins

Esperanza Duarte-Escalante[1], Gerardo Zúñiga[2], Oscar Nava Ramírez[1], Susana Córdoba[3], Nicolás Refojo[3], Roberto Arenas[4], Laurence Delhaes[5], María del Rocío Reyes-Montes[1]/+

[1]Laboratorio de Micología Molecular, Departamento de Microbiología-Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México, 04510 México, DF, México [2]Departamento de Zoología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México, DF, México [3]Departamento Micología INEI ANLIS Dr. Carlos G. Malbrán, Buenos Aires, Argentina [4]Departamento de Dermatología, Hospital General Dr. Manuel Gea González, General Hospital, México, DF, México [5]EA3609, Lille 2 University, Pasteur Institute of Lille, Lille, France

Fifty-five clinical and environmental Aspergillus fumigatus *isolates from Mexico, Argentina, France and Peru were analyzed to determine their genetic variability, reproductive system and level of differentiation using amplified fragment length polymorphism markers. The level of genetic variability was assessed by measuring the percentage of polymorphic loci, number of effective alleles, expected heterozygocity and by performing an association index test (I_A). The degree of genetic differentiation and variation was determined using analysis of molecular variance at three levels. Using the paired genetic distances, a dendrogram was built to detect the genetic relationship among alleles. Finally, a network of haplotypes was constructed to determine the geographic relationship among them. The results indicate that the clinical isolates have greater genetic variability than the environmental isolates. The I_A of the clinical and environmental isolates suggests a recombining population structure. The genetic differentiation among isolates and the dendrogram suggest that the groups of isolates are different. The network of haplotypes demonstrates that the majority of the isolates are grouped according to geographic origin.*

Key words: *Aspergillus fumigatus* - AFLP - genetic diversity - polymorphisms

Aspergillus fumigatus Fresenius is the causal agent of aspergillosis, a widely distributed fungal disease. It lives in soil, where it grows among organic remains. This fungus contributes to the decomposition of organic matter, such as compost (humus) and fodder, while also playing an active role in the recycling of carbon and nitrogen sources (Latgé 1999, 2003).

A. fumigatus produces large amounts of spores on its conidial head. The dispersion mechanism of these spores is very simple, as the conidia are released into the environment through air currents. The conidia range between 2-3 µm in diameter (Latgé 1999, 2003). On average, humans inhale hundreds of these infectious propagules daily, yet inhalation of the conidia by immunocompetent hosts rarely shows adverse effects since their immune systems are able to eliminate the fungus. Disease occurs, however, when the host response is either too strong or too weak. In immunocompromised hosts, *A. fumigatus* represents a major cause of morbidity and mortality. In the last decade, this patient population is expanding, even in developed countries, due to the increased use of transplantation, to the development of immunosuppressive and myeloablative therapies for autoimmune and neoplastic disease, and to the human immunodeficiency virus/AIDS pandemic (Hohl & Feldmesser 2007). While the clinical epidemiology, human risk factors, and treatment strategies for fungal opportunists such as *Candida* spp. and *Aspergillus* spp. are currently well codified (Eggiman et al. 2003, Singh & Paterson 2005, Ullmann & Cornerly 2006), relatively little is known about *Aspergillus* environmental circulation, reproduction or molecular epidemiology.

Molecular studies characterizing aspergillosis in others countries, especially in European countries (Fridkin & Jarvis 1996, Menotti et al. 2005), have been increasing in recent years, but few data are available from Latin or South American countries. Several molecular methods have been evaluated for *A. fumigatus* strain typing and these methods include randomly amplified polymorphic DNA typing (Aufauvre-Brown et al. 1992), sequence-specific DNA primer analysis (Lin et al. 1995), polymorphic microsatellite markers (Bart-Delabesse et al. 1998, 1999, de Valk et al. 2005, 2007) and analysis of hybridization profiles with the dispersed, repetitive DNA probe *Afut1* restriction fragment length polymorphism (RFLP) (Neuveglise et al. 1996, Chazalet et al. 1998). Those molecular typing techniques allow elucidation of the epidemiology of *Aspegillus* infections and investigation of potential case clusters, but they can also provide information to analyze whether the micromycete reproduction mode affects antifungal susceptibility evolution, especially in terms of resistance gene dissemination.

Although numerous papers were published recently on the application of several typing techniques to *A. fumigatus*

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+ Corresponding author: remoa@servidor.unam.mx
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isolates, only some authors attempted to examine the reproductive mode of *A. fumigatus* populations and the conclusions drawn by them were controversial (Girardin et al. 1994, Fridkin & Jarvis 1996, Lasker 2002, Levdansky et al. 2007). In Latin America, there is very little information on these different aspects of the epidemiology of aspergillosis. The most outstanding epidemiological data are summarized and reported in retrospective studies on patients diagnosed with fungal infections or correspond to case reports. The observation of high levels of genetic diversity within this supposedly asexual species (Varga & Tóth 2003).

To address these concerns, we performed a molecular analysis of *A. fumigatus* isolates to examine genetic variation among clinical and environmental *A. fumigatus* isolates from different geographical origins [Mexico (MX), Argentina (AR), France (FR) and Peru (PE)] to determine mode of fungal reproduction and the contribution of each variable to the disease epidemiology, in order to improve our control and prevention therapy guide lines.

We choose the AFLP (amplified fragment length polymorphisms) technique, because it represents a highly discriminatory method at the intraspecific level and previously has been shown to have good discriminatory power for fungal strain differentiation (O'Donnell et al. 2004, Delhaes et al. 2008). In AFLP analyses, fragments are amplified from random locations throughout an organism's genome in a highly reproducible manner (Vos et al. 1995, O'Donnell et al. 2004, Delhaes et al. 2008).

MATERIALS AND METHODS

Isolates - Fifty five isolates of *A. fumigatus* were used (Table I). *A. fumigatus* isolates were identified by their macroscopic or microscopic appearance and their ability to grow at 48°C. The fungal strains were cultured on YPD at 37°C for 18 h and DNA extraction was performed as described by Reyes-Montes et al. (1999).

AFLP - AFLP analysis was performed as described by Vos et al. (1995). Briefly, DNA was restricted with the endonucleases *Eco*RI and *Mse*I. After restriction, adaptors were ligated to the resulting fragments. The resulting fragments were preamplified with primers E (5′-GACTGCGTACCAATTC-3′) and M (5′-GACGATGAGTCCTGAGTAA-3′), followed by a second, selective PCR. The selective primers were identical to primer E or M but were extended with specific two or three nucleotides at the 3′- terminus. Eight primer combinations were used: E+AA:M+CTC; E+AC:M+CAC; E+AA:M+CAT; E+AC:M+CTC; E+AA:M+CTG;

TABLE I

Source and geographic origin of *Aspergillus fumigatus* isolates

Isolate	Source	Geographic origin	Isolate	Source	Geographic origin
MM-7	Environment	MX	951740a	Clinical	AR
MM-8	Environment	MX	951741	Clinical	AR
MM-9	Clinical	MX	951744	Clinical	AR
MM-10	Clinical	MX	951745	Clinical	AR
MM-11	Clinical	MX	951746	Clinical	AR
MM-32	Clinical	MX	951747	Clinical	AR
MM-33	Clinical	MX	951748	Clinical	AR
MM-34	Clinical	MX	88248	Clinical	AR
MM-35	Clinical	MX	90370	Clinical	AR
MM-36	Clinical	MX	982928	Clinical	AR
MM-37	Clinical	MX	993315	Clinical	AR
MM-38	Clinical	MX	9272	Clinical	AR
MM-39	Clinical	MX	8571	Clinical	AR
MM-45	Clinical	MX	6578	Clinical	AR
MM-46	Clinical	MX	51435	Clinical	PE
Amb III	Environment	AR	51594	Clinical	PE
Amb V	Environment	AR	53027	Clinical	PE
Amb VIII	Environment	AR	53097	Clinical	PE
951740	Clinical	AR	Af-8	Clinical	FR
951744	Clinical	AR	Af-11	Clinical	FR
951746	Clinical	AR	Af-15	Clinical	FR
951722	Clinical	AR	Af-22	Clinical	FR
951733	Clinical	AR	Af-26	Clinical	FR
951734	Clinical	AR	Af-29	Clinical	FR
951736	Clinical	AR	Af-34	Clinical	FR
951737	Clinical	AR	Af-35	Clinical	FR
951738	Clinical	AR	Af-41	Clinical	FR
951739	Clinical	AR			

AR: Argentina; FR: France; MX: Mexico; PE: Peru.

E+AC:M+CAT; E+AA:M+CAC; E+AC:M+CTG. Primer E was radioactively labelled with ATP-P^{32} and the amplified material was analyzed on 5% polyacrylamide slab gels.

Statistical analysis - Reproducible bands with sizes between 100-400 bp in different samples across the gel were scored. AFLP markers were scored visually, compared with a 50 bp DNA Ladder (Invitrogen Life Technologies, Carlsbad, CA), manually coded and analyzed as binary data of presence (1) or absence (0). Genetic diversity of AFLP markers was calculated by Shannon's Index (*I*), assuming that each phenotypic marker represents a distinct locus (Allnutt et al. 1999), and Nei's genetic diversity, using allelic frequencies (Nei 1973). Additionally, expected heterozygosity per population and average heterozygosity were calculated using allele frequencies according to Zhivotovsky's Bayesian method (Zhivotovsky 1999). Analysis of molecular variance (AMOVA) using FAMD v 1.1β (Schlüter & Harris 2006) was performed to partition the molecular variance into different hierarchical levels. AMOVA analysis was carried out to test differences among isolations, among isolations within countries and between isolation sources (clinical and environmental).

The statistical significance of the molecular variance partitioning and the associated estimates of φ-statistics were assessed by conducting 10,000 random permutations of the data (Excoffier et al. 1992). Pairwise genetic distances among groups of isolates were calculated using Nei´s method (Nei 1973). The groups were formed according to their geographic origin (countries) and isolation source (clinical and environmental). The genetic relationship among them was established by unweighted paired group method using the arithmetic mean (UPGMA - PopGene 1.31 program) (Yeh et al. 1999). The distortion of the inferred tree was estimated by means of the cophenetic correlation coefficient (CCCr) using Mantel's test (Manly 1997). In addition, a haplotype network was built to evaluate the genealogical relationships among AFLP haplotypes by the Median-Joining method (Bandelt et al. 1999) implemented in NETWORK 4.2.0.1 (htt://www.fluxus-engineering.com, Polzin & Daneschmand 2003). The parameters used were epsilon = 0, 1/1 transitions-transvertions weight, 10 characters weight, and connexion criteria. To distinguish between clonal and recombinant structures, we used the index of association (I_A) (Maynard-Smith et al. 1993), a statistical test that measures the degree of non-random association between alleles at different loci (linkage disequilibrium), implemented in LIAN v 3.5 (Haubold & Hudson 2000).

RESULTS

Using the eight combinations of primers, a total of 105 AFLP markers were amplified. Genetic variation levels of isolates are shown according to geographic and isolation source in Table II. In general, the estimates of genetic variability indicate that the clinical isolates are more variable than the environmental ones. Average polymorphism of isolates varied from 81.90% (AR) to 22.86% (PE) and from 18.10% (AR) to 6.67% (MX), respectively, for clinical and environmental isolates. The number of effective alleles, as well as values for genetic diversity estimated from the *I* - an estimator not sensible to the number of isolates analyzed - and the average heterozygosity showed the same tendency for both clinical and environmental isolates. The AMOVA shows the structure of the genetic variation of the group of isolates analyzed. The percentage of variation shared among the clinical and environmental isolates was very low (9.05%) and the genetic differentiation was statistically significant (Fst = 0.253, $p < 0.001$). Between these two groups, the shared variation among geographically grouped isolates was slightly greater (16.22%), but the differentiation was also statistically significant (Fst = 0.178, $p < 0.001$). Finally, the shared variation among the isolates from the same country was high (74.73%) and their genetic differentiation did not differ from zero (Fst = 0.091, p = 0.046).

The I_A value estimated from all of isolates indicates that *A. fumigatus* has a recombinant reproductive system (Table III). The analysis conducted on partial isolate groups by geographic origin and isolation source showed similar results.

The dendrogram shows the integration of two well-defined groups (Fig. 1). The first is comprised of isolates from AR and MX, which are separated into two subgroups according to the isolation source of each isolate. The second group clustered the isolates from FR and PE. The CCCr (0.70, $p < 0.005$) indicates both that the tree is a good representation of genetic relationships of the isolates and that the different groups are consistent. Lastly, the network of the total group of AFLP haplotypes shows that the number of mutations ranges between one-20 (Fig. 2). The network of haplotypes shows a large number of reticula-

TABLE II

Polymorphism, effective number of alleles, genetic diversity and expected heterozygosity of *Aspergillus fumigatus* isolates

Population	P (%)	n_e	*I*	h
MX[a]	6.67	1.0471 ± 0.1253	0.0403 ± 0.1071	0.0276 ± 0.0733
MX[b]	72.38	1.5197 ± 0.1083	0.4243 ± 0.0785	0.2912 ± 0.0559
AR[a]	18.10	1.1225 ± 0.1644	0.1038 ± 0.1305	0.0704 ± 0.0898
AR[b]	81.90	1.6128 ± 0.0761	0.4890 ± 0.0524	0.3379 ± 0.0379
FR[b]	70.48	1.4726 ± 0.1292	0.3945 ± 0.0951	0.2683 ± 0.0678
PE[b]	22.86	1.1657 ± 0.1624	0.1361 ± 0.1278	0.0935 ± 0.0888

a: environmental isolates; *b*: clinical isolates; AR: Argentina; FR: France; h: heterozygosity; *I*: genetic diversity, Shannon´s index; MX: Mexico; n_e: effective number of alleles; P: polymorphism; PE: Peru.

TABLE III

Reproductive structure of *Aspergillus fumigatus* isolates

Groups	Isolates	I_A
MX, AR	Enrironmental	0.0111 (< 0.05)
MX, AR, FR, PE	Clinical	0.0378 (< 0.05)
MX	Clinical and environmental	0.0650 (< 0.05)
AR	Clinical and environmental	0.0469 (< 0.05)
FR	Clinical	0.0586 (< 0.05)
PE	Clinical	0.0087 (< 0.05)

AR: Argentina; FR: France; I_A: index of association; MX: Mexico; PE: Peru.

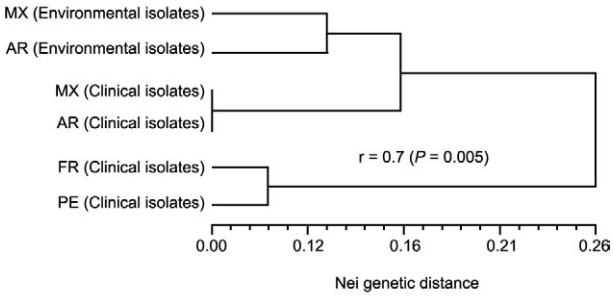

Fig. 1: the dendrogram generated between the environmental and clinical isolates from the different countries constructed using UPGMA pairwise genetic distance (Nei 1973). AR: Argentina; FR: France; MX: Mexico; PE: Peru.

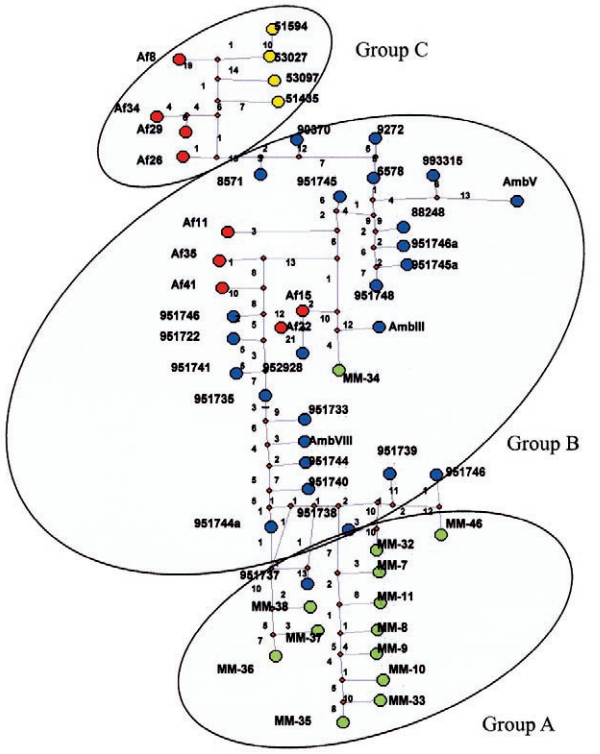

Fig. 2: median-joining network analysis of *Aspergillus fumigatus* isolates. The green circles correspond to the isolates from Mexico; blue circles correspond to the isolates from Argentina; red circles correspond to the isolates from France; yellow circles correspond to the isolates from Peru.

tions among the isolates studied, exhibiting a clear association among the majority of isolates from MX (group A), AR (group B) and PE (group C) to their geographic origin, with some exceptions. The isolates from FR and some from AR are highly variable, which determines their position to be more irregular. However, all the isolates from PE are in the same group.

DISCUSSION

In this study, the AFLP technique used with eight selective base combinations, was found to be useful for distinguishing among *A. fumigatus* strains obtained from the environment and patients, as well as for identifying the structures of populations and their relationships with their geographical origin, according to previous published results (Warris et al. 2003, Bonin et al. 2007, de Valk et al. 2007).

The results of this study demonstrate that the levels of genetic variation between the *A. fumigatus* clinical isolates had the highest values, with the exception of those from Peru. In contrast, the environmental isolates had the lowest genetic variation values. When we analyzed the clinical and environmental isolates of *A. fumigatus* from different geographic origins, the genetic differentiation values found for the clinical isolates were greater than those found for the environmental ones, showing that they are genetically different groups. The dendrogram, based on the Nei genetic distance, groups the MX and AR isolates in a single group, but subclusters the environmental isolates separately from the clinical isolates, indicating that the environmental isolates from MX and AR are genetically different from the clinical ones. On the other hand, the clinical isolates from FR and PE constitute another group, showing that they do not share many genetic characteristics with the MX and AR isolates. Despite the low number of environmental isolates (n = 5) as compared with the large number of clinical isolates (n = 50), genetic differences observed in our study may be explained by (i) a colonization prior to a patient's hospitalization that develops into an infection after the patient becomes immunosuppressed or (ii) concomitant infection by multiple strains where only one survived or has been isolated and typed. Alternatively, genetic differences could be explained by studies reporting that patients inhaling hundreds of *Aspergillus* genotypes from the environment have developed polyclonal invasive aspergillosis more frequently than expected. According to these studies, the percentage of polyclonal infections could be established in 50-75% of the cases; in certain patients, up to five different genotypes could be detected (Bart-Delabesse et al. 1999, Bertout et al. 2001). This also confirms that individuals could be exposed to a great variety of *Aspergillus* genotypes in the environment (Chazalet et al. 1998, Menotti et al. 2005).

These findings suggest that only isolates that adapt to the host will grow. In addition, genetic differences can result from the ability of the isolates that enter the patient to reactivate their virulence, which changes their genotype and generates more variability in relation to the original strain (Latgé 2003). Another explanation is that environmental isolates recovered in the laboratory are not necessarily those that infect patients.

The AMOVA analysis suggests that the genetic variation of *Aspergillus* isolates is highly structured by geographic origin and isolation source. This structure could be by natural selection, as suggested by the observed recombinant reproductive structure and revealed by the I_A estimated in each isolate group, where all I_A values were found to be approximately zero (Table III). It was previously thought that the variability seen in *A. fumigatus* could be attributed to the possibility of parasexual genetic recombinations (Pál et al. 2007). However, these are infrequent and are limited to isolates within the same vegetative compatibility group. Another possibility, which most authors prefer, is the existence of unknown sexual forms or variability produced by previous sexual recombinations at the time when *A. fumigatus* was selected as a species (Varga 2003). These explanations, supported by meta-analysis carried out with conglomerate analysis and association indexes, show that the strains that are geographically related are more genetically similar than those that are not. These findings reaffirm the hypothesis that variability is due to an ancient recombination and some selective pressure (Varga 2003). These hypotheses are reinforced by the work of Paoletti et al. (2005), which shows that *A. fumigatus* isolates containing the complementary alpha-domain MAT gene were identified, and showed the expression of MAT1-1 and MAT1-2 and of genes encoding for sex pheromones and pheromone receptors. These results indicate that *A. fumigatus* has a recent evolutionary history of sexual recombination and might have the potential for sexual reproduction. This form of reproduction has now been confirmed with the finding that a sexual reproductive cycle in this fungus causes the production of cleistothecia and ascospores. When found in its teleomorphic (sexual) state, it was included in the *Neosartorya* genus on the basis of the phylogenetic and morphologic relation of its sexual forms (Rydholm et al. 2006, Samson et al. 2007) and named *Neosartorya fumigata* O´Gorman, Fuller & Dyer sp. nov. (O´Gorman et al. 2009). The recent confirmation of a sexual cycle in *A. fumigatus* explains the great genotypic diversity found among the isolates studied.

When a review of the relationship between isolate haplotypes was performed, it was found that the majority of the isolates were grouped according to their geographic origin, and could be defined by three large groups. Group C contained the majority of the isolates from FR and all of those from PE, which seem to cluster into their own sub-group. On the other hand, group B showed that the AR isolates cluster with some isolates from FR and MX, leading to a relationship close to AR isolates' paraphyletic behaviour. Many reticulations with one-20 mutational changes are seen in the network of isolates, reflecting again the large variability among them, similar to published data by Warris et al. (2003). The current study showed that both clinical and environmental isolates of *A. fumigatus* display genetic diversity, reinforcing conclusions reached by other investigators (Warris et al. 2003). These facts could have repercussions for the diagnosis and treatment of patients with aspergillosis, since it has become more common to find great variation within this species. Changes due to mutations or recombinations can occur and do reflect the *Aspergillus* resistance to anti-mycotic treatments (Díaz-Guerra et al. 2000, Trama et al. 2005).

As *Aspergillus* infections remain difficult to treat (Denning et al. 1997), and the outcomes, especially in immunocompromised patients, are very poor, prevention is ever more important. Therefore, understanding the epidemiology, evolution and ability of this fungus to adapt should help in its future management. Moreover, it is pertinent to point out that in order to obtain greater knowledge about genetic and structural diversity of *A. fumigatus* populations it is important to obtain a greater number of environmental samples. Further molecular studies analyzing clinical and environmental *Aspergillus* isolates will be useful in determining the genetic relationships between isolates responsible for infections occurring in the same institution, in revealing whether this filamentous fungus is able to develop different routes of transmission (air and/or water) as has been proposed (Warris et al. 2003) and which reproduction mode is primarily used by *Aspergillus*.

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