

Genetic structure of *Aspergillus flavus* populations in human and avian isolates

I. Hadrich · I. Amouri · S. Neji · N. Mahfoud · S. Ranque ·
F. Makni · A. Ayadi

Received: 3 July 2012 / Accepted: 20 August 2012
© Springer-Verlag 2012

Abstract *Aspergillus flavus* is the second leading cause of allergic, invasive, and colonizing fungal diseases in humans, and also the second most frequent organism associated with avian infections. Currently, it is not known whether there is a link between the environmental isolates and/or human isolates of *A. flavus* and those responsible for aspergillosis in birds. Microsatellite typing was used to analyze 29 *A. flavus* clinical and environmental avian isolates and 63 human clinical isolates collected from patients with a variety of aspergillosis diseases. The combination of all six markers yielded 77 different genotypes with a 0.98 D value. *A. flavus* genotypes obtained from avian isolates were compared with those obtained from human clinical and environmental samples. The standardized indices of association I_A and $rBarD$ were significantly different from zero ($p < 0.01$), suggesting a prevailing clonal reproduction. There was high genetic diversity between the hospital and poultry environments of *A. flavus* isolates. The human environmental population was significantly differentiated from environmental and clinical avian

populations ($F_{st} > 0.25$). The avian clinical subpopulation exchanged few strains with the environmental human ($N_m = 7.24$) and avian ($N_m = 6.60$) populations. The minimum spanning tree analysis identified three *A. flavus* genotype clusters that were highly structured according to the isolation source ($p < 10^{-4}$).

Introduction

Aspergillus is a ubiquitous, saprophytic mold that can be isolated from a variety of habitats worldwide. In humans, conidia can colonize the respiratory tract, causing pulmonary infections, including bronchopulmonary aspergillosis, aspergilloma, and invasive aspergillosis (IA). In birds, respiratory aspergillosis is considered as a major cause of morbidity and mortality. *A. fumigatus* is a common pathogen in poultry and captive wild birds, and an emerging opportunistic fungal pathogen in immunocompromised humans.

A. fumigatus is the predominant etiological agent of aspergillosis, followed by *A. flavus* [1–3]. *A. flavus* is the second leading cause of allergic, invasive, and colonizing fungal diseases in humans, and also the second most frequent organism associated with avian infections. However, in certain countries such as India, Saudi Arabia, Sudan, and Tunisia, *A. flavus* is the predominant etiological agent [3–5]. To investigate the genetic and epidemiological relationship between environmental and clinical isolates, typing methods with high discriminatory power must be applied.

The epidemiology of human IA hospital outbreaks has been investigated by several groups [6–8]. Moreover, previous molecular typing studies have showed that there was a high variability among *A. flavus* avian isolates and multiple genotypes recovered from healthy and diseased birds [9–11]. However, the relationship between environmental isolates and/or human isolates of *A. flavus* and those responsible for

I. Hadrich · I. Amouri · S. Neji · F. Makni · A. Ayadi (✉)
Laboratoire de Biologie Moléculaire Parasitaire et Fongique,
Faculté de Médecine de Sfax,
Rue Magida Boulila,
3029 Sfax, Tunisia
e-mail: ali.ayadi@rns.tn

N. Mahfoud
Service du Laboratoire, CHU Hédi-Chaker,
3029 Sfax, Tunisia

S. Ranque
Parasitology & Mycology, CHU Timone-Adultes,
Assistance Publique—Hôpitaux de Marseille,
13005 Marseille, France

S. Ranque
Aix-Marseille Université, UMR MD3,
13885 Marseille, France

aspergillosis in birds remain unknown. This study aimed to examine the genetic structure of human and avian populations of *A. flavus* isolates using microsatellite DNA markers.

Methods

Human isolates

Sixty-three human clinical (HC) *A. flavus* isolates were isolated from samples collected from 35 patients with aspergillosis (19 with IA, six with sinusitis, eight with otomycosis, and two with keratomycosis) hospitalized in Habib Bourguiba or Hédi Chaker Hospital, Sfax, Tunisia. Eleven human environmental (HE) isolates were isolated from samples collected in the room (tables, air conditioners, etc.) of patients with IA hospitalized in the hematology ward as described elsewhere [12]. Briefly, in four patients (P2, P8 [12] and P34, P27) with IA, an identical genotype was found in clinical and environmental isolates. Isolates from P2 were collected during the same month 3 days apart (15, 18, and 21.05.09). The isolates from P8 were collected the same day (11.06.05). Isolates collected with an interval of 2 and 3 days had a similar genotype in P34 and P27, respectively.

Avian isolates

Seven clinical (AC) and 22 environmental (AE) *A. flavus* isolates were cultured from 25 lung biopsy samples and 150 environmental (egg shells, food, and litter) samples, respectively. These samples were collected from chickens in a poultry farm located about 15 km north of Sfax. One gram of each litter or food sample was introduced in a tube containing 10 mL of sterile water. After vigorous shaking, 1 mL was inoculated onto a Sabouraud–chloramphenicol agar and Czapek medium. Culture plates were incubated at 25 °C for 4 days. Samples from eggs were collected by swabbing.

Avian clinical isolates were isolated post mortem by plating lung material from deceased chicks (birds number=25) obtained during aseptic necropsy.

Identification of the isolates and DNA isolation

These *A. flavus* strains were identified according to macroscopic and microscopic morphological characteristics and the DNA sequence analysis of the rRNA ITS1, 5.8S, ITS2 region, as previously described [13].

Microsatellites typing

Six microsatellites (AFLA1, AFLA2, AFLA3, AFPM3, AFPM 7, and AFPM1) were used to type *A. flavus* isolates, as we have previously described [12].

Statistical analyses

The Simpson's index of diversity (D) was computed for each marker [14]. The genetic differentiation between both populations was measured using the pairwise fixation index (F_{st}) computed with the Fstat V2.9.3 software [15]. The indirect estimates of gene flow were calculated using Wright's estimate $N_m = (1 - F_{st}) / 4 F_{st}$ [16].

To determine the extent of clonality and recombination in the different populations, the index of association (I_A) and $rBarD$ were computed with Multilocus 1.3b software [17]. Both test the null hypothesis of no linkage disequilibrium, which would indicate the absence of recombination. They are expected to be zero if populations are freely recombining and greater than zero if there is an association between alleles (clonality). The $rBarD$ statistic takes into consideration the number of loci tested and is considered to be a more robust measure of association.

Phylogenetic relationships were estimated based on multilocus genotypes using the minimum spanning tree (MSTree) method as implemented at <http://pubmlst.org/>. The effect of covariates on the cluster distribution was tested with the Chi-square test.

Results

Upon the analysis of 105 *A. flavus* clinical isolates, we found a very heterogeneous genetic background and a total of 77 distinct genotypes were obtained. Fifty-seven genotypes were found once, five genotypes were found twice, two genotypes were found three times, and one four times. Ten to 19 distinct alleles were detected for each microsatellite marker. The highest discriminatory power for a single locus was obtained with the AFLA1 marker, which had 19 distinct alleles and a 0.91 D value. The combination of all six markers yielded 77 different genotypes with a 0.98 D value. The mean gene diversity over the microsatellite loci was 0.79 and 0.77 for human and avian *A. flavus* populations, respectively.

Multilocus linkage disequilibrium

The standardized indices of association I_A and $rBarD$ (Table 1) were significantly different from zero ($p < 0.01$), indicating a strong linkage disequilibrium between loci in each population. Our results suggest that the prevailing mode of reproduction of *A. flavus* in natural populations is clonal.

Population structure

In order to assess genetic diversity among the populations, Wright's fixation index (F_{st}) was calculated for each locus

Table 1 Multilocus linkage disequilibrium analysis in each *Aspergillus flavus* population

Population	No. of isolates	No. of haplotypes	Value for all isolates	
			I_A^a	$rBarD^a$
Human clinical	65	43	1.68	0.34
Human environmental	11	9	0.93	0.18
Avian environmental	22	19	0.76	0.15
Avian clinical	7	6	0.77	0.19
All isolates	105	77	1.50	0.30

^a $p < 0.001$ (1,000 randomizations)

for all pairwise combinations of the four populations of *A. flavus* isolates (Table 2). *A. flavus* strain dispersal was also estimated by the gene flow (N_m) (Table 2). N_m can be interpreted as the effective number of migrants exchanged between demes (or subpopulations) per generation [18]; N_m is inversely related to F_{st} .

A little to moderate differentiation was observed between environmental and clinical *A. flavus* strains isolated from either human or avian populations ($F_{st}=0.029$ and 0.06, respectively). According to the N_m values (Table 2), human clinical and human environmental subpopulations shared more genotypes with a high migration rate (blastospores dispersion) ($N_m=11.38$). The avian (AC) and human (HC) clinical *A. flavus* populations showed important differences in their population structure and exchanged very few *A. flavus* strains, as indicated by their F_{st} ($0.182 > 0.15$; $p=0.00017$) and N_m values ($N_m=2.49$). However, the human environmental (HE) population was significantly differentiated from both environmental (AE) and clinical (AC) avian populations ($F_{st} > 0.25$ [0.251 and 0.375, respectively]; $p=0.00017$) with a low migration rate ($N_m=2.09$ and 7.24, respectively). We can, thus, suggest that no clear differences exist between strains isolated in the same localization (hospital or poultry farm). In contrast, hospital environment and poultry *A. flavus* isolates were highly differentiated.

Table 2 Pairwise F_{st} and N_m values for the studied populations

	HC	HE	AE	AC
HC	–	0.029 $p=0.143^*$	0.109 $p=0.00017^*$	0.182 $p=0.00017^*$
HE	11.38	–	0.251 $p=0.00017^*$	0.375 $p=0.00017^*$
AE	5.72	2.09	–	0.060 $p=0.10267^*$
AC	2.49	7.24	6.60	–

HC human clinical; HE human environmental; AC avian clinical; AE avian environmental

F_{st} (above diagonal), N_m (below diagonal)

* p -values calculated from 6,000 permutations

Minimum spanning tree

In order to visualize the genotypic diversity, an MStree analysis of the 77 distinct multilocus microsatellite genotypes was performed (Fig. 1). Three major clusters were observed. The first cluster comprised 33 genotypes corresponding to 14 HC isolates, 13 AC isolates, and six AE isolates. The second cluster includes mainly HC (19) and HE (8) isolates. The third cluster was composed of both HC (6) and AE (7) isolates. In the dendrogram, genotypes corresponding to human environmental isolates were clearly separated from avian isolates.

The Chi-square test results of the effect of covariates on the cluster distribution are presented in Table 3. The isolation site was significantly associated with genotype clusters ($p < 10^{-4}$); particularly, cluster II included only human isolates. Neither invasive disease (human and avian IA) versus other sites (otomycosis, sinusitis, and keratomycosis) nor the sampling site (poultry farm vs. hospital ward) were significantly associated to the genotype cluster distribution. Finally, the genotype clusters were significantly associated ($p < 10^{-4}$) to the year when they were collected.

Discussion

Our study is the first investigation comparing the genetic population structure of *A. flavus* provided by human and avian aspergillosis infection. Since *A. flavus* is the most common *Aspergillus* species in tropical countries to cause *Aspergillus* infection [3–5, 19], there is a clear need for improved molecular typing methods in order to study the genetic structure of this fungus. Therefore, understanding the epidemiology, evolution, and ability of this fungus to adapt should help in its future management. The panel of six markers presented in this study provides excellent discriminatory power for *A. flavus* strains, dividing our 105 *A. flavus* strains into 77 genotypes. Thus, microsatellite polymorphism analysis revealed a very heterogeneous *A. flavus* population. Microsatellite-based typing has been described as a reproducible and discriminatory typing technique for *A. flavus* strains [12].

Our study has some limitations. First, the number of isolates included in this study is relatively low, thus, our findings should be reproduced in further studies in order to increase their evidence level. Another limitation of our approach is that the high mutation rate among polymorphic microsatellites markers may lead to underestimating subpopulation divergence, because: (i) the maximum value that F_{st} can attain is reduced when there are numerous alleles, as in microsatellite markers, and (ii) the genetic homogeneity among subpopulations can be overestimated (and the gene flow underestimated) because of microsatellite allele homoplasy that results from reversion mutation. Because single nucleotide polymorphism (SNP) loci are less susceptible to these effects,

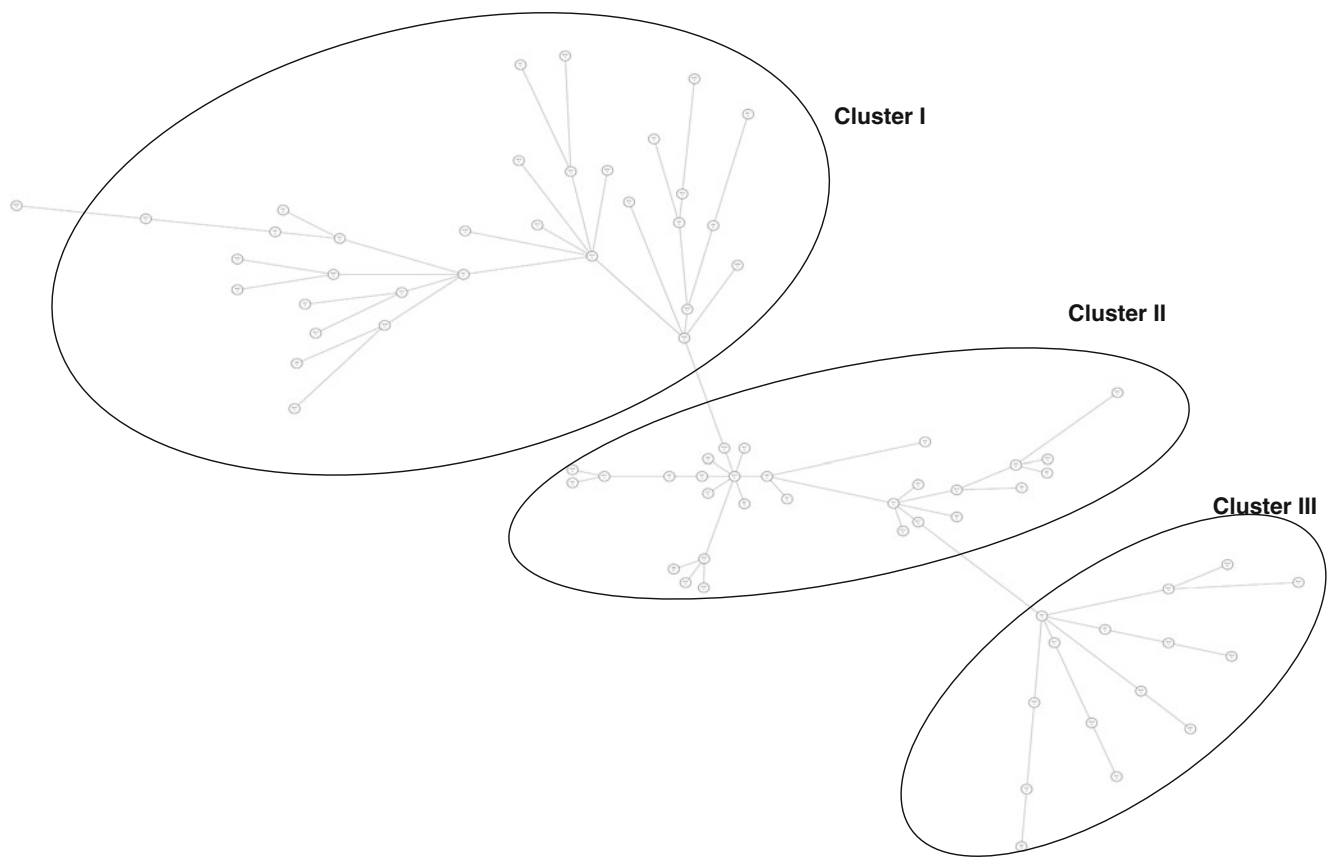


Fig. 1 Minimum spanning tree (MSTree) generated from the analysis of 29 avian and 63 human *Aspergillus flavus* isolates

Table 3 Association of covariates with the three *A. flavus* genetic clusters identified by minimum spanning tree (MSTree) analysis

Clusters	I	II	III	<i>p</i> -value
<i>n</i> (%)	36 (35)	48 (46)	11 (15)	–
Human isolates	44 %	100 %	55 %	<10 ^{−4}
Environmental isolates	36 %	23 %	45 %	0.16
Clinical isolates	64 %	77 %	55 %	0.16
Global site effect				<10 ^{−4}
Poultry farm	69 %	0 %	31 %	–
Hédi Chaker Hospital	9 %	82 %	9 %	–
Habib Bourguiba Hospital	58 %	11 %	32 %	–
IA (human and avian)	15 %	75 %	11	–
Global year effect				<10 ^{−4}
2005	0 %	56 %	0 %	–
2006	0 %	19 %	0 %	–
2007	3 %	21 %	0 %	–
2008	11 %	2 %	25 %	–
2009	6 %	0 %	10 %	–
2010	8 %	2 %	15 %	–
2011	28 %	0 %	15 %	–
2012	44 %	0 %	35 %	–

they may provide better estimates of F_{st} and may give more relevant information on reproduction [20]. However, to our knowledge, SNPs have not yet been used to study the population genetics in fungi. It would, thus, be useful to develop an SNP-based typing scheme for further population genetic studies in *A. flavus*.

Genetic population structure analysis of avian and human clinical and environmental *A. flavus* isolates was carried out in a geographic area restricted to the region of Sfax, Tunisia. According to the F_{st} and NF_m results, a low genetic diversity in *A. flavus* with high migration rates (due to the airborne dispersion of conidia) was observed between strains isolated in the same localization (hospital or poultry farm). However, *A. flavus* isolates collected from the hospital environment were genetically different from these collected from farms. This result was supported by the MSTree, showing that multilocus microsatellite genotypes corresponding to human environmental isolates were clearly separated from avian ones. Cell surface protein (CSP) typing and variable number of tandem repeats (VNTR) analysis of *A. fumigatus* isolates reported a clear separation according to their geographic origin, rather than their respective hosts [21, 22]. However, the VNTR analysis did not show a clear separation of *A. fumigatus* isolates collected from birds living in two different farms in the same department [21]. Recently, Wang et

al., using the VNTR technique, evaluated 55 isolates from Chinese avian farms and from a Tunisian hospital. The clustering analysis revealed a clear geographical structuring of *A. flavus* isolates [23].

In the hospital setting, the patient environment (tables, air conditioners, etc.) is exposed to chemical or physical disinfection procedures aiming at destroying pathogens, including fungi. This could exert a selective pressure on *A. flavus* genes and might contribute to the genetic differentiation between human and avian environmental isolates. Although, as discussed before, SNPs might give more relevant information on the mode of reproduction in these populations, this hypothesis is supported by standardized indices of association I_A and $rBarD$ being significantly different from zero ($p < 0.01$), suggesting a prevailing clonal mode of reproduction of *A. flavus*. However, the human clinical isolates were widespread among the three clusters and throughout the MStree diagram, suggesting that any environmental or avian clinical isolate of *A. flavus* could be infectious to humans. Van Waeyenberghe et al. found, by comparing human and avian *A. fumigatus* genotypes, that there was no specific association between the observed genotypes and the origin of the isolates (environment, human, or bird) [24]. Our results indicate the great variety of *Aspergillus* genotypes in the environment. The statistical analysis suggests that MStree genetic clusters of *A. flavus* are associated with the isolation source ($p < 10^{-4}$). The statistically significant association of genotypes with the year of isolation (Table 3) might be due to confounding factors, chiefly because of the relatively low number of time-point measures within each population and the relatively short time period studied.

Conclusion

The present study describes a specific population structure of human environmental *Aspergillus flavus* isolates which was clearly distinct from environmental and clinical avian populations. Further studies are needed in order to better understand the epidemiology of *A. flavus* at a wider geographical scale and in particular situations, such as in hospitals or avian farms, where invasive aspergillosis (IA) outbreaks occur.

Acknowledgments We thank Mr. Fouazi Mseddi for graciously providing the chicken used in this study.

Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

- Denning DW (1998) Invasive aspergillosis. Clin Infect Dis 26 (4):781–803
- Balajee SA, Kano R, Baddley JW, Moser SA, Marr KA, Alexander BD, Andes D, Kontoyiannis DP, Perrone G, Peterson S, Brandt ME, Pappas PG, Chiller T (2009) Molecular identification of *Aspergillus* species collected for the Transplant-Associated Infection Surveillance Network. J Clin Microbiol 47 (10):3138–3141
- Pasqualotto AC (2009) Differences in pathogenicity and clinical syndromes due to *Aspergillus fumigatus* and *Aspergillus flavus*. Med Mycol 47(Suppl 1):S261–S270
- Chakrabarti A, Shivaprakash MR, Singh R, Tarai B, George VK, Fomda BA, Gupta A (2008) Fungal endophthalmitis: fourteen years' experience from a center in India. Retina 28(10):1400–1407
- Hadrich I, Makni F, Sellami H, Cheikhrouhou F, Sellami A, Bouaziz H, Hdiji S, Elloumi M, Ayadi A (2010) Invasive aspergillosis: epidemiology and environmental study in haematology patients (Sfax, Tunisia). Mycoses 53(5):443–447
- Bertout S, Renaud F, Barton R, Symoens F, Burnod J, Piens MA, Lebeau B, Viviani MA, Chapuis F, Bastide JM, Grillot R, Mallié M; European Research Group on Biotype and Genotype of *Aspergillus* (2001) Genetic polymorphism of *Aspergillus fumigatus* in clinical samples from patients with invasive aspergillosis: investigation using multiple typing methods. J Clin Microbiol 39 (5):1731–1737
- Chazalet V, Debeaupuis JP, Sarfati J, Lortholary J, Ribaud P, Shah P, Cornet M, Vu Thien H, Gluckman E, Brückner G, Latgé JP (1998) Molecular typing of environmental and patient isolates of *Aspergillus fumigatus* from various hospital settings. J Clin Microbiol 36(6):1494–1500
- Rosehart K, Richards MH, Bidochka MJ (2002) Microsatellite analysis of environmental and clinical isolates of the opportunist fungal pathogen *Aspergillus fumigatus*. J Med Microbiol 51 (12):1128–1134
- Lair-Fuller S, Guillot J, Desterke C, Seguin D, Warin S, Bezille A, Chermette R, Bretagne S (2003) Differentiation between isolates of *Aspergillus fumigatus* from breeding turkeys and their environment by genotyping with microsatellite markers. J Clin Microbiol 41(4):1798–1800
- Olias P, Gruber AD, Hafez HM, Lierz M, Slesiona S, Brock M, Jacobsen ID (2011) Molecular epidemiology and virulence assessment of *Aspergillus fumigatus* isolates from white stork chicks and their environment. Vet Microbiol 148(2–4):348–355
- Alvarez-Perez S, Mateos A, Dominguez L, Martinez-Nevado E, Blanco JL, Garcia ME (2010) Polyclonal *Aspergillus fumigatus* infection in captive penguins. Vet Microbiol 144(3–4):444–449
- Hadrich I, Makni F, Ayadi A, Ranque S (2010) Microsatellite typing to trace *Aspergillus flavus* infections in a hematology unit. J Clin Microbiol 48(7):2396–2401
- de Hoog GS, Nishikaku AS, Fernandez-Zepkenfeldt G, Padín-González C, Burger E, Badali H, Richard-Yegres N, van den Ende AH (2007) Molecular analysis and pathogenicity of the *Cladophialophora carrionii* complex, with the description of a novel species. Stud Mycol 58:219–234
- Hunter PR, Gaston MA (1988) Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol 26(11):2465–2466
- Goudet J, Raymond M, de Meeüs T, Rousset F (1996) Testing differentiation in diploid populations. Genetics 144(4):1933–1940
- Wright S (1943) Isolation by distance. Genetics 28(2):114–138
- Agapow P-M, Burt A (2001) Indices of multilocus linkage disequilibrium. Mol Ecol Notes 1:101–102
- Wright S (1969) Evolution and the genetics of populations. Volume 2, the theory of gene frequencies. The University of Chicago Press, Chicago
- Hedayati MT, Pasqualotto AC, Warn PA, Bowyer P, Denning DW (2007) *Aspergillus flavus*: human pathogen, allergen and mycotoxin producer. Microbiology 153(Pt 6):1677–1692

20. Coates BS, Sumerford DV, Miller NJ, Kim KS, Sappington TW, Siegfried BD, Lewis LC (2009) Comparative performance of single nucleotide polymorphism and microsatellite markers for population genetic analysis. *J Hered* 100(5):556–564
21. Thierry S, Wang D, Arné P, Deville M, De Bruin B, Nieguitsila A, Pourcel C, Laroucau K, Chermette R, Huang W, Botterel F, Guillot J (2010) Multiple-locus variable-number tandem repeat analysis for molecular typing of *Aspergillus fumigatus*. *BMC Microbiol* 10:315
22. Balajee SA, Tay ST, Lasker BA, Hurst SF, Rooney AP (2007) Characterization of a novel gene for strain typing reveals substructuring of *Aspergillus fumigatus* across North America. *Eukaryot Cell* 6(8):1392–1399
23. Wang DY, Hadj-Henni L, Thierry S, Arné P, Chermette R, Botterel F, Hadrich I, Makni F, Ayadi A, Ranque S, Huang WY, Guillot J (2012) Simple and highly discriminatory VNTR-based multiplex PCR for the typing of the emerging fungal pathogen *Aspergillus flavus*. *PLoS One* (in press)
24. Van Waeyenberghe L, Pasmans F, Beernaert LA, Haesebrouck F, Vercammen F, Verstappen F, Dorrestein GM, Klaassen CH, Martel A (2011) Microsatellite typing of avian clinical and environmental isolates of *Aspergillus fumigatus*. *Avian Pathol* 40(1):73–77