

CHARACTERIZATION OF AIRBORNE *ASPERGILLUS* SERIES *VERSICOLORS* COLLECTED IN FRENCH BIOAEROSOLS

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ABSTRACT

Indoor air quality and exposure to fungal bioaerosols is a major concern in Europe where people spend most of their time indoors. 30–50% of European homes have moisture problems facilitating mold growth, with significant health and economic consequences. *Aspergilli* series *Versicolores* are molds recurrently found in these homes which are known to cause allergies, aggravate asthma and produce sterigmatocystin, a potential human carcinogen. Recent phylogenetic studies highlighted new *Versicolores* species which led us to characterize fungal isolates of this section to better understand their repartition in French bioaerosols. Air samples were taken with a cyclonic biocollector indoor (hospital and mold-damaged homes) and outdoor (agricultural environment), temperature and relative humidity were recorded during sampling. Collection liquid was cultured on malt extract agar (MEA) medium supplemented with chloramphenicol (0.05%, w/v). Each colony was isolated and then purified on MEA. A total of 93 isolates belonging to the series *Versicolores* were characterized both macroscopically, microscopically and were also identified using molecular approach. We identified eight different species from bioaerosols: *Aspergillus creber* (n = 40), *A. jensenii* (n = 37), *A. protuberus* (n = 6), *A. puulaauensis* (n = 4), *A. sydowii* (n = 2), *A. tabacinus* (n = 2), *A. fructus* (n = 1), and *A. amoenus* (n = 1). The phylogenetic tree performed from the *BenA* sequences shows homologies between isolates of the same species recovered from bioaerosols collected in the same environment which confirms the interest of Bt2a/b primers set for the identification of species of the *Versicolores* series. This work constitutes the first characterization of *Aspergilli* of the series *Versicolores* from French bioaerosols.

Keywords: *Aspergillus*, series *Versicolores*, bioaerosols.

1 INTRODUCTION

In France, as in most European countries, air quality has become a major public health issue [1]. Indeed, it is estimated that air pollution causes 48,000 deaths each year in France, which represents nearly 8% of total annual mortality between 2016 and 2019 [2]. This air quality can be altered by different factors from natural or anthropic origin [3]. Among the factors of natural origin, molds are the most frequent in indoor environments [4] where they proliferate easily in the presence of humidity, which affects 30 to 50% of European homes [5], explaining the visible presence of molds belonging mainly to the genera *Aspergillus*, *Penicillium* and *Cladosporium* [6] in 14–20% of French homes [7]. These are mainly responsible for allergies [8] and sick building syndrome [9], can aggravate asthma [10] and are likely to cause infections in immunocompromised patients [11], [12]. The frequency and intensity of these symptoms are related to the level of contamination of the habitat and the duration of exposure of the population [13], which can reach up to 22 hours spent indoors per day in industrialized countries [14], [15]. However, as the relations between dampness, microbial exposure and health effects cannot be quantified precisely, no quantitative health-based guideline values or thresholds can be recommended for acceptable levels of contamination with microorganisms [5]. Some molds are also capable of producing cytotoxic



[16], [17] and/or genotoxic mycotoxins [18], [19]. This explains the important cost related to medical and renovation expenses in France which amount to 19 billion euros [20].

The molds of the genus *Aspergillus* belonging to the series *Versicolores* section *Nidulantes* are cosmopolitan. They have been found in the soil [21], in various foodstuffs for humans and animals [22], in hypersaline water [23] and in indoor environments [24]. On average, they are found in 70% of bioaerosols [6]. In addition to causing, allergies [25], aggravate asthma [26] and being linked to sick building syndrome [9], they can occasionally be responsible for onychomycosis [27] or pulmonary aspergillosis [28]. Their presence has also been shown to be an explanatory variable for various symptoms experienced by the inhabitants of dwellings damaged by moulds, such as fever, itching, headaches, dizziness or abnormally abundant expectorations [6]. *Aspergilli* of the series *Versicolores* except *A. sydowii* produce sterigmatocystin [29], a mycotoxin involved in the biosynthetic pathway of aflatoxins [30] and recognized as potentially carcinogenic to humans by IARC (group 2B) [31]. *Aspergillus versicolor* was considered until a few years ago as the most abundant species of the series *Versicolores* in bioaerosols. However, several taxonomic revisions have revealed a total of 12 new species within the series *Versicolores* through different phylogenetic studies. Among these, *Aspergillus creber* from the Latin word “creber” meaning “numerous” or “frequent” is now considered to be the most frequent species and was misidentified as *Aspergillus versicolor* [32], [33]. Studies on the diversity and ecology of the species of the series *Versicolores* confirm this hypothesis [34]. Furthermore, the different species of the series *Versicolores* produce different metabolites from each other and do not all have the same pathogenicity [35]–[37].

This work focuses on the molecular characterization of 93 environmental isolates of *Aspergillus* series *Versicolores* isolated from French bioaerosols in order to evaluate their diversity and to contribute to the assessment of fungal exposure to these molds.

2 MATERIAL AND METHODS

2.1 Bioaerosols collection

Bioaerosols were collected in a hospital (Centre François Baclesse, Caen, France) (n = 24) [38], in *Serpula lacrymans*- and mold-damaged homes (n = 65) [13], [39] and outdoor in agricultural environments (silage and hay) (n = 4) [40], [41].

Samples were cultured on malt extract agar (MEA) medium with 0.02% chloramphenicol (Cooper, Melun, France). Plates were incubated at 25°C and checked daily. Each fungal colony was isolated and purified on MEA. All *Aspergillus* isolates belonging to the series *Versicolores* (n = 93) were stored on slant agar at 4°C and in a cryoprotective agent composed of sterile water (Fresenius Kabi AG, Bad Homburg, Germany) and 10% glycerol (Carlo Erba, Val-de-Reuil, France) at –80°C before molecular characterization.

2.2 Molecular characterization

DNA extraction was performed using a modified protocol of the Nucleospin™ Plant II kit (Macherey-Nagel, Duren, Germany). Fungal colony was introduced in a 2 mL microtube with glass beads. The microtube underwent two incubation cycles of 15 min at 80°C and then 15 min at –80°C. It was placed into a Qiagen Tissue Lyser with 400 µL of lysis buffer PL1 (Macherey-Nagel, Duren, Germany) for 15 min at 20 Hz, and incubated with 10 µL of RNase (Macherey-Nagel, Duren, Germany) and 20 µL of proteinase K at 10 mg/mL (Sigma-Aldrich, St. Louis, MO, USA) at 65°C for 15 min. Then, 400 µL of chloroform (Sigma-Aldrich, St.



Louis, MO, USA) was added to the mixture. The aqueous phase was recovered and extracted using the precipitation kit and washing buffer according to the protocol described by the supplier.

DNA was purified using the NucleoSpin gDNA Clean-up kit (Macherey-Nagel, Duren, Germany) following the instructions of the manufacturer. For each isolate, quantification and quality of purified DNA were realized using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) [42].

In this study molecular characterization was performed by amplification of the beta-tubulin gene (*BenA*) using Bt2a/Bt2b (5'-GGTAACCAAATCGGTGCTGCTTTC-3'/5'-ACCCTCAGTGTAGTGACCCTTGGC-3') primers [32]. Sequences obtained were compared using BLAST (Basic Local Alignment Search Tool, NCBI) to the reference sequences of the 17 *Aspergillus* species of the series *Versicolores* publicly available by Houbraken et al. [43]. Identification was considered reliable only by having a query cover $\geq 98\%$ and a % ID $\geq 99\%$.

Phylogenetic tree was generated using CLC Workbench 21.0.4. Tree construction was based on neighbor-joining method. Branch supports of the best maximum likelihood tree were estimated by 500 bootstrap replicates.

3 RESULTS

3.1 Relative abundance in French bioaerosols

Among all mold species recovered from bioaerosols, *Aspergilli* series *Versicolores* were the fifth most common species in bioaerosols from the hospital, the first in *Serpula lacrymans*- and mold-damaged homes, and the third most common in agricultural environments, with concentrations ranging from 2.48 CFU/m³ in agricultural environments to 3.44×10^5 CFU/m³ in mold-damaged homes. *Aspergilli* series *Versicolores* were associated with 109, 100, and 28 other fungal species in bioaerosols collected from hospital, *Serpula lacrymans*- and mold-damaged homes, and agricultural environments, respectively. The most represented genera in all environments were *Aspergillus* (*A. fumigatus*, *A. niger*, *A. melleus*, *A. flavus*, *A. pseudoglaucus*), *Penicillium* (*P. chrysogenum*, *P. brevicompactum*, *P. crustosum*) and *Cladosporium* (*C. cladosporioides*, *C. herbarum*, *C. sphaerospermum*) [18], [32]–[34].

As shown in Fig. 1, among all the isolates collected in French bioaerosols (n = 93), we were able to molecularly identify eight species of the *Versicolores* series: *Aspergillus creber* (n = 40) comes first, followed by *A. jensenii* (n = 37), *A. protuberus* (n = 6), *A. puulaauensis* (n = 4), *A. sydowii* (n = 2), *A. tabacinus* (n = 2), *A. amoenus* (n = 1) and *A. fructus* (n = 1). Bioaerosols collected at the hospital (n = 24) contained more *A. jensenii* (n = 14), followed by *A. protuberus* (n = 6), *A. creber* (n = 3) and *A. puulaauensis* (n = 1). *Aspergillus protuberus* was only found in bioaerosols collected at the hospital. Bioaerosols collected from houses damaged by *Serpula lacrymans* and molds (n = 65) were mainly composed of *A. creber* (n = 37) and *A. jensenii* (n = 22). *A. puulaauensis* (n = 3), *A. sydowii* (n = 2), and *A. amoenus* (n = 1) were also present although much less abundant. Of the four isolates obtained from bioaerosols collected from agricultural environments, we identified two isolates of *A. tabacinus*, one isolate of *A. jensenii* and one isolate of *A. fructus*.

3.2 Phylogenetic study

As shown in Fig. 2, the *Sydowii* subseries is much more represented within the bioaerosols than the *Versicolores* subseries. Indeed, *Aspergillus creber*, *A. jensenii*, *A. puulaauensis* and



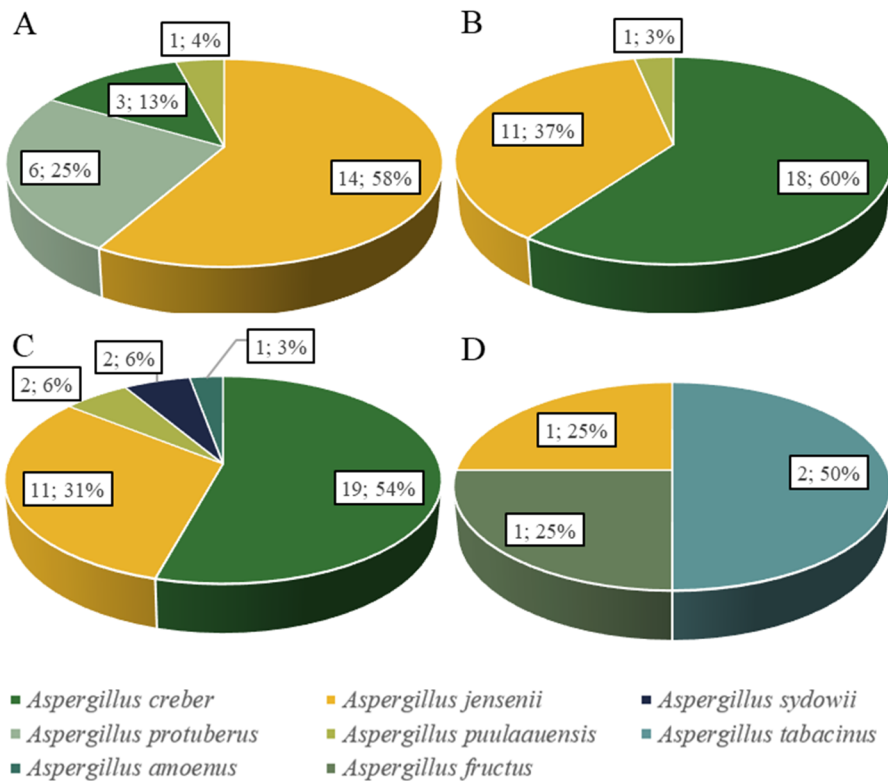


Figure 1: Relative abundances (number of isolates; percentage) of *Aspergillus* species belonging to the *Versicolores* series in bioaerosols from (A) hospital; (B) *Serpula lacrymans*-damaged homes; (C) mold-damaged homes; and (D) agricultural environments.

A. sydowii species represent 83 isolates (89.2%) of the isolates found in French bioaerosols. The species belonging to the *Versicolores* subseries (*Aspergillus protuberus*, *A. tabacinus*, *A. fructus* and *A. amoenus*) are much less present in bioaerosols. Indeed, these represent only ten isolates (10.8%) although *Aspergillus protuberus* is the third most frequently isolated species of the *Versicolores* series from bioaerosols. The use of the Bt2a/b primers set allowed the construction of a phylogenetic tree with a clear distinction of the eight species belonging to the *Versicolores* series found in French bioaerosols and to determine the specific richness for each environment where they were collected. In descending order, the highest species richness was found in bioaerosols from mold-damaged homes ($n = 5$), followed by those collected at the hospital ($n = 4$) and tied for third place *Serpula lacrymans*-damaged homes and in agricultural environments ($n = 3$). *Aspergillus tabacinus* and *A. fructus* were only found in bioaerosols collected from agricultural environments. *Aspergillus jensenii* was the only species found in all environments. We also observed a similar distribution of *Versicolores* species in contaminated homes regardless of whether they were contaminated by *Serpula lacrymans* or molds. The amplification of the *BenA* sequence has allowed the identification of nucleotide variations sufficient to discriminate between different strains within the same species and environment (especially for *A. jensenii*).

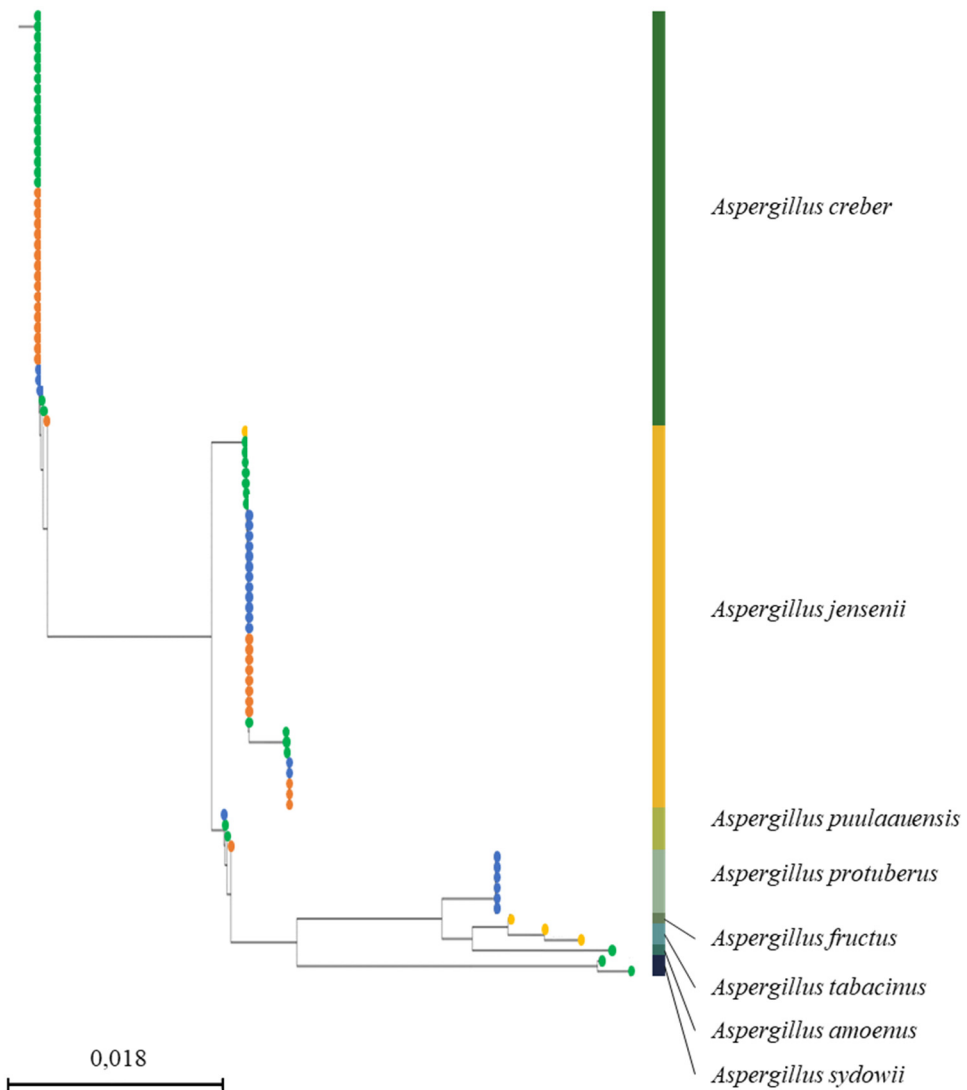


Figure 2: Phylogenetic tree of *Aspergillus* series *Versicolores* species found in French bioaerosols calculated from DNA sequence data from *BenA* (isolates from mold-damaged homes bioaerosols in green, from *Serpula lacrymans*-damaged homes bioaerosols in orange, from hospital bioaerosols in blue and from agricultural environments bioaerosols in yellow).

4 DISCUSSION

Ninety-three fungal isolates recovered from bioaerosols collected in a hospital, in contaminated homes and in agricultural environments were molecularly identified as belonging to species of the *Aspergillus Versicolores* series. This study constitutes the first report on the distribution of these species in French bioaerosols molecularly characterized by *benA* gene amplification. We were able to group these isolates under eight different species,

which constitutes one of the highest specific richness reported to date. Indeed, other studies conducted on isolates collected from indoor environments (air and dust samples) identified by *CaM* gene amplification show that species richness within the *Versicolores* series varies from three (*Aspergillus creber*, *A. jensenii* and *A. protuberus*) to 11 species (*A. creber*, *A. jensenii*, *A. puulaauensis*, *A. tennesseensis*, *A. venenatus*, *A. amoenus*, *A. fructus*, *A. griseoaurantiacus*, *A. pepii*, *A. protuberus* and *A. sydowii*) [36], [44]. *Aspergillus creber* and *A. jensenii* were the most frequently isolated species from French bioaerosols (83% of isolates), which is consistent with studies on *Aspergillus* species belonging to the *Versicolores* series that always find these species, and especially with the study by Jakšić et al. where these two species represent 62% of isolates [36], [45]. However, we did not find *Aspergillus cvjetkovicii*, *A. griseoaurantiacus*, *A. pepii*, *A. tennesseensis*, or *A. venenatus*, which have been isolated from indoor air bioaerosols in the United States and Croatia [33], [36], [45] but are more frequently found in various foods and feeds and in soil [36], [46]. *A. protuberus* and *A. puulaauensis* remain quite recurrent in indoor air and dust, as reported by Micheluz et al. [44] and Jakšić et al. [36]. *Aspergillus sydowii* was only isolated from two bioaerosols collected from contaminated homes, confirming its presence at a rather low frequency in air compared to food matrices or human pathology [33], [37], [46]. *Aspergillus amoenus* was isolated by Jurjevic et al. [33] from different sources (mammary gland, cured meat, *Berberis* sp. fruit) but also from indoor dust in Japan [46], which may explain why we found so few among all the collected isolates. This is the first time that *Aspergillus tabacinus* has been isolated from bioaerosols, whereas it is more frequently isolated from tobacco, cured meat, or plants (corn) [33]. We also found an isolate of *Aspergillus fructus* that had never been identified in bioaerosols, although it had been previously recovered from dust of water-damaged and control houses in Croatia [24]. However, this species seems to be more frequently identified from fruits [33]. *Aspergillus versicolor* stricto sensu was not found in our bioaerosols, confirming the observations of Jurjevic et al. [33] and Kobayashi et al. [46], who reported its presence only in food products (dairy feeds, rice and noodles). Our work shows a large preponderance of species of the *Sydowii* subseries in indoor air bioaerosols which is in agreement with all studies that have investigated *Aspergillus* species of the *Versicolores* series in bioaerosols whether in Croatia [36], the United Kingdom [32], the USA [33], Italy [44] or Japan [46]. Although we have no other point of comparison to date and despite the small number of isolates recovered, it is interesting to note that species of the *Versicolores* subseries seem to be more frequent in outdoor air collected from agricultural environments. The creation of the phylogenetic tree from the *BenA* sequences shows homologies between isolates of the same species recovered from bioaerosols collected in the same environment which confirms the interest of the use of the Bt2a/b primers set for the identification of species of the *Versicolores* series.

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