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Abundance of *Pseudallescheria/Scedosporium* species in the Australian urban environment suggests a possible source for scedosporiosis including the colonization of airways in cystic fibrosis

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Members of the *Pseudallescheria/Scedosporium* species complex are emerging opportunistic fungal pathogens which have the capacity to colonize patients with damaged airways, including those with cystic fibrosis (CF). Assuming human infection is acquired via inhalation of fungal spores from the environment, we performed a qualitative environmental survey encompassing 25 urban, semirural and rural sites in the greater Sydney region to determine the prevalence of *Pseudallescheria/Scedosporium* species. Soil sampling revealed an abundance of *Pseudallescheria/Scedosporium*, particularly in locations associated with high human activity. No variation was noted during repeated sampling at different times of the year. Strains of *Scedosporium aurantiacum* were most frequently isolated (54.6%), followed by *Scedosporium prolificans* (43%), *P. boydii* (2.1%) and *S. dehoogii* (0.3%). The findings coincide with the relatively high prevalence of *Scedosporium* infections in Australia and their presence as colonizers in CF patients. They emphasize the importance of environmental studies to assess the clinical risk of infection.

Keywords *Scedosporium*, soil sampling, environmental isolation, *Scedosporium aurantiacum*

Introduction

Members of the genera *Pseudallescheria* and *Scedosporium* are increasingly encountered as emerging fungal pathogens [1–5]. Disease is predominantly caused by four species, i.e., *Scedosporium apiospermum*, *Pseudallescheria boydii*, *Scedosporium prolificans* and the recently described, *Scedosporium aurantiacum* [3–5]. Infections occur in both immunocompromised and immunocompetent individuals, ranging from localized, superficial infections to fatal disseminated disease. Notably, isolates of *Pseudallescheria/Scedosporium* are also associated with long-term colonization of the respiratory tract of individuals with chronic lung diseases, such as cystic fibrosis (CF) [6,7]. A recent study of patients from a major Sydney adult CF clinic in Australia revealed that *S. prolificans*, *S. apiospermum* and *S. aurantiacum* were collectively the third most frequent airway colonizers (17.4% patients) after *Aspergillus* and *Penicillium* species [8].

Despite antifungal therapy, mortality from *Scedosporium* infections, particularly *S. prolificans*, remains high (up to 87.5% in disseminated disease) [9]. Antifungal treatment is problematic since these pathogens are resistant to most antifungal agents, and alternate approaches to improve patient outcomes, such as pre-emptive and preventive strategies have yet to be evaluated. A better understanding of the epidemiology, the mode of the transmission of the fungal agents, as well as delineation of likely environmental sources of *Scedosporium* infections is thus important to better understand the risk factors for colonization and/or infection in high risk patient groups such as those with CF.

Sidot *et al*. recovered *S. apiospermum* from home environments of CF patients. The fungus was obtained in

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culture from 41 of 57 (71.9%) houseplant or surface soil samples, but none from water or air highlighting the occurrence of the pathogen in the vicinity of the patients as a potential risk factor [10]. Other studies have documented the occurrence of *Pseudallescheria/Scedosporium* species in soil from industrial, urban playgrounds and agricultural areas, garden soil, sewer, and polluted ponds [11,12]. This suggested the presence of these fungi is strongly associated with organic contamination, such as hydrocarbons from human, animal and industrial waste or crude oil, in the environment as a consequence of human and animal activities [11,12]. This association is most likely due to a number of factors including the ability of *P. boydii* to survive at very low oxygen partial pressure and to its ability to tolerate 5% NaCl [13]. They are able to utilize natural gas and/or aromatic compounds as carbon sources [12,14,15] as reported in a study on bioremediation of aged polycyclic aromatic hydrocarbons (PAHs)-contaminated soil by filamentous fungi. This investigation showed that *Scedosporium* spp. were able to significantly degrade PAHs, along with other fungi such as *Cladosporium* spp. and *Fusarium* spp. [16]. A positive correlation between ammonium concentrations and *Pseudallescheria* density in soil from industrial areas, parks and playgrounds was demonstrated by Kaltseis *et al*. [11]. These results confirmed earlier observations [17], suggesting that high nitrogen levels in soil are required for the growth of these species [11]. In addition they found that *Pseudallescheria* spp. were abundant in soils with a pH range of 6.1–7.5 [11].

Taking into account that recent Australian clinical studies have documented a relatively high prevalence of *Scedosporium* infections [5,18] and assuming that infections are acquired via inhalation of fungal spores from the environment, we undertook a qualitative environmental survey in the greater Sydney region, including rural and urban sites, to estimate the prevalence of strains of the *Pseudallescheria/Scedosporium* complex in the environment.

Materials and methods

Soil sampling

Environmental sampling sites within the greater Sydney region were selected to represent different types of human activity (Table 1). These included areas of high human activity within 3 km of the central Sydney area (city center), those of lower human impact or activity (various Sydney suburban areas within 20 km from the city center), as well as remote rural areas in the Blue Mountains and the Central Coast, Australia (45–100 km from Sydney) (Table 1, Fig. 1). Soil samples were collected using a metal spatula, which was cleaned with 70% ethanol between samplings to avoid cross contamination. Soil of sufficient quantity to fill a 50 ml sterile Falcon tube was obtained at a depth of approximately 10 cm, avoiding plant debris, roots, and gravel and then stored at 4ºC until processed. With the exception of the Blue Mountains, all sites were sampled twice, i.e., the first time in December (Australian summer), and the second time in July (Australian winter).

Isolation of Scedosporium from soil

Fungal isolation from soil samples was performed as previously described [19]. Briefly, the total soil sample was carefully mixed and air-dried for 3-4 days at room temperature. One gram of air-dried soil sample was placed in a sterile 50 ml Falcon tube and mixed with 10 ml sterile water, thoroughly vortexed, and left to stand for 10 min. The mixture was then streaked onto ten plates of dichloran rose bengal chloramphenicol (DRBC) media (Oxoid, UK), supplemented with benomyl (methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate; Sigma, USA) at a concentration of 10 µg/ml [19]. The plates were incubated at 37°C and examined daily for fungal growth. Portions of suspected *Pseudallescheria* or *Scedosporium* colonies, as illustrated in Fig. 2a, were stained with lactophenol cotton blue on glass slides and examined with light microcopy. Colonies that showed microscopic features of *Pseudallescheria* and *Scedosporium* (Fig. 2b–e) were subcultured onto potato dextrose agar (Oxoid, USA) to obtain pure colonies.

Determination of fungal burden

All *Pseudallescheria* and *Scedosporium* colonies recovered during the isolation process (see above) on ten inoculated plates were counted. The same procedure was repeated for the soil collected from the same sites at the time of the second sampling (see above). Fungal burdens were plotted using the program GraphPad Prism version 5.0b (GraphPad Software Inc., USA).

Identification of Scedosporium isolates

Pure *Pseudallescheria/Scedosporium* colonies were subcultured for morphological identification [20]. Species identification was confirmed by internal transcribed spacer region restricted fragment-length polymorphism (ITS-RFLP) analysis and ITS sequencing [4].

Extraction of fungal DNA

One-week-old pure cultures were scraped and suspended in sterile water in a 1.5 ml Eppendorf tube and the DNA was extracted as previously published [21].

Table 1 Sampling locations included in the study, characteristics, species obtained and corresponding soil fungal burdens.

Sampling location	Area type	Species isolated	*Scedosporium/ Pseudallescheria* fungal burden in (CFU/g dry weight)[#]
Circular Quay, Sydney	**Inner Sydney Areas** with high human activity	*S. aurantiacum, S. prolificans*	527
Darling Harbour, Sydney		*S. aurantiacum, S. prolificans, S. dehoogii, P. boydii*	905
Hyde Park, Sydney		*S. aurantiacum, S. prolificans*	60
Royal Botanic Garden, Sydney		*S. aurantiacum, S. prolificans*	62
Sydney Olympic Park, Homebush Bay	**Outer Sydney Areas** with a wide range of human activities (suburban areas, including gardens, parks, playgrounds, quiet neighborhoods, suburban train stations)	*S. aurantiacum, S. prolificans*	40
Remembrance Park, Lidcombe		*S. aurantiacum, S. prolificans*	20
Winspear Avenue, Bankstown		*S. prolificans, P. boydii*	40
Paul Keating Park, Bankstown		*S. aurantiacum, S. prolificans*	260
Auburn Botanical Garden, Auburn		*nil*	0
Westmead Hospital, Westmead		*S. prolificans*	20
Merrylands Swimming Centre, Merrylands		*nil*	0
Davy Robinson Drive, Milperra		*nil*	0
Bennet Park, Roseland		*nil*	0
Light Horse Park, Liverpool		*nil*	0
Adams Park, Cabramatta		*nil*	0
Chatswood Railway Station, Chatswood		*S. prolificans*	110
Boronia Park, Epping		*S. prolificans*	40
William Street, Hornsby		*S. prolificans*	20
Campbelltown Railway Station, Campbelltown		*nil*	0
Remembrance Park, Woy Woy	**Sydney Surrounding Areas** with low human activity (Central Coast and Blue Mountains National Park including bush-walking tracks, parks)	*S. prolificans*	60
Gordon Falls Park		*S. prolificans*	3
Frank Walford Park		*nil*	0
Federal Pass A		*S. prolificans*	2
Federal Pass B		*S. prolificans*	2
Katoomba Park		*S. prolificans*	4

[#]CFU/g dry weight = Colony forming unit per gram of dry soil.

ITS-RFLP and ITS sequencing

Amplification of the ITS1, 5.8S, and ITS2 regions of the rDNA gene cluster was performed, followed by double digestion with two endonucleases *Sau*96I and *Hha*I (New England Biolabs, USA), as previously reported [4]. The resulting ITS-RFLP patterns were separated on 3% agarose gels and identification was obtained by comparing the generated RFLP profiles with those obtained from type cultures of the *Pseudallescheria*/*Scedosporium* species. In addition the ITS1/2 region was also sequenced using commercially available sequencing services. DNA sequence data were compared against the GenBank database using BLASTn searches, for the identification of the strains to the species level. Species were named according to the recently proposed taxonomic nomenclature for the *Pseudallescheria*/*Scedosporium* species complex [19,22].

Statistical analysis

Statistical analysis was performed using the program GraphPad Prism version 5.0b (GraphPad Software Inc., USA). Comparison between sampling areas (Inner Sydney Areas, Outer Sydney Areas and Sydney Surrounding Areas) was performed using one-way ANOVA and Mann Whitney U test. P-values < 0.05 were considered as statistically significant.

Results

Environmental samples were collected from a total of 25 sites in the greater Sydney region (Table 1, Fig. 1). *Pseudallescheria/Scedosporium* strains were recovered from all four sites sampled in the central Sydney region compared to eight of 15 sites in the Sydney suburban region. Samples from five of the 6 remote sites (Blue Mountains, Central Coast) yielded *Pseudallescheria/Scedosporium* species (Table 1, Fig. 3).

The fungal burden in soil samples was determined and expressed as colony-forming unit per gram of dry weight (CFU/g dry weight) of soil. The CFU counts ranged from 60–905 CFU/g dry weight from samples recovered at the high human impact sites, inner city of Sydney, and from

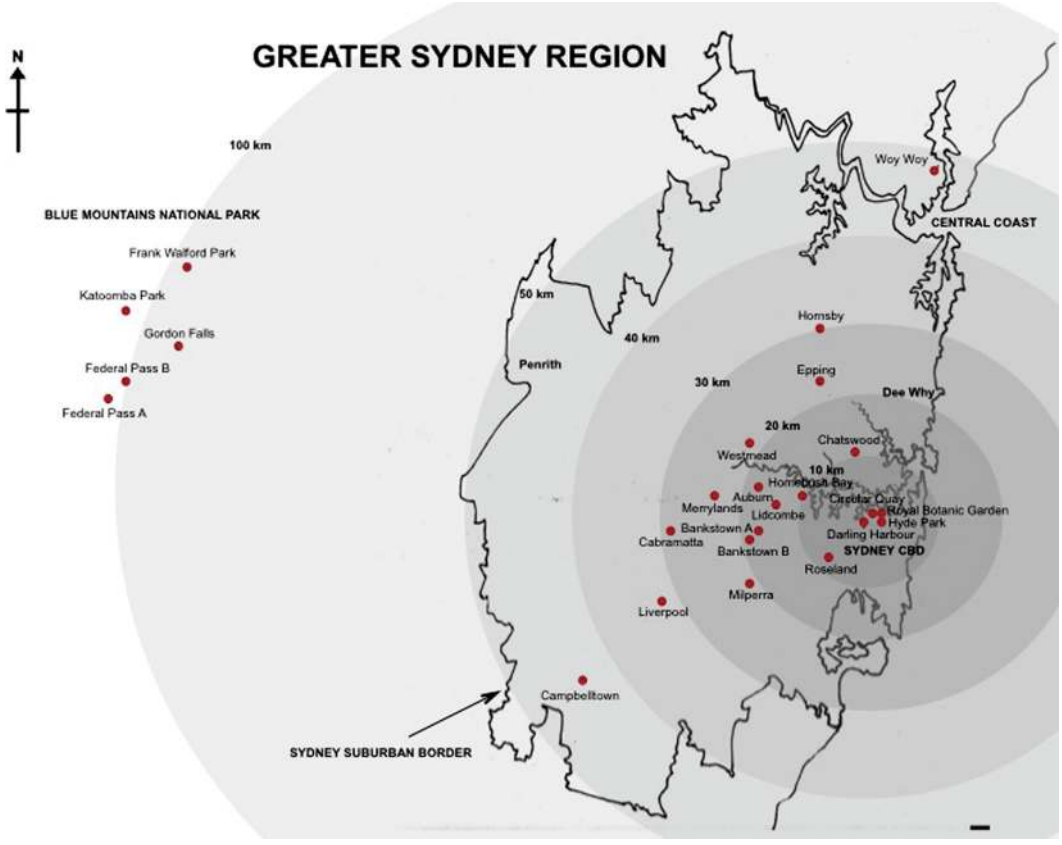

Fig. 1 Location of the sampling sites studied within the greater Sydney region.

0–260 CFU/g dry weight in the suburban/rural areas (Table 1). There were significant differences ($P = 0.002$) in the number of *Pseudallescheria/Scedosporium* colonies recovered from the samples of urban, high populated areas (Inner Sydney Areas) compared to the samples taken from suburban areas (Outer Sydney Areas and Sydney Surrounding Areas). Significant differences in the fungal burden were also evident when comparing Inner and Outer Sydney Areas ($P = 0.008$). The highest fungal burden was obtained from samples collected from Darling Harbour, a central inner city tourist/entertainment area in Sydney (Table 1).

There were no significant differences in the CFU counts between soil samples collected per sampling site at different time points (December and July) ($P = 0.726$).

A total of 2,175 *Pseudallescheria/Scedosporium* colonies were isolated from all sampling sites. ITS-RFLP analysis after double digestion with *Sau*96I and *Hha*I followed by ITS sequencing identified a subset of isolates obtained on two of the 10 primary isolation plates for each sampling site as either *S. aurantiacum* ($n = 194$), *S. prolificans* ($n = 153$), *Scedosporium dehoogi* ($n = 1$) or *P. boydii* ($n = 7$) (Fig. 3). *S. aurantiacum* was the most frequently isolated species, comprising 54.6% of the total isolates. *S. prolificans* was found in all environmental sites from which *Pseudallescheria/Scedosporium* strains were recovered, while *S. aurantiacum* and *P. boydii* were recovered mainly in urban areas (Fig. 3). One *S. dehoogii* strain was recovered from one site in the Inner Sydney Area at Darling Harbour.

Discussion

Given the emerging nature of *Pseudallescheria* and *Scedosporium* infections [12], and their ubiquitous presence in the environment [11], a better understanding of their environmental niche(s) is crucial to understand the possible relationship (if any) between the prevalence of these species in the environment and the observed increased incidence of *Scedosporium* infections in Australia. In an earlier report by our group we showed that *S. prolificans, S. apiospermum* and *S. aurantiacum* are collectively the third most frequent lung colonizers in CF patients [8]. The current study was undertaken to perform a qualitative environmental survey in the greater Sydney region for the presence of *Pseudallescheria/Scedosporium* species to set the stage

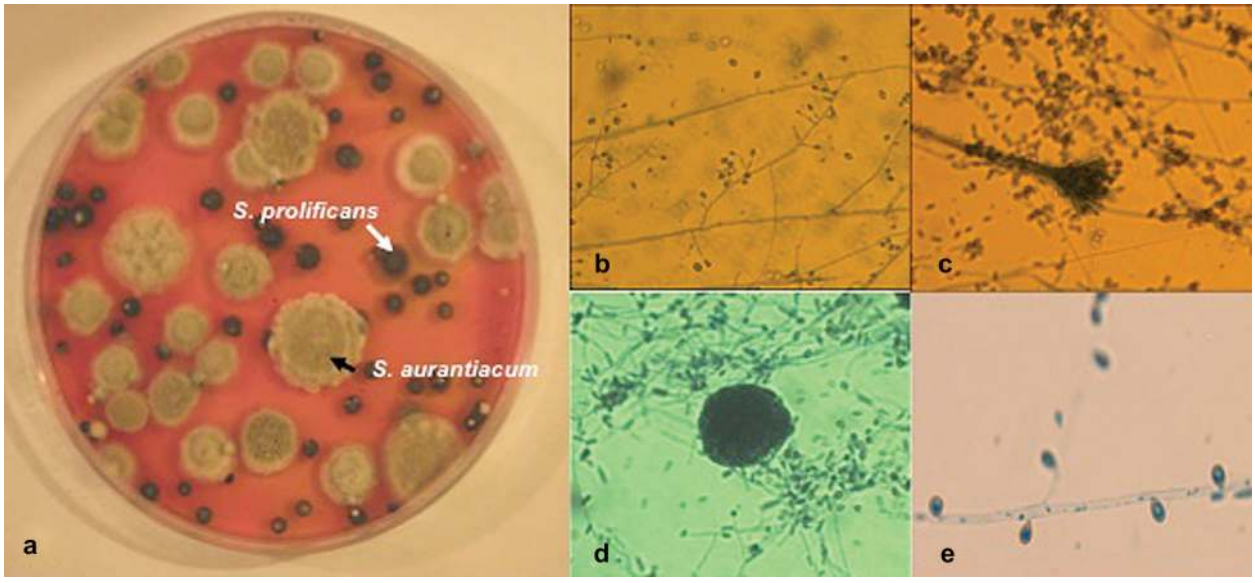

Fig. 2 *Pseudallescheria/Scedosporium* species isolated from soil: (a) DRBC + benomyl plate showing colonies of *Scedosporium* (arrow), (b) *Scedosporium prolificans*, (c) *Scedosporium aurantiacum*, (d) *Pseudallescheria boydii*, and (e) *Scedosporium dehoogii*.

for future detailed studies focusing on the investigation of a possible connection (if any) between environmental sources and clinical colonization/infection.

Environmental surveys have been performed to identify and characterize the ecological niche(s) of *Pseudallescheria/Scedosporium* species that favor their growth [11]. Studies have also been undertaken to establish a source of infection in presumed outbreak episodes [4,18]. However, often *Pseudallescheria/Scedosporium* colonies were not detected due to the overwhelming growth of other rapid-growing filamentous fungi such as *Aspergillus* spp. and *Mucor* spp. [3,18]. In a report by Rainer *et al*., DRBC and *Scedosporium* semi-selective media (SceSel+), both supplemented with benomyl, were noted to be most suited for isolating *Pseudallescheria/Scedosporium* species from soil [23]. Kaltseis *et al*. and Tintelnot *et al*. also used SceSel+ media to recover *Pseudallescheria/Scedosporium* species from environmental samples [11,24]. Based on these experiences we used DRBC medium supplemented with benomyl [23] for the primary isolation of *Pseudallescheria/Scedosporium* species from soil. This selective medium supported specifically the growth of *Pseudallescheria* and *Scedosporium* species (Fig. 1a) and adequately prevented overgrowth of other molds.

Our current environmental survey in the greater Sydney Region revealed that the highest number of *Pseudallescheria* and *Scedosporium* isolates was recovered from Circular Quay and Darling Harbour, two sites which are the main tourist attractions in the middle of downtown Sydney with high human activities (Table 1, Fig. 1). The presence of waterfronts and ferry wharfs nearby, as well as numerous water birds, suggests the probability of hydrocarbon contamination as has been previously reported [11]. Two other sites, Hyde Park and the Royal Botanic Garden, showed less of a fungal burden even though they are located in the same vicinity of central Sydney (Table 1, Fig. 1). These two sites cover a large park area and have relatively lower human traffic. Therefore, the fungal burdens in these two areas were similar to those seen in other parks in the suburban Sydney areas (Table 1, Fig. 3).

Sampling sites in the suburban Sydney areas were selected to cover a wide range of areas with different human activity. These areas, which obviously have much less human impact than downtown Sydney, were also expected to differ from one another with regard to the degree of human activity and hence differ with regards to their fungal burden. *S. prolificans* was found to predominate in various suburban parks and in nine of the sites, *S. prolificans* was the only *Scedosporium* species isolated (Table 1, Fig. 3). Of note is the fact that Paul Keating Park in Bankstown in the South West of the greater Sydney region had a significantly higher fungal burden (260 CFUs/g dry weight) (Table 1) than the rest of the remaining suburban sites sampled, with *S. aurantiacum* (86%) being the predominant species recovered (see below, Table 1, Fig. 3). The high fungal burden identified at this site could be due to the fact that the park is located in the local centre of this suburban neighborhood and is frequently visited by large numbers of the local population for recreation, supporting the notion that high human activity and resulting organic pollution, including decaying organisms, manure, mineral oil or the use of mineral fertilizers is associated

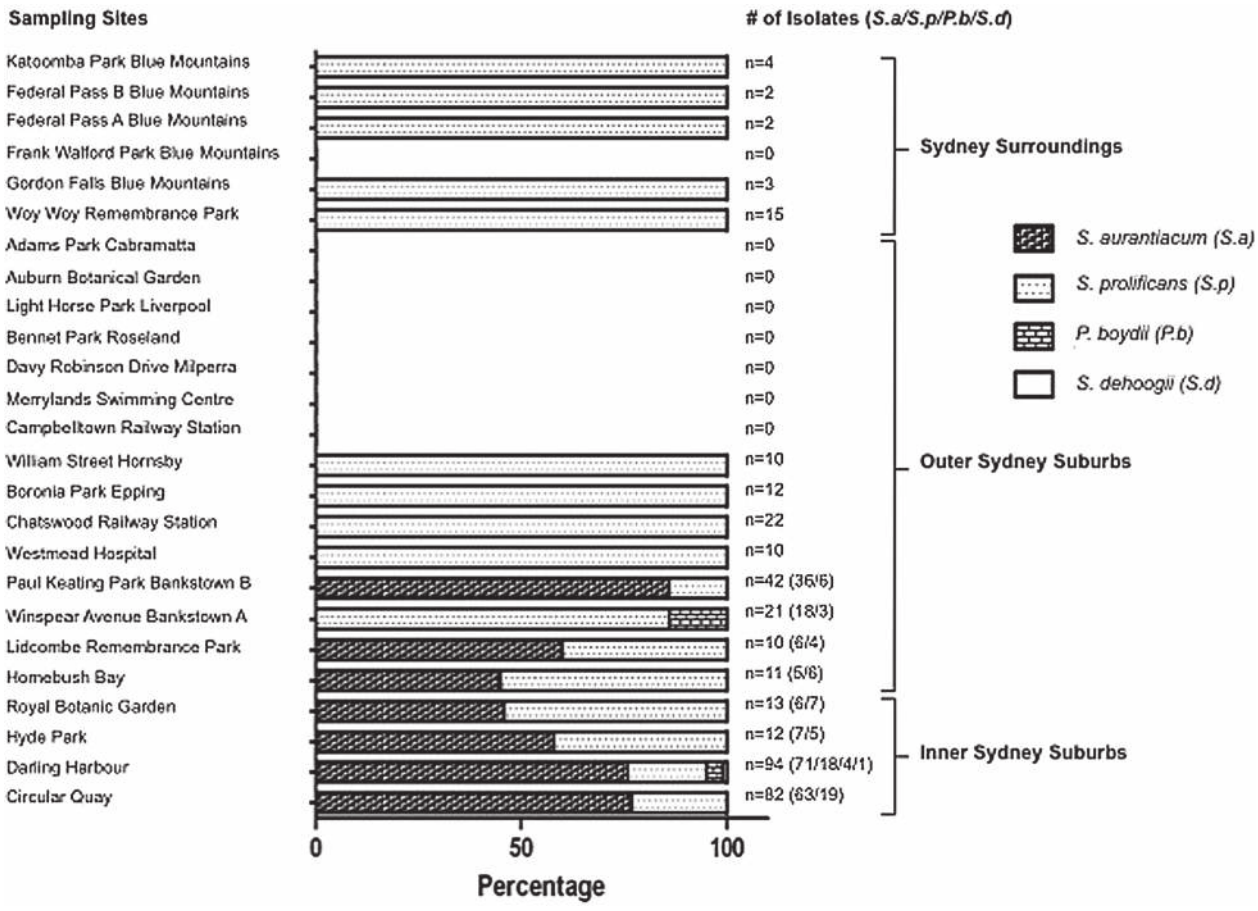

Fig. 3 Distribution of *Pseudallesheria/Scedosporium* species at the studied sampling sites.

with the presence of *Pseudallescheria* and *Scedosporium* species in the environment [11].

Previous studies in Europe have found that *S. apiospermum* was the most frequently isolated species from industrial areas, parks and playgrounds, as well as the agricultural areas, ditches, streams, puddles and cow dung [11,24]. In contrast, the present survey found *S. aurantiacum* to be the most frequent environmental species in the greater Sydney region (54.6% of all isolates). This finding is interesting, as isolation of *S. aurantiacum* in clinical specimens has been recently found to be associated with colonization (as well as infection) in patients with chronic lung disease including those with lung transplantation, bronchiectasis and CF [4,5,8]. The high presence of *S. aurantiacum* in the Australian environment may be an explanation for the relatively high proportion of *Scedosporium* infections in Australia including colonization in CF patients, due to environmental exposure to this fungus. The second most common species recovered in our survey was *S. prolificans* (43%). Of note, *P. boydii* and *S. dehoogii* were isolated in small numbers ($n = 7$ (2.1%) and $n = 1$ (0.3%), respectively) (Fig. 3). *S. dehoogii* has been previously recovered from the environment in Austria and Spain [11,22].

The present survey showed a consistent presence of strains of the identified *Pseudallescheria/Scedosporium* species at a certain sampling site at two different sampling times, validating the observed fungal burdens and the actual presence of those species in the environment. The repeated sampling excluded the possibility of sampling artifacts and gave no indication of climatic variation. However, seasonal variation has been reported for other filamentous fungi such as *Cladosporium, Penicillium* and *Aspergillus* [25,26], but has not been investigated in the current study. Further studies within the *Pseudallescheria/Scedosporium* species complex, looking at the presence of such variations are warranted. This is particularly relevant since allergic bronchopulmonary mycosis has been ascribed to these fungi [27].

In summary, *Pseudallescheria/Scedosporium* species were ubiquitous in soil in the greater Sydney region. There was a close association between density of fungi recovered and degree of human activity. However, the clinical implications of the environmental presence of *Pseudallescheria/*

Scedosporium are not yet known. The data herein presented suggest that the presence of high numbers of *S. aurantiacum* and to a lesser extent, *S. prolificans* isolates in the Australian environment may be relevant to their relative prevalence as causative agents of infection [4,5,8]. Further surveys targeted at the environments of particular at-risk groups, e.g., lung transplant recipients, or CF patients, are indicated as are studies examining the genetic relatedness of environmental and clinical isolates using robust tools such as multilocus sequence typing (MLST) analysis.

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