

Next-generation sequencing revealed dominant fungal populations in collected dust from selected public school classrooms in Metro Manila

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Abstract Fungal contaminants inside classrooms may increase the chance of health-related problems for school children and teachers, reducing their learning and productivity. Recent initiatives have utilized next-generation sequencing (NGS) technology in order to understand dust ecology and were used to significantly correlate some genera with health-related conditions. To our knowledge, this paper is the first report, describing the fungal community profile of collected dust using 454 pyrosequencing of the ITS region of the 18S rRNA gene from public school classrooms in Metro Manila, Philippines. Culture-dependent technique was done

by gravimetric sampling on Sabouraud Dextrose Agar (SDA) to note the importance of existing viable spores present in the rooms. Composite samples of settled dust from each classroom were collected and pooled to represent one sample per school. The fungal ITS rRNA genes amplified from genomic DNA with barcoded primers were sent for pyrosequencing on a 454 GS FLX titanium platform, and sequences were analyzed using the ITScan pipeline. Fungal sequences from the collected dust samples clustered in 108 operational taxonomic units (OTUs), most of which occur as singleton. The number of OTUs that correspond to fungal species varied from 16 to 29 per sample. Rarefaction curves indicated that sampling coverage was partial and that the remaining fraction of the species diversity remains to be discovered. Genera that were detected by both NGS and by cultivation on SDA include *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium*.

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1 Introduction

In the Philippines, most students spend at least half of a day inside classrooms. This environment can contain vehicles such as dust to expose them to various sources of chemical and microbial contaminants (Jurado et al.

2014; Papadopoulos et al. 2013). Some of these rooms may even lack proper ventilation, thereby compromising the health status of children. Dust in homes, offices and other human environments contains a lot of particles carrying biological and chemical pollutants which have been associated with lead poisoning, cancer, allergy, asthma, nervous system damage and sickness building symptoms (Roberts and Dickey 1995). Therefore, there is a need to study the biological components of accumulated dusts inside these classrooms to get an idea of their possible health implications.

Fungal spores and mycelia fragments are the most common contaminants of indoor dust (Butte and Heinzow 2002), and high indoor fungal concentrations may lead to the development of sickness building syndrome or SBS (Burge 2004). This syndrome includes a group of mucosal or skin-related symptoms that are linked to working in certain building environments such as headaches, nausea, sore throat, nasal congestion and irritated eyes. Such symptoms are reported especially from cases involving cohabitation of large number of people in a given room (Cooley et al. 1998; Herbarth et al. 2003; Wang et al. 2008; Zhang et al. 2011). Children are particularly susceptible due to the large amount of time they spend inside a school building, and they can have significant contact with microbes of potential harm (Jones 1998; Smith et al. 2000).

Culture-based methodology in studying communities of dust-borne fungi has often been biased toward selecting dominant and cultivable species making evaluation of their diversity limited. Recent advances in genomics and sequencing approaches utilize culture-independent strategies in investigating fungal communities to expand the identification beyond the <1 % cultivable fraction of the population in a given sample (Lindahl et al. 2013). More importantly, the accurate identification of fungi is a prerequisite for the assessment of their potential health effects in a given indoor environment.

Pyrosequencing technique has recently been used to characterize fungal diversity (Liu et al. 2015). The internal transcribed spacer (ITS) region in rRNA genes, on the other hand, has been recently designated as a universal marker for molecular barcoding of fungi serving as the default region for species identification. Ferro et al. (2014) introduced ITScan, an online and user-friendly automated pipeline for fungal diversity

analysis and identification based on ITS sequences. As a routine analysis for pyrosequencing data, microbial diversity from samples is determined by grouping the generated sequences into operational taxonomic units or OTUs using MOTHUR program coupled with an OTU-based approach analysis (Schloss et al. 2009; Schloss and Westcott 2011; Links et al. 2013). Compared to the usual algorithm in analyzing data, ITScan, therefore, lessens the use of multiple programs and stages of analysis that can make the process laborious and time-consuming.

Here, we described the fungal community profile of collected indoor dusts from public school classrooms in Metro Manila, Philippines, using a more robust platform, the 454 pyrosequencing of the ITS region of the 18S rRNA gene. Culture-dependent technique was also conducted to determine the viable spores present in the rooms that may have impact on respiratory health of the children. To our knowledge, this is the first reported data of dust-borne fungi community profiling by 454 pyrosequencing in the Philippines.

2 Materials and methods

2.1 Dust collection

Five public elementary schools in Metro Manila were purposively selected as sampling sites based on a previously related study conducted on chemical pollution and were coded 1–5 for anonymity (Fig. 1). Permits to schedule and collect indoor dust were secured from the school head or principal. They were informed of the implications of the study, its objectives and significance on the health of public school students prior to obtaining a written permit from each school.

Composite samples of settled dust from each classroom were collected using a *JLM Blast Vac Turbo* powered hand-held vacuum cleaner during daytime and pooled to represent one sample per school and were processed according to Pitkaranta et al. (2008). Collection of dust samples across sites was done during the months of November 2014–January 2015 characterized by sunny weather with no apparent clouds and no rainshowers.

Culturable fungi from dust were enumerated by gravimetric sampling utilizing Sabouraud Dextrose Agar (SDA) as culture medium. Four plates were

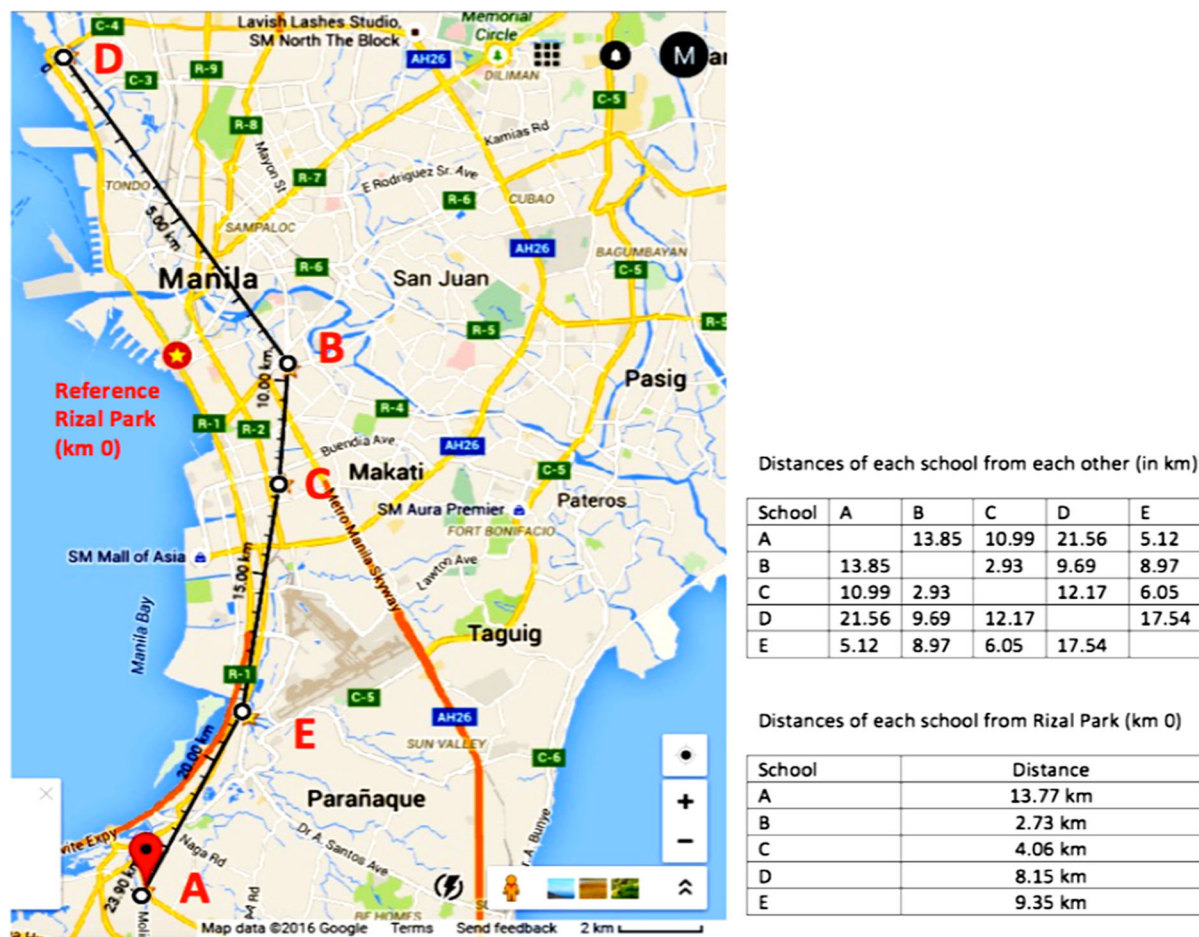


Fig. 1 Map showing the locations of the schools, their distances from one another, and their distances from the reference (Rizal Park, Manila as km 0)

placed at the corners of the classroom. The plates were exposed for 45 min to allow airborne dust to settle on the media. A total of twelve plates were obtained from each school. Afterward, culture plates were incubated for 7 days followed by colony and microscopic examination as described by Pitkaranta et al. (2008).

3 Pyrosequencing

Genomic DNA was extracted using the CTAB method following Suárez-Martínez et al. (2006). PCR amplification of the fungal ITS rRNA genes from genomic DNA was performed with barcoded primers upon submission to Macrogen (KOREA) in preparation for pyrosequencing on the 454 GS FLX titanium platform (Roche, Basel, Switzerland).

3.1 Processing of pyrosequencing data

Pyrosequencing data were processed using ITScan (Ferro et al. 2014), a web-based pipeline, which automates the analysis of fungal ITS sequences generated either by Sanger or next-generation sequencing (NGS) platforms. Third-party programs ChimeraChecker (Nilsson et al. 2010), MAFFT (Kato and Kuma 2002), MOTHUR and BLAST (Altschul et al. 1997) that are routinely utilized in analyzing pyrosequencing generated data were integrated in the ITScan pipeline steps. ChimeraChecker was used to classify all sequences as chimeric, non-chimeric or not evaluated using default parameters. Non-chimeric ITS sequences were then aligned to each other in the MAFFT software. Aligned sequences were run into the MOTHUR package, which clusters

similar sequences to each other to generate operational taxonomic units (OTUs), and calculated diversity indices and richness estimators (Schloss et al. 2009). Dissimilarity value was set at 3 %. MOTHUR then selected a representative sequence having the smallest distance from all remaining sequences within a given OTU. The selected representative sequence (or centroid) was used in a BLASTN search, and the first hit is used to identify the OTU.

4 Results

4.1 Fungal community diversity

Fungal sequences from the collected dust samples clustered in 108 OTUs, most of which occur as singleton. The NGS data further revealed that the highest diversity was found in School 3, with a Shannon–Wiener index and Simpson index of 1.3 and 0.40, respectively (Table 1). The number of OTUs that correspond to fungal species varied from 16 to 29 per sample. Rarefaction curves indicated that sampling coverage was partial and that remaining fraction of the species diversity remains to be discovered through additional sampling (Supplementary Fig. 1).

4.2 Fungal community profile

Seven genera were identified from the dust colonies obtained from SDA plates. These colonies were further identified belonging to the genera *Absydia*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Penicillium*, *Rhizopus* and *Trichoderma* (Fig. 2). Of the seven genera detected by cultivation, only *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium* were also detected by NGS.

Of the 108 OTUs, 36 % were annotated to species, 13 % to genus, 1 % to family, 2 % to order and 1 % to class. The list of phylotypes and the frequency of detection across samples is described fully in Table 2 and Fig. 3. We identified representatives of 53 OTUs that were annotated to species or genus level (Table 2) majority of which are Ascomycetes (Fig. 1) belonging to the genus *Aspergillus*. On the other hand, most of the unknown OTUs (OTUs not annotated to species or genus) shared a high (>98 %) sequence similarity with environmental sequences previously published in GenBank. Sequences affiliated with yeast-like and mushroom-like species were also present. Only *Aspergillus* sp. SL2 and the uncultured fungal clone B2B_33 were found in dust collected from all schools. Noticeable dominant groups were of the filamentous ascomycete *Aspergillus* and the yeasts *Candida* and *Cryptococcus* which appeared dust collected from all schools and more than one school, respectively.

5 Discussion

Seven genera of cultivable fungi were found in this study including *Absydia*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Penicillium*, *Rhizopus* and *Trichoderma*. Previous reports using culture-dependent methods have also mentioned the presence of these genera with Ascomycetes predominating the populations (Pitkäranta et al. 2011). The list commonly includes *Acremonium*, *Aureobasidium*, *Chaetomium*, *Eurotium*, *Fusarium*, *Mucor*, *Phoma*, *Pithomyces*, *Scopulariopsis*, *Unocladium* and *Wallemia* (Jo and Seo 2005) to occur commonly in indoor environments as shown by environmental data from culture-dependent methods (Rintala et al. 2012; Araujo et al. 2008) and supported by culture-independent studies (Cooley

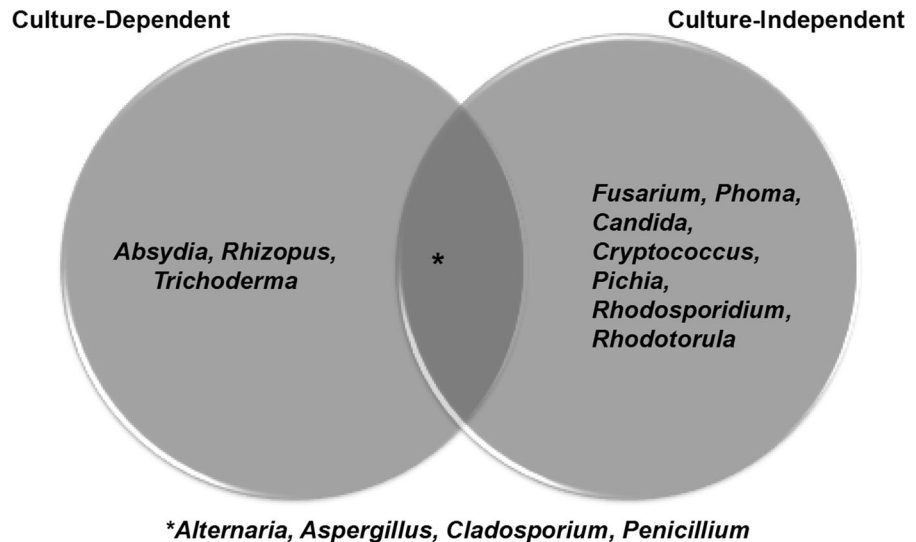
Table 1 Shannon–Wiener and Simpson biodiversity indices of fungi in collected dust samples from primary public schools in Metro Manila

Biodiversity index	School 1	School 2	School 3	School 4	School 5
Shannon–Wiener (H)	0.15	0.20	1.30	0.50	0.15
Simpson (SID)	1.11	1.11	2.5	1.25	1.11

$H = \sum p_i \ln p_i$, where p_i is the frequency of the i th species

$SID = 1/D_s$, where $D_s = \sum (n_i(n_i - 1))/(N(N - 1))$ where n_i is the total number of individuals in i th species and N is the total number of individuals in all species

Fig. 2 Genera of fungi obtained from culture-dependent and culture-independent approaches. The *asterisk* indicates that the genera were recovered in both methods



et al. 1998; Herbarth et al. 2003). Culture-dependent methods in studying fungal communities from environmental samples such as dust have always been the primary choice, but limit the data to cultivable and dominant strains. *Aspergillus* dominated the fungal population probably due to its ability to inhabit various substrates in a given favorable tropical and subtropical setting. *Aspergillus*, *Penicillium*, *Cladosporium* and *Mucor* have been considered to be the most common fungi in school environments (Meklin et al. 2005; Ramachandran et al. 2005; Dotterud et al. 1995). Their ability to easily disperse conidia added to their large size also helps to suspend their structures in culture media for long periods of time (Abarca et al. 2004). *Trichoderma*, *Penicillium*, *Rhizopus* and *Cladosporium* are frequently isolated as well. Occurrence of the isolated fungal species is consistent with the studies of other researchers on fungal assessment contained in indoor environments (Rintala et al. 2012; Karbowsa-Berent et al. 2011). The ability to form spores (Mandal and Brandl 2011) and metabolic capacities to withstand harsh environmental conditions (Hoesini et al. 2013) make them resistant to desiccation and lack of nutrients supporting their usual presence. Although *Absidia* and *Alternaria* are less occurring species, they also form significant proportion of total counts (Dixit et al. 1996).

Philippine public school classrooms in Metro Manila normally hold an average range of 40–70 students per room forcing them to share a small amount of space for approximately 8 h of stay per day.

And being a tropical country, high level of humidity is common that can provide an ideal growth condition for indoor microbes such as fungi (Bayer et al. 2000). Fungi are ubiquitous elements of indoor environments where humans are consistently exposed. Although most fungal strains may apparently be commensal or harmless, some species may be a possible health risk (Amend et al. 2010). To our knowledge, this is the first time that fungal communities were studied using next-generation sequencing in the Philippines to profile settled dust-borne fungi. However, resource limitation has restricted the conduct of multiple sampling; thus, this paper is a baseline information and only focused on describing the fungal profile found from the dust samples collected from five different schools.

Fungal sequences from the dust samples clustered in 108 OTUs, mostly singletons, and characterized by consistently low diversity. The OTUs that correspond to fungal species varied from 16 to 29 per sample with the rarefaction curves suggesting a partial coverage and a need for follow-up studies to discover rare or less dominant fractions of the species diversity that may not be represented here. Nonetheless, our results aligned with previous studies conducted using next-generation sequencing although the analyses were more multivariate (Dannemiller et al. 2014; Katra et al. 2014; Visagie et al. 2014; Pitkäranta et al. 2011). For instance, factors such as the numbers of occupants (both human and non-human), outdoor climate, degree of urbanization and geographical location were considered on how they may influence the quantity and the

Table 2 Affiliation of the unclassified and classified clones obtained from the collected dust samples showing their frequency of occurrence

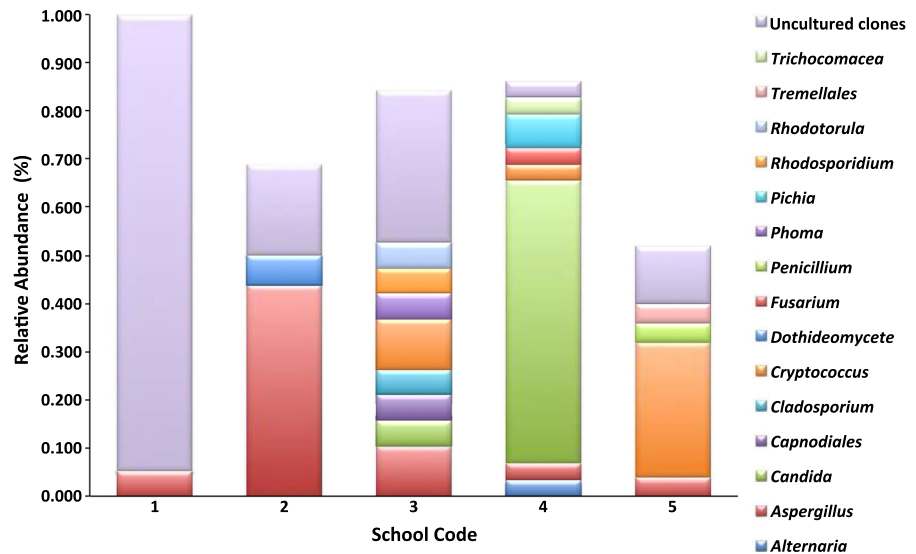
Identification	School 1	School 2	School 3	School 4	School 5
Ascomycetes					
<i>Alternaria MBPBA</i>	ND	ND	ND	0.034	ND
<i>Aspergillus caelatus</i>	ND	0.063	ND	ND	ND
<i>Aspergillus carbonarius</i>	ND	0.125	ND	ND	ND
<i>Aspergillus flavus CBE2</i>	ND	0.063	ND	ND	ND
<i>Aspergillus niger AN 01</i>	ND	0.063	ND	ND	ND
<i>Aspergillus</i> sp. 245A	ND	0.063	ND	ND	ND
<i>Aspergillus</i> sp. SL2	0.053	0.250	0.053	0.034	0.040
<i>Aspergillus tamarii DQ113</i>	ND	ND	0.053	ND	ND
<i>Capnodiales</i> sp. TR 006	ND	ND	0.053	ND	ND
<i>Cladosporium MB C003</i>	ND	ND	0.053	ND	ND
<i>Dothideomycete</i> sp. 7408	ND	0.063	ND	ND	ND
<i>Fusarium equiseti</i>	ND	ND	ND	0.034	ND
<i>Penicillium CCF2941</i>	ND	ND	ND	ND	0.040
<i>Phoma P9E4</i>	ND	ND	0.053	ND	ND
<i>Trichocomacea</i> sp. LM248	ND	ND	ND	0.034	ND
Yeasts					
<i>Candida orthopsilosis</i>	ND	ND	0.053	0.586	ND
<i>Cryptococcus heveanensis CBS 569</i>	ND	ND	0.105	0.034	ND
<i>Cryptococcus rajasthanensis</i>	ND	ND	ND	ND	0.280
<i>Pichia</i> sp. 1 TMS-2011	ND	ND	ND	0.069	ND
<i>Rhodospiridium toruloides 2241</i>	ND	ND	0.053	ND	ND
<i>Rhodotorula minuta LEMI 2795</i>	ND	ND	0.053	ND	ND
Basidiomycetes					
<i>Tremellales LM 195</i>	ND	ND	ND	ND	0.040
Uncultured fungal clones					
<i>Fungal</i> sp. G2-2010b	ND	ND	0.053	ND	ND
<i>UFC 00-304-39</i>	ND	ND	0.105	ND	ND
<i>UFC ABP_25</i>	ND	ND	ND	ND	0.080
<i>UFC f1HSc209</i>	ND	ND	0.158	ND	ND
<i>UFC f4Fc75</i>	ND	ND	0.053	ND	ND
<i>UFC L042884-122-064-C03</i>	ND	0.125	ND	ND	ND
<i>UFC T8-1-1</i>	ND	ND	0.053	ND	ND
<i>UFC TH04</i>	ND	ND	ND	ND	0.040
<i>UFC B2B_33</i>	0.947	0.063	0.053	0.172	0.480
<i>Uncultured Alternaria clone CEgs 101159</i>	ND	0.063	ND	ND	ND

ND not detected

identity of fungi found in collected dust (Barberán et al. 2015). It will be interesting to discover how our low community diversity in tandem with the mentioned factors in Philippine setting affects respiratory-related health status of exposed children inside the classroom.

Lowest diversity indices were calculated for schools 1 and 5. Fungal diversity is influenced by environmental factors (Adams et al. 2014). Schools 1 and 5 are located along a river implying higher rates of ventilation compared to School 3, which is located in a closed environment. Lower air exchange rate is highly

Fig. 3 Relative abundances of clones in the collected dust samples classified into genera of fungi based on the ITSScan database (Ferro et al. 2014)



associated with higher fungal diversity (Weigl et al. 2016) supporting School 3's claim for the highest diversity. Since plants are also sources of airborne fungi (Weigl et al. 2016), and trees surround School 3, it might also explain the higher diversity found in this school.

Recent approaches using culture-independent analysis of sequences have updated our understanding of microbial diversity. Previously, we conducted profiling of fungal communities using culture-independent method such as denaturing gradient gel electrophoresis (DGGE) using environmental samples from aquaculture and non-aquaculture sites (Galan et al. 2013). These advances in next-generation sequencing (NGS) technology, such as pyrosequencing, enable a more robust and quick characterization of desired microbial communities with greater sequence depth compared to cloning or Sanger sequencing (Sogin et al. 2006). Methodological comparison in this study indicated that cultivation when combined with molecular approach, such as next-generation sequencing, could detect abundant species following a cross-sectional sampling. Our results confirmed the detection of *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium* really came from settled dust. In addition, NGS data have expanded our knowledge of the fungal genera that were present but have been missed by the plating method including *Fusarium* and *Phoma* along with the yeasts *Candida*, *Cryptococcus*, *Rhodospiridium* and *Rhodotorula*. Previous reports have identified the three most abundant allergenic genera (i.e., *Alternaria*,

Aspergillus and *Phoma* (Barberán et al. 2015) from indoor environments. They were careful to note, however, that the species in these genera differ in their levels of allergenicity and that linking the abundances with rates of allergic diseases will be worthy of follow-up study.

Interestingly, a large proportion of unknown OTUs were detected by the NGS in this study. The non-identification may be due to the database that we used (ITSScan) or probably we can have a candidate for the discovery of novel species from collected dust. This is possible since nearly 90 % of fungal biodiversity may as yet be unidentified (Schmit and Mueller 2007). However, additional experiments may confirm such possibility.

Previous initiatives have utilized next-generation sequencing (NGS) technology in order to understand dust ecology, more importantly its influence to human health. Pitkäranta et al. (2011) and Visagie et al. (2014) have used the tandem of cultivation and NGS in order to describe the mycobiome from collected dust from indoor environment such as the household (Barberán et al. 2015). Furthermore, NGS was used to significantly correlate some genera with health-related conditions such as asthma (Dannemiller et al. 2014; Kutra et al. 2014). Realizing the importance of diversity as well as the genera present in collected dust inside classrooms, it will be an important venture for us to follow up on the relationship of these fungal genera and the status of the respiratory health of the children concerned particularly in our country's tropical setting.

6 Conclusion

This paper is the first report, describing the fungal community profile of collected dust using culture-dependent method in tandem with 454 pyrosequencing of the ITS region of the 18S rRNA gene from public school classrooms in Metro Manila, Philippines. Fungal sequences from the collected dust samples clustered in 108 operational taxonomic units (OTUs) as analyzed by the ITScan software. Pyrosequencing identified more specimens than culture-dependent approach and confirmed the identity viable spores that can be found indoors. The viable spores may be considered threats to public health, but further investigation is merited. Genera that were detected by both NGS and by cultivation on SDA include *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium*. This baseline information would be helpful in planning preventive measures on preventing respiratory-related problems of exposed school children.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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Supplementary Figure 1

Rarefaction Curves

