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Diversity and abundance of human-pathogenic fungi associated with pigeon faeces in urban environments

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Abstract

Pathogenic fungi are a growing health concern worldwide, particularly in large, densely populated cities. The dramatic upsurge of pigeon populations in cities has been implicated in the increased incidence of invasive fungal infections. In this study, we used a culture-independent, high-throughput sequencing approach to describe the diversity of clinically relevant fungi (CRF) associated with pigeon faeces and map the relative abundance of CRF across Seoul, Korea. In addition, we tested whether certain geographical, sociological and meteorological factors were significantly associated with the diversity and relative abundance of CRF. Finally, we compared the CRF diversity of fresh and old pigeon faeces to identify the source of the fungi and the role of pigeons in dispersal. Our results demonstrated that both the composition and relative abundance of CRF are unevenly distributed across Seoul. The green area ratio and the number of multiplex houses were positively correlated with species diversity, whereas wind speed and number of households were negatively correlated. The number of workers and green area ratio were positively correlated with the relative abundance of CRF, whereas wind speed was negatively correlated. Because many CRF were absent in fresh faeces, we inferred that most species cannot survive the gastrointestinal tract of pigeons and instead are likely transmitted through soil or air and use pigeon faeces as a substrate for proliferation.

KEYWORDS
Columba livia, culture-independent, high-throughput sequencing, public health

1 | INTRODUCTION

Pathogenic fungi are a major human health concern with annual mortality rates of some invasive fungal diseases exceeding 50% (Brown et al., 2012). The health risks associated with fungal pathogens are often overlooked, yet diseases caused by the most common pathogenic fungi (e.g., candidiasis, aspergillosis and mucormycosis) cause at least as many deaths as tuberculosis or malaria (Brown et al., 2012; WHO Fact Sheet Report 2016a,b). The incidence of fungal disease is rising due to the growing population of immunocompromised individuals (Underhill & Iliev, 2014), the expanding geographic distribution of pathogenic fungi as a result of climate change (Garcia-Solache & Casadevall, 2010), economic globalization and the accompanying increase in human population densities in and around cities (Gushulak & MacPherson, 2004; Wilson, 1995). As most cases of pathogenic fungal disease go unreported to health monitoring agencies such as the Centers for Disease Control and the World Health Organization, epidemiological approaches to prevention and treatment are limited (Brown et al., 2012).

Pigeons (*Columba livia domestica*) have been implicated in the increased incidence of human fungal infection in cities as their populations dramatically increased with urbanization (Jerolmack, 2008). Pigeons are suspected carriers of potentially pathogenic fungi, including *Cryptococcus neoformans* (Emmons, 1955), which causes human cryptococcosis, a disease that causes over one million serious illnesses annually (Harris et al., 2011; Kronstad et al., 2011). Recent studies, however, suggest a more serious health threat appears to be their excrement, which is ubiquitous in urban areas. The chemical

composition of pigeon faeces, specifically the availability of nitrogen from uric acid, creates an excellent substrate for fungal spores to propagate, and several studies have shown that pathogenic fungi metabolize faecal compounds during various developmental processes (Frazzita et al., 2013; Lee, Chow, Morrow, Djordjevic, & Fraser, 2011; Lee et al., 2013; Xue, 2012).

To understand the importance of pigeons as potential vectors or facilitators in the spread of pathogenic fungi in human populations, the diversity and abundance of potentially pathogenic fungi in pigeon faeces must be further investigated. To date, approximately 48 species of potentially pathogenic fungi from 28 genera have been isolated from pigeons globally (Chae et al., 2012; Haag-Wackernagel & Moch, 2004; Jang et al., 2011; Khosravi, 1997; Ramirez, Robertstad, Hutchinson, & Chavez, 1976; Soltani, Bayat, Hashemi, Zia, & Pestechian, 2013; Tokarzewski, 2007). This is likely a substantial underestimate because most reports were based on culture-dependent methods, and the majority of fungi are not readily cultured (Pace, 1997; Rappe & Giovannoni, 2003). Recently, a study on fungal diversity on human skin compared culture-based sampling with a culture-independent method using high-throughput sequencing. They reported over 130 fungal operational taxonomic units (OTUs) using culture-independent methods compared to just five using culture-based methods (Findley et al., 2013). Thus, it is reasonable to project that the diversity of fungi associated with pigeons is far more complex than is currently described.

In this study, we used a cross-sectional, culture-independent analysis to evaluate the potential health risk associated with pigeon faeces in a densely populated city, Seoul, Korea. Seoul is an ideal place to evaluate the relationship between pigeons and potentially pathogenic fungal diversity because it has a large, dense human population (>24 million in the greater metropolitan area: the fourth largest in the world) and, as is true with most other major cities in the world, pigeon populations have dramatically increased in tandem with the increase in human urbanization (Moller et al., 2012). In addition, previous culture-based studies in the Seoul area have identified several potentially pathogenic fungi in pigeon faeces (Chae et al., 2012; Chee & Lee, 2005; Jang et al., 2011).

Identifying environmental factors that are statistically associated with pathogenic fungal diversity and abundance is a critical step for causal inference. Fungal diversity in air and soil is known to be influenced by season, weather, human activity, urbanization and habitat type (Fierer et al., 2008; Findley et al., 2013; Newbound, McCarthy, & Lebel, 2010; Wu, Su, & Lin, 2000; Shelton, Kirkland, Flanders, & Morris, 2002; Summerbell et al., 1992). The abundance of pathogenic fungi (particularly *Cr. neoformans*) has been linked to weather (humidity, temperature and radiation), vegetation, microbes and the chemical properties of pigeon faeces (pH, uric acid and nitrogen) (Granados & Castaneda, 2006; Hubalek, 1975; Ishaq, Bulmer, & Felton, 1968; Levitz, 1991; Ruiz, Neilson, & Bulmer, 1982; Walter & Yee, 1968). Interestingly, recent studies revealed a strong association between biogeographical (species diversity, land area, etc.) and sociological factors (human population size, health expenditure, etc.) with global distribution of human infectious diseases (Han, Kramer, & Drake, 2016; Murray et al., 2015; Semenza, Rocklov, Penttinen, & Lindgren, 2016; Stephens et al., 2016). Until now, however, no study has assessed the relationship between these environmental factors and pathogenic fungal diversity or abundance.

In this study, we provide the first non-culture-based molecular study of the diversity and geographic distribution of fungi found in pigeon faeces in a highly urbanized environment. We focused on those fungi that are potentially pathogenic to humans, hereafter referred to as clinically relevant fungi (CRF). In addition, we describe environmental and demographic factors that were associated with CRF diversity and abundance. Finally, we investigated whether urban pigeons are true vectors of potentially pathogenic fungi, or whether their role is limited to providing substrate for already existing spores, by comparing species diversity found in fresh versus old pigeon faeces.

2 | METHODS

2.1 | Study location, sample collection and meta data acquisition

Pigeon (*Co. livia)* faecal samples were collected from 38 sites across Seoul, Korea, in January 2014. Three to five faecal droppings from multiple pigeons were considered as one sample and two different types of samples were collected: old (completely dried, decolorized) and fresh (wet, coloured). We collected old samples from each locality, while fresh samples were collected from 18 of these sites (Table S1). Samples were collected using sterilized forceps and placed in sterile 15-ml Falcon tubes (BD Bioscience, Canaan, CT, USA). To minimize soil contamination, all samples were collected from rooftops, window ledges or sidewalks. Fresh faeces were collected immediately after excretion. All samples were transported on ice and stored at −80°C until subsequent analysis.

Data were collected for variables potentially influencing fungal diversity and abundance, including geography (latitude, longitude and altitude), weather (precipitation, wind speed, maximum/mean/minimum temperature and humidity), site conditions (pigeon population size, green area ratio, impermeability and habitat type) and sociological variables (distance to city centre, number of households, number of workers, number of apartments, number of multiplex houses and number of restaurants). The geographic data were recorded while sampling using the WGS84 coordinate system with an EXPLORIST 210 GPS (Magellan, San Dimas, CA, USA). All weather data were collected from the Korea Meteorological Administration database and were spatially interpolated between 29 meteorological stations across Seoul in December 2013 and January 2014 (http://sts.kma.go.kr). Pigeon population size was estimated by observing the number of pigeons present during sampling. Data on the green area ratio (a weighted score for development sites based on the types of landscape, site design features that are implemented and the amount of area they cover), impermeability (the sum of building coverage ratio and impermeable pavement ratio) and habitat type (biotope) were provided by Seoul Metropolitan Government (http://urban.seoul.go.kr). Sociological data were provided by BIZGIS (http://www.biz-gis.com/XsDB).

2.2 | DNA extraction, PCR amplification and pyrosequencing

Samples were homogenized (via grinding or mixing), and total DNA was isolated from 0.2 g (dry weight) or 0.3 g (wet weight) of old and fresh samples, respectively. To remove inhibitory substances commonly found in stool and recover DNA of the highest purity possible, extractions were performed with the PowerSoil DNA Isolation Kit (MO BIO Laboratory, Carlsbad, CA, USA) according to the manufacturer's protocol with two modifications: for old pigeon faeces, an additional 200 μl of PowerSoil DNA Kit Bead Solution (MO BIO Laboratory, Carlsbad, CA, USA) was added to each tube before vortexing (according to the recommendation of the manufacturer for treating extremely dry samples), and all samples (both fresh and old) were incubated at 65°C in a dry oven for 15 min before further processing.

The internal transcribed spacer (ITS) region has been formally proposed as the primary fungal barcoding gene for the Kingdom Fungi because its high sequence variation resolves closely related species across the fungal tree of life (Ciardo, Schar, Bottger, Altwegg, & Bosshard, 2006; Schoch et al., 2012). The ITS region was amplified using the primers ITS1F and ITS4 (White, Bruns, Lee, and Taylor, 1990; Gardes and Bruns, 1993) modified for pyrosequencing. An "A" pyrosequencing adaptor with a 10-bp multiplex tag was added to ITS4 (Parameswaran et al., 2007), and a "B" pyrosequencing adaptor was added to ITS1F (Table S2). Each PCR contained 2 μl of template DNA, AccuPower PCR PreMix (Bioneer, Daejeon, Korea) (containing 1 unit of Top DNA polymerase; 250 μM dNTPs; 10 mM Tris–HCl; 30 mM KCl; and 1.5 mM $MgCl_2$) and 10 pmol of each primer in a final volume of 20 μl. PCR was performed on a C1000™ thermal cycler (Bio-Rad, CA, USA) under the following conditions: initial denature at 95°C for 5 min, followed by 30 cycles of 95°C for 40 s, 55°C for 40 s and 72°C for 1 min and a final step at 72°C for 10 min. PCR products were electrophoresed through a 1% agarose gel with LoadingSTAR (Dyne Bio, Seoul, Korea). To minimize PCR bias caused by differential amplification efficiencies of the ITS genes in multitemplate PCRs, three amplicons were pooled for each sample (Ibarbalz, Perez, Figuerola, & Erijman, 2014; Pinto & Raskin, 2012). PCR products were purified using the Expin PCR Purification Kit (GeneAll Biotechnology Co., Ltd., Seoul, Korea), and DNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo, DE, USA). The 56 total samples were divided equally, and two PCR libraries were constructed with 28 samples each, pooled in equimolar concentrations and analysed using an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). Quality control analyses were performed with a Caliper LabChip GX using the DNA High Sensitivity Assay (PerkinElmer, MA, USA).

Pyrosequencing reactions were performed with flow pattern B on ¼ plate of a 454 GS FLX+ System (Roche 454 Life Sciences, CT, USA) and sequenced in the reverse direction. All steps after library construction were performed at Macrogen (Seoul, Korea). Raw sequence data have been deposited in the Sequence Read Archives (Leinonen, Sugawara, & Shumway, 2011) of NCBI under the Accession No SRR2045733.

2.3 | Sequence processing, taxonomic assignment and diversity analyses

Sequence reads from the two libraries were trimmed, filtered and merged with the following adjustments using QIIME v1.8.0 (Caporaso et al., 2010): minimum length of 200 bp, maximum length of 1,000 bp, minimum quality score of 25, no ambiguous bases, no mismatches in the primer sequence and maximum homopolymer length of 6. We applied these strict filtering requirements to minimize the effect of amplicon noise. Chimera detection and clustering were performed using USEARCH v5.2.236 (Edgar, 2010) implemented in QIIME. OTUs were constructed with the QIIME clustering algorithm "nearest" at 97% sequence similarity, and representative sequences were selected based on the sequence abundance. Singletons (OTUs represented by a single sequence) were removed from analyses.

Accurate identification is important in any molecular study, but especially so when studying CRF because the results have implications for public health. Thus, we used a conservative, multistep method to identify OTUs to species (Fig. S1). As pathogenicity can be strain-specific, when an OTU was phylogenetically closely related to a CRF, we considered it to be potentially pathogenic. BLAST 2.2.22 (Altschul, Gish, Miller, Myers, & Lipman, 1990) was used to assign representative sequences to two curated taxonomic databases: UNITE (Kõljalg et al., 2013) and the ISHAM ITS Database for Human and Animal Pathogenic Fungi (Irinyi et al., 2015), at a 98% minimum identity level. The preliminary identification of OTUs was performed using FHITINGS 1-2 (Dannemiller, Reeves, Bibby, Yamamoto, & Peccia, 2014). Next, neighbour-joining phylogenetic analyses (with 500 bootstrap replicates) were carried out with type strains and verified strains from the literature for each genus. Identifications were only considered valid if the representative sequence was monophyletic with sequences of a single species. The final identification of each OTU was based on the combined results of FHiTINGS and the phylogenetic analyses. To be conservative, we only categorized OTUs as CRF when the species-level identification was strongly supported by both methods. Low-resolution identification often overestimates risk factors by lumping clinical and nonclinical species into a genus-level identification. We followed the fungal taxonomy in Index Fungorum (http://www.indexfungorum.org/names/names.asp) and the classification of CRF in the Atlas of Clinical Fungi v. 4.0 (de Hoog, Guarro, Gené, & Figueras, 2014).

Alpha-diversity analyses were performed on the subsampled data set: OTU diversity was estimated using Chao 1 (Chao, 1987) calculated in EstimateS (Colwell & Elsensohn, 2014) based on 100 randomizations, and Good's coverage (Good, 1953), calculated in QIIME.

2.4 | Statistical analyses

To test for spatial autocorrelation in CRF species diversity and absolute abundance, we used Moran's I correlograms with 500 Monte Carlo simulations in SAM v. 4.0 (Rangel, Diniz-Filho, & Bini, 2010).

We preprocessed metadata before linear regression modelling. As most pigeons in cities have a foraging area within 0.3 km of their

home lofts (Rose, Nagel, & Haag-Wackernagel, 2006), all metadata were averaged to one value for a circle with a radius of 0.3 km using the Spatial Analyst tool in ARCGIS 10.2.2 (ArcGIS Desktop, ESRI, CA, USA). Highly correlated variables (>0.7) were combined using linear regression to avoid overfitting and bias (Tabachnick & Fidell, 2013), and the remaining variables were standardized (Marquandt, 1980) using the Caret package (Kuhn, 2008) in R v. 3.1.0 (Team, 2013).

The multiple linear regression model was built with a permutation test to identify the significant predictors of the two dependent variables: number of CRF species and relative abundance of CRF. The best model was selected according to the corrected Akaike information criterion (Tables S4 and S5). All multivariate analyses were run in R. The coefficient for each of the significant variables in the linear model is reported as the mean ± 95% confidence interval. Variables were considered significant if $p < .05$.

3 | RESULTS

A total of 302,629 raw reads were obtained from the 56 samples; after filtering, 209,780 reads remained for community analysis. We obtained 303 sequence reads from one fresh faecal sample (PD19F, Table S3), which represented 50% of the read count of the sample with the next lowest read count, and thus, this sample was discarded. Discarding this sample resulted in greater OTU detection and higher precision in the beta diversity analyses. After this adjustment, the average read count was 3,808 (range 663–7,313) (Table S3), and the total number of samples was 55, including 38 old faecal samples and 17 fresh faecal samples from a total of 38 sites. The average sequence length of the ITS region was 620 bp (range: 200–866 bp). Chao 1 estimators and Good's coverage confirmed that our sampling and sequencing depth were sufficient; Chao 1 estimators approached asymptotes (Fig. S2), and Good's coverage indicated that our sequencing detected between 94.4% and 99.8% of the total diversity (Table S3).

A total of 431 fungal OTUs were identified across all samples using sequence clustering. These OTUs were assigned to 63 fungal genera (Fig. S3). Using our multistep identification method, 42 of these OTUs were assigned to 35 CRF species.

3.1 | The diversity and distribution of CRF

We did not find spatial autocorrelation among properties of sampling sites (data not shown). The species diversity of CRF associated with pigeon faeces was heterogeneous between sites (2–17 species per site; Figure 1). *Candida bovina* was the most widely distributed CRF species found in old pigeon faeces (32/38 sites). *Alternaria alternata* (31 sites), *Cr. magnus* (28 sites) and *Rhodotorula mucilaginosa* (26 sites) were also widely distributed (Table 1).

The best linear model explained 85% of the total variance and included the following factors: the green area ratio, the number of multiplex houses, wind speed, number of households and habitat

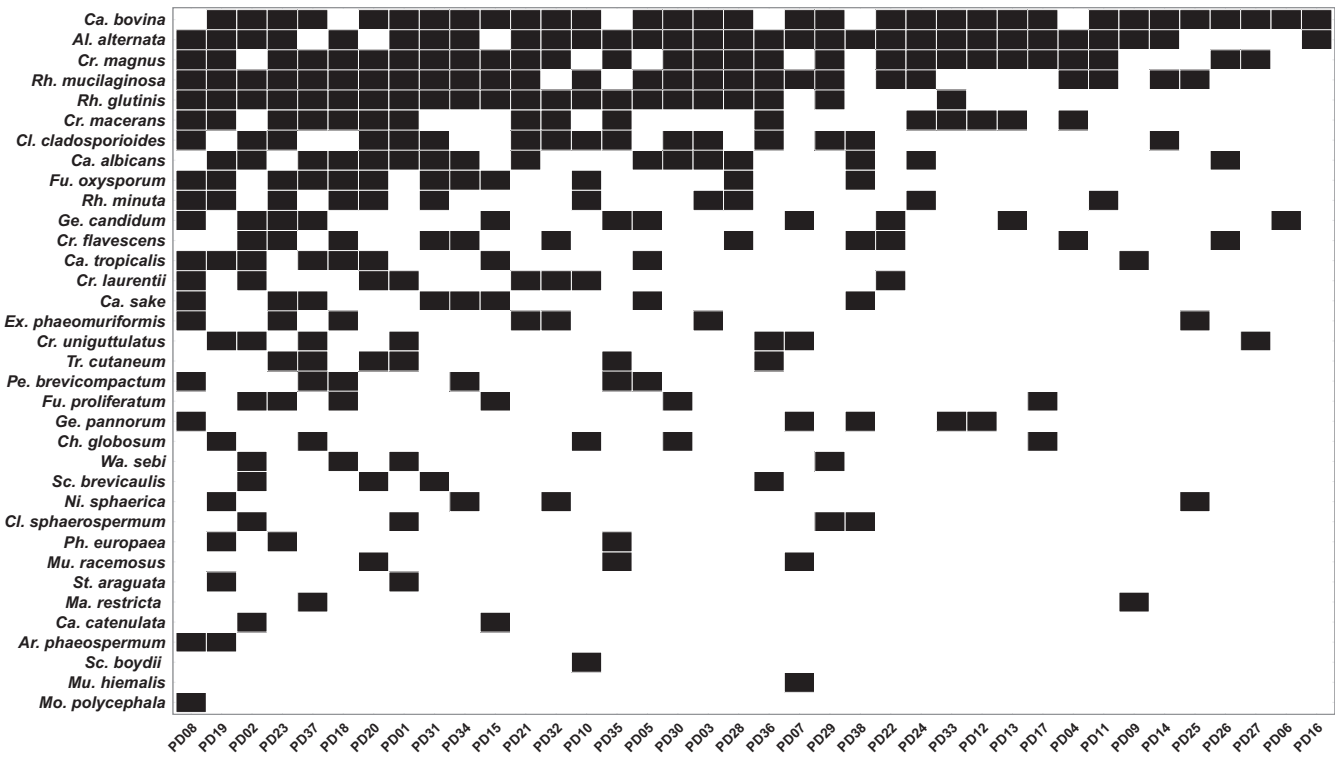

FIGURE 1 The distribution of clinically relevant fungi across sites. Cells in black represent the presence of a species at a site. Rows towards the top represent species with wider distribution, while columns towards the left represent sites with higher diversity. Site locations refer to Figure 2

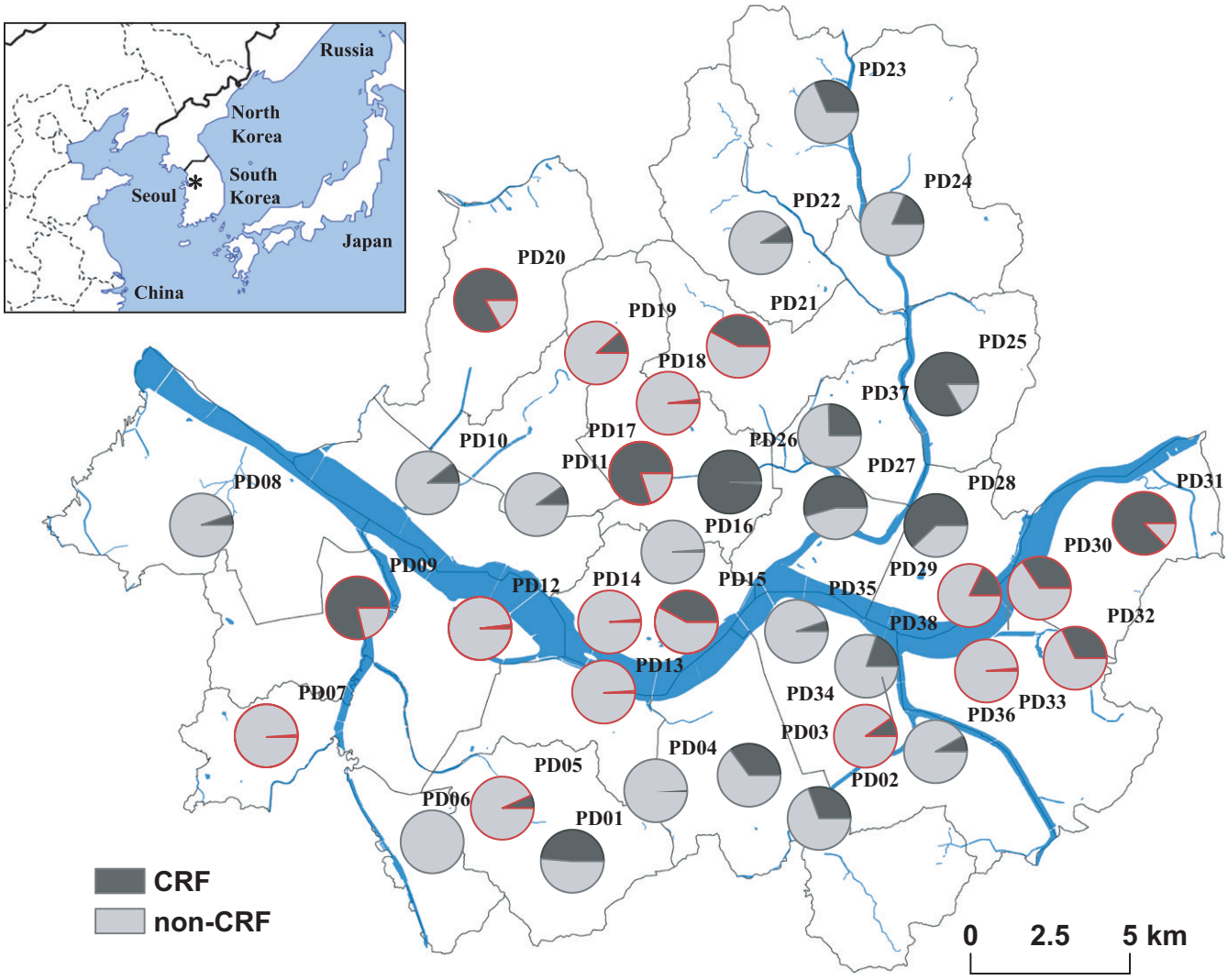

FIGURE 2 The distribution pattern of the clinically relevant fungi (CRF) associated with pigeon faeces across Seoul, Korea. Pie charts represent the proportion of sequences denoted CRF relative to total number of fungal OTUs. Charts with red borders indicate sites where both fresh and old faeces were sampled

type (Table 2). While the green area ratio and the number of multiplex houses were positively correlated with CRF diversity, wind speed and number of households were negatively correlated. An analysis of habitat type (categorical variable) revealed that the "commercial," "industrial/urban," and "residential" biotopes displayed significantly higher CRF diversity compared with the other five biotopes (raw data shown in Table S1).

3.2 | The relative abundance of CRF

The relative abundance of CRF is the relative proportion of sequence reads assigned to CRF species among the total number of sequence reads. The relative abundance of CRF among pigeon faeces differed greatly across sampling sites (ranging from 0.095% to 98.88%; Figure 2, Table S1). Four variables (number of workers, green area ratio, wind speed and habitat type) were statistically significant predictors of the relative abundance of CRF, explaining 59.8% of the total variance (Table 3). Sites with higher number of workers and green area ratio tended to have a higher abundance of CRF compared with the other parts of the city, whereas higher wind speed was associated with lower abundance of CRF. For habitat type the "residential," "industrial/urban" and "commercial" biotopes had a significantly higher relative abundance of CRF compared with the four other biotopes. The relative abundance of CRF and the number of CRF species identified in each sampling site were not related to each other. Relevant data for this analysis are provided in Table S1.

3.3 | Comparison between fresh and old faeces

From the 38 collection sites, 35 CRF species were identified in old faeces while 18 CRF species were identified in fresh faeces from 17 sites; however, when considering sites where both fresh and old faeces were collected (17 sites), all CRF species detected

TABLE 1 List of clinically relevant fungi (CRF) species with biosafety level (BSL) and number of sites that CRF were detected

CRF (strain)[a]	Acc. no.	BSL	Number of sites		
			Fresh[c]	Old[c]	Total
Alternaria alternata[b] (CBS916.96^T)	KF465761	1	8	16	31
Arthrinium phaeospermum[b] (CBS142.55^T)	KF144908	1	0	1	2
Candida albicans (CBS562^T)	JN944029	2	1	8	16
Candida bovina (CBS2760^T)	AJ223020	1	14	17	32
Candida catenulata[b] (CBS565^T)	GU246267	1	0	1	2
Candida sake (CBS159^T)	AJ549822	1	4	4	8
Candida tropicalis (ATCC750^T)	AY939810	2	0	6	9
Chaetomium globosum[b] (CBS164.62^T)	JN209920	1	0	3	5
Cladosporium cladosporioides[b] (CBS169.54^T)	AJ300335	1	1	7	16
Cladosporium sphaerospermum[b] (CBS193.54^T)	DQ780343	1	1	1	4
Cryptococcus flavescens (CBS942)	AB035046	1	0	4	11
Cryptococcus laurentii (CBS139^T)	AF410468	1	0	3	8
Cryptococcus macerans[b] (CBS6532^T)	EU082230	1	4	8	16
Cryptococcus magnus[b] (CBS140^T)	AF190008	1	11	14	28
Cryptococcus uniguttulatus (CBS1730^T)	AF335938	1	0	2	7
Exophiala phaeomuriformis[b] (UTHSC88-471)	EF025401	2	0	3	7
Fusarium oxysporum[b] (NRRL62960)	KM30313	2	0	6	12
Fusarium proliferatum (CBS216.76^T)	AB587006	1	0	4	6
Geomyces pannorum[b] (S9A418)	AJ509868	1	2	3	5
Geotrichum candidum (CBS182.33^T)	AY788300	1	4	4	11
Malassezia restricta (CBS7877^T)	AF522062	1	1	1	2
Mortierella polycephala[b] (CBS456.66^T)	HQ630335	1	2	0	1
Mucor hiemalis[b] (CBS201.65^T)	EU484277	1	1	1	1
Mucor racemosus[b] (CBS260.68^T)	FN650642	1	1	2	3
Nigrospora sphaerica[b] (xsd08093)	FJ478134	1	0	3	4
Penicillium brevicompactum[b] (NRRL868^T)	AY484927	1	0	3	6
Phialophora europaea[b] (CBS129.96)	JQ766440	2	0	1	3
Rhodotorula glutinis (CBS20^T)	AF444539	1	2	12	22
Rhodotorula minuta (JCM3777^T)	AB026016	1	1	4	11
Rhodotorula mucilaginosa (CBS316^T)	AF444541	1	6	12	26
Scedosporium boydii[b] (CBS101.22^T)	AY877350	2	0	0	1
Scopulariopsis brevicaulis[b] (FMR12235)	KP132734	2	0	2	4
Stenella araguata[b] (ATCC24788^T)	EU019250	1	0	1	2
Trichosporon cutaneum (CBS6864^T)	AF444437	2	1	1	6
Wallemia sebi (CBS196.56)	AY302526	1	0	2	4

[a]Strain id numbers ending with a "^{T}" denote type strains.
[b]New global records associated with pigeon faeces.
[c]Number from 17 sites where both fresh and old faeces were collected.

in fresh samples, with the exception of *Mortierella polycephala*, were also present in old samples (Table 1). Therefore, it was not possible to unambiguously differentiate CRF species that used pigeons as a vector and those that used their faeces as a substrate. The sporadic occurrence of *M. polycephala* in pigeon faeces could be due to its rarity in this urban environment. Alternatively, *M. polycephela* spores may have settled on fresh faeces between excretion and sampling.

4 | DISCUSSION

In this study, we combined culture-independent, high-throughput sequencing and rigorous species identification methods to describe the diversity and distribution of CRF in pigeon faeces collected in a densely populated urban environment. We identified 35 CRF species, 23 of which are new records associated with pigeon faeces (Table 1). Statistically significant environmental variables associated

TABLE 2 Factors affecting the diversity of clinically relevant fungi among pigeon faeces based on the highest scoring linear model: GRE + MUL + Wsp + HOU + HAB (adjusted R^2 = .850)

Variable	Beta ± *SE*[a]	Lower 95% CI	Upper 95% CI	*p*-Value
Green area ratio (GRE)	4.492 ± 1.642	2.850	6.134	<.001
Number of multiplex houses (MUL)	2.977 ± 1.951	1.026	4.928	.004
Wind speed (Wsp)	−2.668 ± 1.505	−4.173	−1.164	.001
Number of households (HOU)	−2.724 ± 1.889	−4.613	−0.835	.006
Habitat type (categorical) (HAB)				
Commercial and business biotope	11.877 ± 2.281	9.596	14.158	<.001
Industrial and urban infrastructure biotope	11.296 ± 3.664	7.632	14.961	<.001
Residential biotope	8.762 ± 3.041	5.722	11.803	<.001

Multiplex houses are dwellings with multiple separate units (Jung et al., 2015).
[a]Coefficient and standard error for each variable.
SE, standard error; CI, confidence interval.

TABLE 3 Factors affecting the relative abundance of clinically relevant fungi among pigeon faeces based on the highest scoring linear model: JOB + GRE + Wsp + HAB (adjusted R^2 = .598)

Variable	Beta ± *SE*[a]	Lower 95% CI	Upper 95% CI	*p*-Value
Number of workers (JOB)	0.161 ± 0.049	0.065	0.257	.003
Green area ratio (GRE)	0.155 ± 0.051	0.055	0.255	.005
Wind speed (Wsp)	−0.122 ± 0.049	−0.218	−0.026	.017
Habitat type (categorical) (HAB)				
Residential biotope	0.495 ± 0.089	0.321	0.669	<.001
Industrial and urban infrastructure biotope	0.385 ± 0.111	0.167	0.603	.002
Commercial and business biotope	0.204 ± 0.073	0.061	0.347	.009

[a]Coefficient and standard error for each variable.
SE, standard error; CI, confidence interval.

with CRF diversity were the green area ratio, the number of multiplex houses, wind speed, the number of households and habitat type. In addition, we found that three variables (number of workers, green area ratio and habitat type) were significantly correlated with geographic patterns of abundance. The majority of CRF were found in old rather than fresh faeces, and thus, while pigeons are potentially direct vectors for some pathogenic fungi, it is likely that the primary role of pigeons in disease transmission is the provision of substrate (faeces) for fungal spores from other sources.

4.1 | The diversity and distribution of CRF

We observed high overall fungal diversity among pigeon faeces, with 431 OTUs assigned to 63 fungal genera (Fig. S3). Of these, 42 OTUs representing 35 species were identified as potentially pathogenic (CRF). A comparison of these results with previous, culture-dependent studies of pigeon faeces fungal diversity in the same location (Chae et al., 2012; Jang et al., 2011) showed that 15 species were unique to these previous studies, 28 were unique to our study, and seven were found in both studies.

Although culture-independent, high-throughput sequencing approaches are extremely sensitive for detection of rare or unculturable species, they are dependent on sequencing depth. Based on Chao 1 estimators and Good's coverage, our sampling and sequencing depth were sufficient. However, this may still not be sufficient for detecting all rare fungal species. At relatively low sequencing depth, some singletons may be informative for recovering unique lineages (Zhan et al., 2013); however, these sequences are usually considered artefacts and removed from downstream analyses (Tedersoo et al., 2010). Accordingly, some rare *Candida* species (*Ca. krusei*, *Ca. lusitaniae*, *Ca. magnoliae* and *Ca. zeylanoides*) were initially detected in our samples yet removed from the final list during singleton removal. In addition, three species (*Cr. albidus*, *Sa. cerevisiae* and *Tr. asahii*) that were detected from our samples with BLAST were filtered out of the analysis during multistep confirmation. Six other fungal species (*Ca. ciferrii*, *Ca. colliculosa*, *Ca. famata*, *Ca. magnoliae*, *Ge. klebahnii* and *Ma. furfur*), while frequently isolated from sources other than pigeon faeces worldwide (e.g., soil, plant, insects and wild bird droppings) (Cafarchia, Romito, Coccioli, Camarda, & Otranto, 2008; Guz, 2011; Lo Cascio et al., 2007; Mancianti, Nardoni, & Ceccherelli, 2002; Mendes et al., 2014; Wojcik, Kurnatowski, & Blaszkowska, 2013), have been isolated from pigeon faeces in only two studies, both conducted in Seoul (Chae et al., 2012; Jang et al., 2011). Whether this represents simple sampling bias or specialization of these species in Seoul requires further investigation. Furthermore, some CRF species may have strong seasonal preferences. For

example, *Ca. glabrata* was isolated from pigeons only between April and July (Jang et al., 2011; Kocan & Hasenclever, 1974).

Our linear model identified five statistically significant predictors of CRF species diversity: green area ratio, number of multiplex houses, wind speed, number of households and habitat type, which explained 85.0% of the variance (Tables 2 and S1). If we consider CRF dissemination as an early stage of the infection process, three important factors emerge: source of CRF, method of transmission and availability of the reservoir (Brachman, 1996). As most CRF are opportunistic fungi which originated from natural environments such as soil and plant debris (de Hoog et al., 2014), the fact that green area ratio had the largest effect on CRF diversity suggests that green areas in urban environments may serve as a source of CRF.

Wind speed was negatively correlated with species diversity; high wind speed has been demonstrated to decrease species abundance (Guinea, Pelaez, Alcala, & Bouza, 2006; Hasnain, 1993) and influence species composition (Yamamoto et al., 2012) of aerial fungi, reducing the probability that CRF species will colonize pigeon faeces. Based on our results, wind appears to be an important method of transmission of CRF from the source to the host substrate.

While CRF diversity was positively correlated with the number of multiplex houses, a negative correlation was observed with respect to the number of households. Areas with high household density were residential areas with high-rise apartment buildings (defined as apartment buildings with 10 or more stories) (Lee & Jo, 2006). These areas have limited green space (source) and well-established food waste management systems (less food available for pigeons) (Hong, Song, Byun, Yoo, & Nakagoshi, 2005). On the other hand, residential areas with multiplex houses have more green spaces and food waste available for pigeons (Lee & Paik, 2011). As mentioned above, pigeons empty their bowels while foraging, thus preferred foraging sites have more abundant pigeon faeces. Although further study is required, we conclude that multiplex housing areas, with the associated increased abundance of pigeon faeces, provide sufficient substrate for amplification of CRF species.

Finally, an analysis of habitat type revealed that more urbanized areas ("commercial," "industrial/urban" and "residential" biotopes) had significantly higher ($p < .001$) CRF diversity than other areas ("landscape," "rivers/wetlands," and "woodland" biotopes) (Table 2). Collectively, our data demonstrate that the diversity of CRF isolated from pigeon faeces is significantly affected by the local environment.

4.2 | The relative abundance of CRF

The relative abundance of CRF was unevenly distributed throughout the city (Figure 2). The linear model identified four statistically significant predictors that explained CRF abundance: number of workers, green area ratio, wind speed and habitat type, which collectively explained 59.8% of the variance (Table 3). The two continuous variables (number of workers and green area ratio) were positively correlated with relative abundance, while wind speed was negatively correlated.

It has long been recognized that pigeon faeces can serve as an enrichment medium for certain CRF species (Litvintseva, Kestenbaum, Vilgalys, & Mitchell, 2005; Mantovani, 1978). The physiology of some CRF species enables them to survive and proliferate on pigeon faeces better than some non-CRF species (Ajello, 1967; Littman & Borok, 1968; Nielsen, De Obaldia, & Heitman, 2007; Sorrell & Ellis, 1997). Thus, an increase in overall fungal spores on faeces can selectively increase the relative abundance of CRF over time. If the source of most CRF species associated with pigeon faeces is the surrounding environment rather than the gastrointestinal (GI) tract of pigeons, the relative abundance of CRF in pigeon faeces can be explained as a function of three factors: surface area of the substrate (in this case pigeon faeces), number of CRF agents (e.g., fungal spores) bombarding the pigeon faeces and the physiology of CRF species.

The variables with the highest coefficients were the number of workers and green area ratio. A similar pattern was reported in pigeon populations in Europe (Hetmański, 2008; Sacchi, Gentilli, Razzetti, & Barbieri, 2002), and Przybylska et al. (2012) suggested that a higher density of old, tall buildings in the city centre promotes a higher pigeon density. Higher pigeon density increases the overall amount and surface area of pigeon faeces and thus increases the amount of substrate available for fungal spores. Moreover, there was a positive relationship between green area and the number of both CRF and non-CRF fungi that can colonize pigeon faeces. These two factors, in combination with evidence that some CRF are better adapted to pigeon faeces than non-CRF (Litvintseva et al., 2005; Mantovani, 1978), may selectively increase the relative abundance of CRF species (Frazzita et al., 2013; Lee et al., 2011, 2013; Xue, 2012). Finally, the density of CRF may decrease with increasing wind speeds, because high wind speed can decrease the level of airborne fungi and thus decrease the number of spores settling on faeces (Guinea et al., 2006; Hasnain, 1993).

Among habitat types, more urbanized areas ("residential," "industrial/urban" and "commercial" biotopes) had significantly higher abundance of CRF species ($p < .01$) than other areas ("landscape," "rivers/wetlands" and "woodland" biotopes) (Table 3). This is consistent with our finding that urbanized areas have larger overall number of CRF species than other habitats (Table 2); higher building density in the city centre may promote a higher pigeon density (Przybylska et al., 2012), and thus, more pigeon faeces are available for CRF species to proliferate on.

4.3 | A comparison between fresh and old faeces

By comparing CRF species in fresh and old faeces, we can infer the source of the fungi. If a CRF is found either exclusively or predominantly in fresh faeces, the pigeon is likely a vector for the fungus, as the fungus has passed through the GI tract. If a CRF is found in only old or both old and fresh faeces, either the pigeon or environment could be the source of the fungus. When comparing fresh vs. old faeces collected at the same site, the majority of CRF species likely

originated from the environment, as there were only three instances suggesting that CRFs use pigeons as a vector: *Ca. bovina*, *Cr. magnus* and *Al. alternata* (Table 1). The community of CRF from old pigeon faeces was similar to that observed in studies of airborne fungi (59% of genera shared) (Fang et al., 2005; Oh, Fong, Park, Chang, & Lim, 2014; Shelton et al., 2002; Solomon, 1976; Yamamoto et al., 2012), supporting the hypothesis that these spores may be airborne rather than originating from the pigeons themselves.

Although *Cr. neoformans* is routinely isolated from pigeon faeces worldwide, suggesting that pigeons are an important disease vector for this pathogen (Haag-Wackernagel & Moch, 2004), we did not detect *Cr. neoformans* in our study. Previously, the complete ITS region was used to identify *Cr. neofomans* to the species level (Ciardo et al., 2006). However, formal *Cr. neoformans* serotype groups are now considered as two separate species: *Cr. neoformans* (serotype A and D) and *Cr. gattii* (serotype B and C) (Meyer et al., 2009), and pyrosequencing analysis of the ITS region cannot resolve these species (Montero et al., 2008). Accordingly, BLAST hits for some OTUs in our samples included both *Cr. neoformans* and *Cr. gattii*. These OTUs were eventually removed from the final list of CRF species in this study because the FHITINGS algorithm failed to find a consensus species name for a given OTU. This implies that our samples may have included *Cr. neoformans* and/or *Cr. gatti*, yet our method was not able to identify them to the species level. We suggest that the quality of the fungal ITS database rather than the efficiency of the ITS region and the sensitivity of the pyrosequencing method hindered the species-level identification of *Cr. neoformans* and *Cr. gattii*.

The regular occurrence of three fungal species (*Ca. bovina*, *Cr. magnus* and *Al. alternata*) in fresh faeces demonstrates their potential to survive passage through the pigeon GI tract. Previous studies have suggested that two of these species (*Ca. bovina* and *Cr. magnus*) are disseminated by pigeons (Chae et al., 2012; Hasenclever & Kogan, 1975). *Candida bovina* can survive harsh environments, is thermophilic and colonizes the GI tract of various homeotherms, including humans (Kurtzman et al., 2005) and swine (Van Uden, 1960). In addition, *Cr. magnus* has been isolated from fresh pigeon faeces (Wu, Du, Li, & Lu, 2012). Interestingly, most CRF found in fresh pigeon faeces are not considered major health threats (biosafety level 1), as they tend to cause only mild symptoms and are effectively treated with widely available antifungal products (de Hoog et al., 2014). Highly invasive pathogenic fungal species (e.g., *Ca. albicans* and *Ca. tropicalis*; biosafety level 2) were only found in old faeces (Table 1). The distinction is nuanced, but it appears that pigeons themselves are not important vectors of pathogenic fungi; rather, their faeces are a substrate for environmentally transported CRF (e.g., wind).

5 | CONCLUSION

A culture-independent, high-throughput sequencing approach identified 35 potentially pathogenic fungal species (CRF) in pigeon faeces collected from a highly urbanized, densely populated city. We estimated CRF diversity and abundance based on current knowledge of the pathogenicity of fungi; however, because the pathogenicity of many fungal species is unknown, our results may underestimate the public health consequences of the relationships between CRFs and pigeons. Urban city structures and concentrated population, which are favourable to pigeons' survival and propagation, were significant variables that contributed to the diversity and abundance of CRF. Inferring from the linear models, green areas within an urban city may serve as a source of CRF, while adjacent pigeon faeces promote their spread. Our results suggest that natural processes, such as wind, rather than introduced pigeons, are the critical factors in disseminating CRF; the role of pigeons is primarily to provide a substrate (faeces) for CRF to proliferate. Our results provide valuable information that can be applied to public health policy planning and the development of strategies for the management of pigeons in major metropolitan cities.

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DATA ACCESSIBILITY

Metabarcoding amplicon data and associated metadata are available on NCBI Sequence Read Archive (SRA) under project number: PRJNA285369 (Accession no. SRP058875), Experiment SRX1 044247.

AUTHOR CONTRIBUTIONS

J.J.F., W.D.L. and Y.W.L. conceived and designed the study. J.J.F. and W.D.L. carried out sample collection, processing and bioinformatics. J.J.F., W.D.L., Y.W.L. and J.A.E. analysed the results. J.J.F., W.D.L. and J.A.E. wrote the manuscript. This project was supervised by Y.W.L. All authors participated in editing and refining the analysis and the final version of this manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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