Diversity and Abundance of Club and Coral Fungi in the Upper Lane Cove Valley

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The Kingdom Fungi are central players in the ecology and biogeochemistry of terrestrial ecosystems. Despite this importance, the diversity, distribution and abundance of fungal species are poorly known. Here, we undertook an intensive survey of club and coral fungi in the Upper Lane Cove Valley, Sydney, Australia. Over a two-year period, we collected more than 1100 specimens, and identified these to genus using a combination of DNA barcoding and morphology. The majority of specimens did not match any sequences in GenBank at more than 95% similarity, meaning that many of these fungi are either poorly represented in DNA databases, or are potentially novel species. A number of hotspots for fungal diversity and abundance were identified, largely along creek lines draining southwest through coachwood dominated vegetation. Notably, these hotspots all lie outside the adjacent Lane Cove National Park.

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INTRODUCTION

The Kingdom Fungi is extraordinarily diverse, containing an estimated 2.2 to 3.8 million species. However, it is possibly the least well-known group of multicellular eukaryotes, with only 120,000 currently accepted species names (Hawksworth and Lücking 2017). Fungal species in Australia are likely to be even less well represented, because although Australia has high levels of endemism, many of our fungi have been identified as superficially similar Northern Hemisphere taxa. Estimates suggest that up to 75% of Australian species are undescribed (May 2001).

There are many reasons for this taxonomic deficit (May et al. 2019). Fungi are cryptic, microscopic, and often live as mycelial networks in soil and other substrates. Many niches are unexplored for fungal biodiversity (Naranjo-Ortiz and Gabaldón 2019), and physical surveys have often relied on the appearance of ephemeral sporing bodies. Finally, mycological expertise is declining due to retirement and omission from science curricula (Irga et al. 2018).

DNA-based investigations have been proposed as a solution to speed up the discovery of fungal biodiversity. High throughput sequencing studies have generated hundreds of millions of fungal sequences, discovering many new fungal groups and species (Hibbett 2016). DNA studies suggest that there might be over 6 million fungal species (Baldrian et al. 2021), and have been used to generate updated fungal phylogenies (Spatafora et al. 2017). However, various authors have stressed that molecular data are not necessarily sufficient, and need to be linked to voucher specimens, photographs and other metadata (Lücking et al. 2020; Thines et al. 2018; Truong et al. 2017).

During mid-2020 to mid-2022, we aimed to address some of the deficit in our understanding of fungal biodiversity in Australia by performing intensive field surveys of club and coral fungi in the Upper Lane Cove Valley, Sydney, Australia. We

chose this group of fungi because they exhibit all the characteristics that have previously limited fungal descriptions: they are small, cryptic, morphologically variable, ephemeral, and are found in poorly explored niches. Further, they represent both Ascomycota and Basidiomycota, and have a range of nutritional strategies that include saprotrophs, mutualists and pathogens. Here we present initial findings on their diversity and distribution, with the aims of determining the range of genera in the region and identifying hotspots of fungal diversity worthy of conservation.

MATERIALS AND METHODS

Field work and sampling

Field work was done in accordance with the travel restrictions imposed by the COVID-19 pandemic. Sampling was conducted in the upper Lane Cove Valley, Sydney, Australia, covering all the tributaries and creeks draining into the Lane Cove River, from the watershed bounded by Pennant Hills Road to the north, downstream to Fiddens Wharf in the south. Survey trips were based on the tracks identified on the 1:10,000 Walking Tracks of the Lane Cove Valley map published by STEP Inc, Turramurra, NSW. Individual tracks of 0.4 to 1.7 kilometres in length were surveyed in any one instance, and each track was surveyed between 1 and 16 times between the 1st July 2020 and the 30th June 2022. A total of 208 collection surveys were conducted across the two-year period (Table 1).

Surveys were conducted according to the recommendations in Lindsay et al. (2013). Sides of tracks, leaf litter and adjacent areas, including creek banks, were carefully searched for specimens. Once located, specimens were photographed using a Samsung Galaxy Note 10 mobile phone (Fig. 1), incorporating location data, and note taken of the substrate and associated vegetation. Portions of specimens were collected, cleaned with a paintbrush and re-photographed for scale next to a 1 cm diameter collection vial. Specimens were stored in 4 ml of 100% ethanol for later DNA extraction. Key specimens were collected as vouchers, aiming to collect a range of sporing bodies of different maturities where sufficient specimens were available. These specimens were pressed between absorbent paper for 24 hr, and then dehydrated at 50°C for 1 hr, or until dry. Specimens were then glued to acid-free paper folders, and stored in glassine envelopes held in air-tight boxes containing naphthalene. All collections were made under DPIE Scientific Licence SL 102456, and will be deposited into a registered Herbarium in the future.

DNA extraction and specimen identification

DNA was extracted from 20 mg of ethanol-preserved tissue using a modified salting-out procedure (Sunnucks and Hales 1996). Briefly, tissues were ground in TNES buffer using a sterile plastic pestle, incubated at 57°C overnight with proteinase K, lysates were salted out, and DNA precipitated with ethanol. DNA from selected specimens that were representative of particular morphologies, phenologies or niches were prepared for PCR, with the aim of assigning these morphotypes to genus.

To confirm the identity of fungal specimens, the universal fungal barcode (nuclear ribosomal internal transcribed spacer – ITS) was amplified using PCR (Schoch et al. 2012; Xu 2016). Approximately 20 ng DNA was amplified using primers ITS1 and ITS4 (White et al. 1990). Reactions (50 ul) were performed in 1 x GoTaq Colourless master mix (Promega) with the addition of 1 mg/ml RNAse A and 0.5 uM of primers ITS1 and ITS4. The PCR program consisted of 94°C 3 min; 94°C 30 sec, 50°C 30 sec, 72°C 90 sec, 35 cycles; 72°C 5 min; 4°C hold. Success of PCR was monitored by electrophoresis on 2% agarose/TBE gels post-stained with GelRed (Biotium).

Aliquots (8 ul) of PCR products were dispensed to 96-well microtiter trays and sent to Macrogen, Korea for Sanger dideoxy sequencing, in both directions, using the original amplification primers. Sequence files were edited and contigs generated using GeneStudio. Complete ITS sequences were used to interrogate the NCBI nucleotide database using BLASTn (megablast), excluding uncultured/environmental sample sequences. Search results were scrutinized to identify sequenced specimens to at least genus level. Non-sequenced specimens were identified by morphological comparison to sequenced collections. In this way, all specimens were assigned to at least genus level.

Mapping fungal distributions

GPS data for individual specimens were mapped using ArcGIS Pro 2.8.1. The entire study area was then divided into a regular grid of 100 m^2 cells. Fungal genus richness, genus abundance and the overall number of specimens were then calculated for each grid cell containing at least one specimen. The expected abundance of specimens in each grid cell was displayed as a heatmap (smoothed interpolation, kernel density estimation), overlain with symbols that show the number of genera recorded in each grid cell (Fig. 2).

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Table 1:
Locations surveyed during the study

Acronym	Location	Km	Surveys	Total	Start	End
ALR	Alston Reserve, Carlisle Close	0.8	4	3.2	-33.777648, 151.131242	-33.773820, 151.130432
AVD	Avondale Dam	0.6	6	3.6	-33.753433, 151.118799	-33.751489, 151.120688
BBCK	Blackbutt Creek, Gordon	1.2	5	6.0	-33.758504, 151.141738	-33.770413, 151.141223
BCT	Byles Creek Trail	1.1	2	2.2	-33.743497, 151.066472	-33.751091, 151.084322
BNF	Browns Field, Cooper Crescent	0.8	6	4.8	-33.734293, 151.111565	-33.735730, 151.107528
BPT	Beltana Place Trail	1.1	2	2.2	-33.738983, 151.097501	-33.738814, 151.091174
BWT	Browns Waterhole Track	1.1	15	16.5	-33.759939, 151.108289	-33.761918, 151.105137
CPCK	Terra Ulong Creek	0.9	1	0.9	-33.739759, 151.078694	-33.740491, 151.084466
CPKWY	Comenarra Parkway, Coups Ck	1.4	7	9.8	-33.730131, 151.091642	-33.734788, 151.096813
CRT	Canoon Road Trail	1.6	10	16	-33.747582, 151.104851	-33.748424, 151.098360
CSST	Cove Street Step Track	1.3	6	7.8	-33.752519, 151.105105	-33.756493, 151.104314
DAT	Doncaster Avenue	0.7	2	1.4	-33.758292, 151.123418	-33.762620, 151.119754
DVCK	Devlins Creek, Epping	1.6	2	3.2	-33.751356, 151.084123	-33.753193, 151.099866
FCK	Falls Creek, Gordon	0.9	8	7.2	-33.765457, 151.145600	-33.770520, 151.141394
FID	Fiddens Wharf	1.7	4	6.8	-33.782606, 151.144742	-33.773587, 151.139678
GAFT	Gloucester Avenue Fire Trail	2.0	4	8.0	-33.770983, 151.126116	-33.765513, 151.123241
GCT	Gipps Close Trail	1.6	8	12.8	-33.744230, 151.111291	-33.738572, 151.117803
GSPR	Granny Springs Reserve	0.4	1	0.4	-33.734324, 151.126662	-33.733650, 151.126346
HPT	Howson Park Trail	1.3	2	2.6	-33.742045, 151.106587	-33.736909, 151.113844
KLT	Koombalah Loop Track	1.7	7	11.9	-33.758454, 151.119032	-33.760789, 151.107874
KJTK	Kurrajong Track	1.6	2	3.2	-33.743630, 151.078306	-33.751388, 151.084132
LCNP	Lane Cove National Park	1.5	4	6.0	-33.770519, 151.117877	-33.770947, 151.119851
LCVW	Lane Cove Valley Walk	0.9	3	2.7	-33.758816, 151.105328	-33.756507, 151.104727
LPT	Lorna Pass Track	2.0	5	10.0	-33.733183, 151.091326	-33.733263, 151.092907
LTCK	Lower Terrys Creek	1.5	4	6.0	-33.770618, 151.093789	-33.761918, 151.105137
MAT	Marona Avenue Trail	0.9	3	2.7	-33.741667, 151.097678	-33.739233, 151.091418
MXST	Maxwell Street	0.9	4	3.2	-33.753051, 151.113814	-33.750405, 151.117065
QCK	Quarry Creek	1.0	3	3.0	-33.765242, 151.131524	-33.772409, 151.125902
RAFT	Robin Avenue Fire Trail	0.8	5	4.0	-33.758894, 151.116361	-33.753408, 151.116925
RCFT	Rudder Creek Fire Trail	1.9	4	7.6	-33.768808, 151.135386	-33.772552, 151.126074
RPT	Rofe Park Trail	1.6	16	25.6	-33.744196, 151.121158	-33.745525, 151.124134
SCCK	Scout Creek	1.2	5	6.0	-33.738455, 151.083768	-33.742574, 151.090113
SDMR	Sir David Martin Reserve	0.9	5	4.5	-33.750716, 151.112611	-33.749659, 151.111138
SNF	Sheldon Forest	1.5	11	16.5	-33.738761, 151.131827	-33.745532, 151.124161
TCK	Terrys Creek	1.2	4	4.8	-33.779094, 151.093081	-33.770877, 151.093886
TRP	Troon Place	1.1	8	8.8	-33.747772, 151.127803	-33.745525, 151.124134
TWCT	Twin Creeks Comenarra Trail	1.4	7	9.8	-33.739045, 151.106174	-33.736892, 151.113876
ULCV	Upper Lane Cove Valley	1.8	5	9.0	-33.748424, 151.098360	-33.738175, 151.089974
UTCK	Upper Terrys Creek	0.8	3	2.4	-33.782132, 151.087868	-33.779094, 151.093081
XLCVW	Extra Lane Cove Valley Walk	1.0	3	3.0	-33.753972, 151.118968	-33.762626, 151.119440
TOTAL			**208**	**266.1**		

Figure 1. Representative specimens of common genera examined in this study. Top (L-R) *Clavaria zollingeri*, *Clavulinopsis sulcata*, *Clavulina* cf. *cristata* and *Microglossum* cf. *olivaceum*; Bottom (L-R) *Ramaria capiiata*, *Ramariopsis crocea*, *Thelephora palmata* and *Xylaria* cf. *polymorpha*. Field of view for each panel 4 to 8 cm.

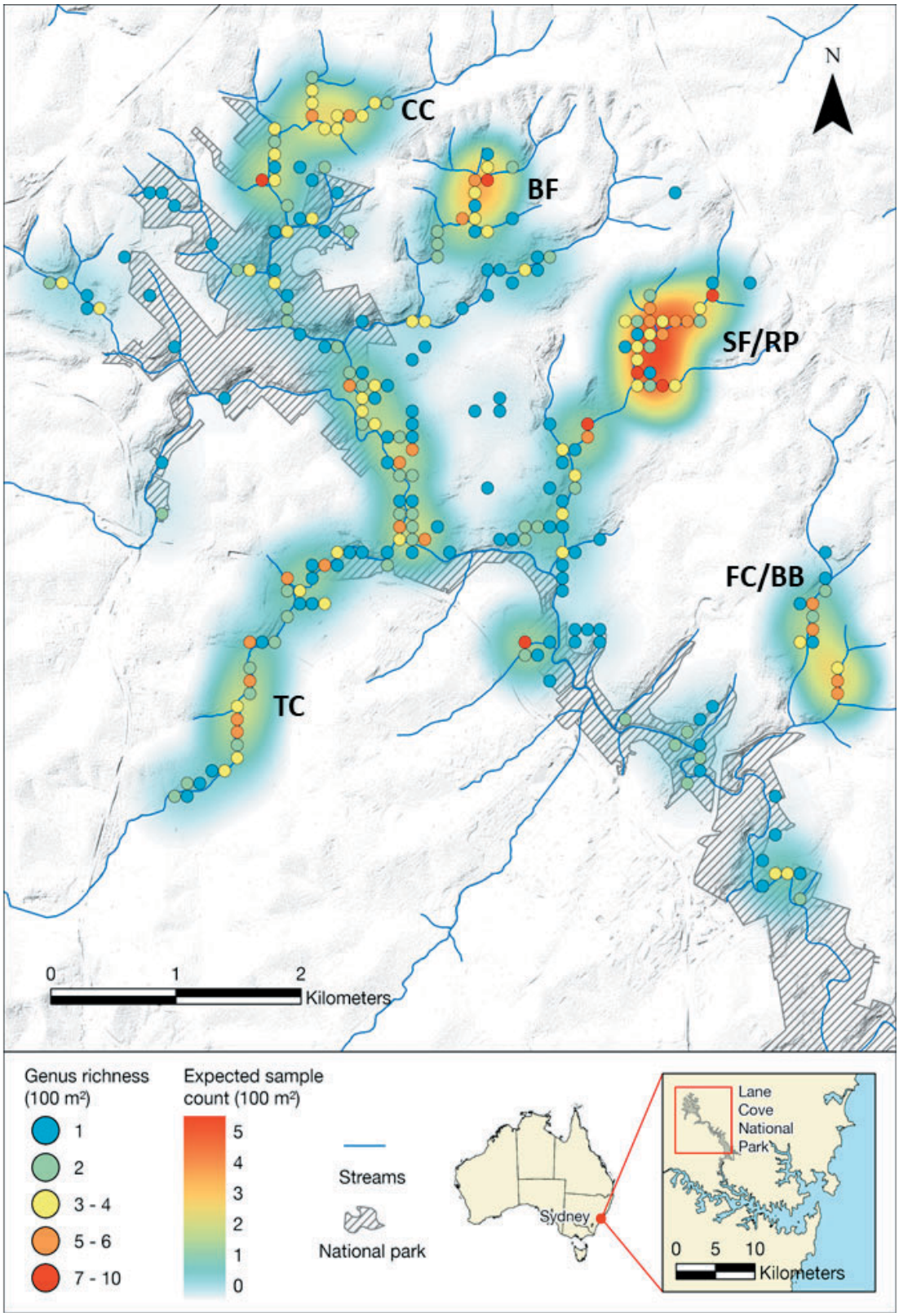

Figure 2. Map of specimen abundance and genus richness for club and coral fruiting bodies in the Upper Lane Cove Valley, Sydney. Coloured circles designate fungal genus richness per 100 m² grid cell. Expected sample counts for the surveyed areas were interpolated using a kernel density estimate based on the total number of specimens collected within each 100 m² grid cell, and are shown as the background heat map. Because not all specimens were collected in any area, predicted sample counts are necessarily underestimates. Locations: SF/RP, Sheldon Forest/Rofe Park; BF, Browns Field; CC, Coups Creek; FC/BB, Falls Creek/Blackbutt Creek.

To identify possible predictors of fungal genera richness and abundance, spatial data on local topography, vegetation communities and soil properties were collated for each 100 m^2 grid cell. Slope gradient and aspect were calculated using a 1 m resolution digital elevation model (New South Wales Spatial Services 2013). Local vegetation communities were derived from regional plant community type maps (New South Wales Department of Planning and Environment 2022). Key soil properties (soil class, pH, nitrogen, carbon and phosphate content) were summarised using digital soil maps modelled from field surveys and environmental data (New South Wales Office of Environment and Heritage 2018). Statistical analyses of these data were performed in Stata 17.

RESULTS

Over the period of July 1st 2020 to June 30th 2022, we conducted 208 fungal surveys covering a total of 266.1 km, with an average of 1.28 km per survey (Table 1). This resulted in a collection of 1177 specimens of club and coral fruiting bodies. A subset of specimens, broadly representative of morphologies, environments and niches, was prepared for DNA sequencing by PCR amplification of the universal fungal DNA barcode: the internal transcribed spacer region of the nuclear ribosomal RNA gene (ITS) (Schoch et al. 2012). A total of 479 specimens, representing 40% of the collections, were successfully sequenced across the entire ITS region, and used to interrogate GenBank depositions via the NCBI BLASTn site.

Sequenced specimens were assigned to genera based on the set of most similar sequences returned by the BLASTn search, and cross checking that the specimen morphology matched that for the genus returned by the search. The highest matches to sequences deposited in the database ranged from 80% to 100%. However, the majority of our specimens exhibited less than 95% similarity to any sequence in the database (Fig. 3). The accepted similarity across the ITS region for a fungal species identity is generally set at 97% (Truong et al. 2017; Xu 2016), and therefore the majority of our specimens are likely to be species that are either not represented in current DNA databases, or are novel species. Among the five most commonly recovered genera, our specimens of *Ramaria* and *Ramariopsis* were particularly poorly represented in DNA databases, with few if any specimens matching reference sequences at similarities more than 97% (Fig. 3). Consequently, for the current paper, we chose to only identify specimens to genus level, and leave more formal species identifications and phylogenies to a series of future publications.

Specimens that had not been sequenced were assigned to genera based on their similar morphology and habit to known, sequenced specimens. Some genera were pooled for subsequent analysis because of the potential difficulty in separating genera based solely on gross morphology. These included the genera *Geoglossum, Trichoglossum* and *Glutinoglossum* in the earth tongues, and the entomopathogens *Cordyceps, Metarhizium* and *Lecanicillium.* This process resulted in a total of 12 taxonomic groups (Table 2). Among the Basidiomycota, the genera *Clavulinopsis, Ramariopsis* and *Ramaria* were most commonly recovered, while in the Ascomycota, the most common specimens were from the *Geoglossales* (Table 2).

The collection location for all specimens was then plotted onto a map of the survey area, assigning each specimen to one of 242 individual 100 m^2 grid cells. The abundance of specimens in each cell was displayed as a heatmap (kernel density estimation), overlain with symbols that show the number of genera recorded in each grid cell. The heatmap portrays the expected total number of club and coral sporing bodies across the survey area, while the overlain coloured symbols show the number of genera actually collected in each grid cell (Fig. 2). Because not all specimens were collected in any one area, absolute abundance is significantly underestimated. Abundance and diversity were highly correlated (r 240 = .87, $p <$ .001). Hotspots for both abundance and diversity are evident on the map, these primarily being: Sheldon Forest/Rofe Park; Browns Field; and Coups Creek. To a lesser extent, Falls Creek/Blackbutt Creek, Terrys Creek, and sections of the Lane Cove River also exhibit some areas with concentrations of club and coral fruiting bodies.

The most notable hotspots for fungal fruiting bodies have some characteristics in common. They are all on creek lines oriented in a south to south-westerly direction, and are often found in Sydney Coastal Coachwood Gallery Rainforest. In general, they lie north of the Lane Cove River, and are outside the boundaries of the Lane Cove National Park. Our general observations suggest that these hotspots were most often located in patches of undisturbed bushland, containing few invasive plant species.

To more formally investigate potential environmental drivers of club and coral distribution, we tested the association of fungal abundance and diversity with vegetation type, soil properties and

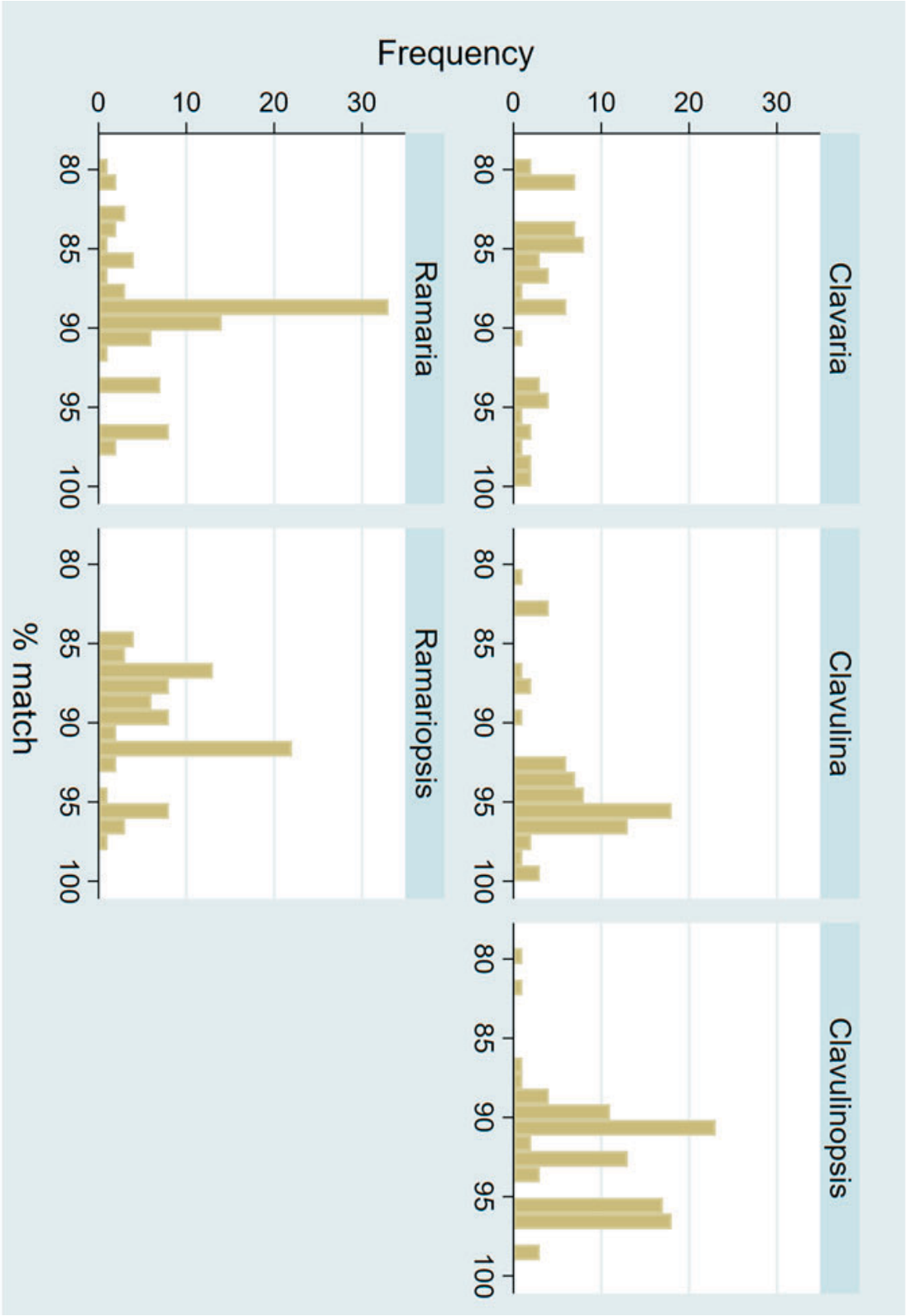

Figure 3. Distribution of percentage similarity to ITS sequences in the NCBI nucleotide database, plotted by genus. Full length ITS sequences generated from fungal specimens collected in the Lane Cove Valley were used to interrogate the NCBI database (excluding environmental sequences). Data for the five most abundant genera are shown. Histograms represent the highest percentage matches recorded for each sequenced specimen. The similarity percentage beyond which specimens are usually ascribed as members of the same fungal species is often set at 97% (Truong et al., 2017; Xu, 2016).

Table 2: Taxa collected during this study.

Division	Order	Genus	No.[A]
Basidiomycota	Agaricales	*Clavaria*	133
		Clavulinopsis	245
		Macrotyphula	4
		Ramariopsis	250
	Cantharellales	*Clavulina*	158
	Gomphales	*Ramaria, Phaeoclavulina*[B]	234
	Thelephorales	*Thelephora*	21
	Tremellodendropsidales	*Tremellodendropsis*	2
Ascomycota	Geoglossales	*Geoglossum*, *Glutinoglossum, Trichoglossum*[C]	62
		Microglossum	5
	Hypocreales	*Cordyceps*, *Lecanicillium, Metarhizium*[D]	27
	Xylariales	*Xylaria*	36

A: Number of specimens; B: *Phaeoclavulina* and *Ramaria* are considered together here; C: *Geoglossum, Glutinoglossum,* and *Trichoglossum* numbers were pooled because of the difficulty separating these genera on visible characteristics alone; D: The entomopathogens *Cordyceps, Lecanicillium* and *Metarhizium* were also pooled in this Table.

elevation (NSW DPE 2022; NSW OEH 2018; NSW Spatial Services 2013). Of these data, the only metrics able to be assigned to individual specimen GPS locations were elevation and aspect. Other metrics, such as soil properties, pH and phosphate concentrations were recorded in the databases at much larger spatial scales than the fungal distribution data. Similarly, vegetation data did not capture the small patches of coachwood forest in which most fungi were recorded.

A Spearman's correlation was used to test the relationship between the logarithms of abundance and diversity using data from the 242 grid cells. As hypothesised, there was a strong positive correlation between abundance and diversity, $r_s = .93$, $p < .001$. Given the high correlation between abundance and diversity and the fact that diversity was the metric of most interest, we concentrated on potential drivers of diversity in subsequent analyses. A multiple linear regression analysis was performed to assess how various environmental factors might predict the natural log of local club and coral diversity. The overall model yielded a statistically significant result, $F_{(11, 230)} = 3.86$, $p < .001$, but with a small effect size of $R^2 = 15.6\%$.

Three of the environmental variables we examined were independently significant predictors of local diversity: Soil Class ($t_{(230)} = 3.45$, $p = .001$), with small to moderate standardized effect size $\beta = .25$; pH at a depth of 30cm ($t_{(230)} = 4.34$, $p < .001$), with moderate standardized effect size $\beta = .38$; and percent nitrogen at a depth of 5cm ($t_{(230)} = 2.57$, $p = .011$), with small standardized effect size $\beta = .19$. All other environmental factors considered in this model did not appear to be significant predictors of local club and coral diversity.

DISCUSSION

In this study, we combined physical survey methods with DNA sequencing to generate an overview of the diversity and distribution of club and coral fungi in the Lane Cove Valley. Because the Australian Fungi are currently very under-described (May 2001), and DNA databases are incomplete, it was not surprising that many of our specimens could not be identified beyond the genus level. Nevertheless, significant conclusions can still be drawn.

The majority of our specimens exhibited less than 95% similarity to any ITS sequence deposited in GenBank (Fig. 3). Fungal species identity using this barcode is usually set at 97% (Truong et al. 2017; Xu 2016). Consequently, few of our specimens could be easily assigned to a species based solely on DNA homology. This was particularly the case for specimens in the genera *Ramaria* and *Ramariopsis* (Fig. 3). Part of the explanation might be that the DNA databases are incomplete, and many Australian species are currently not represented. However, when the gross morphology of specimens was examined, there was often no corresponding species described in guides to Australian fungi (see Hubregtse 2019). This strongly suggests that the Lane Cove Valley contains

multiple undescribed species of club and coral fungi, and underscores the wealth of fungal species yet to be described locally, and in Australia more generally (May 2001).

Nevertheless, all specimens could be assigned to a genus based on morphology and DNA data, allowing the distribution of these genera to be examined across the entire Lane Cove Valley. The abundance of individual specimens and diversity of genera within 100 m^2 grid cells were highly correlated. Hotspots for diversity and abundance of club and coral fungi were evident in the valley (Fig. 2). While many of these locations were in reserves, none of them was within the boundaries of the Lane Cove National Park. This means that a number of potentially undescribed species are currently less well protected than they could be. Conservation of fungal diversity is receiving increasing attention (May et al. 2019), and indeed the Lane Cove Bushland Park has been recognized as an internationally significant site for conservation of waxcap fungi (*Hygrocybe* species) (Kearney and Kearney 2015). Based on our field observations, some species of club and coral fungi are as rare as these endangered waxcaps, and could deserve a similar conservation status.

There are some caveats associated with defining biodiversity hotspots based on opportunistic surveys. Potential biases can arise through variable intensity of sampling, or failure to sample inaccessible areas. In Table 1, we outline each survey route, the number of times it was visited, and the total distance surveyed. Some survey routes were visited only once, or a few times, because there was little evidence of fungal sporing bodies of any kind, even at the peak of fungi season. However, examining the dataset, a significant proportion of survey routes produced only low fungal diversity, despite having high coverage in terms of kilometres surveyed (Table 1). We surveyed all the tracks and fire-trails on the 1:10,000 map of Walking Tracks of the Lane Cove Valley to ensure a comprehensive coverage of the area. Thus, despite the caveats imposed by opportunistic survey methods, we are confident that the diversity data shown in Figure 2 are real representations of the diversity of club and coral genera in the Lane Cove Valley.

To understand the features of potential fungal hotspots, we examined the environmental parameters that could influence the distribution of these fungi. Most available environmental data were recorded at a lower spatial resolution than the 100 m^2 cells we employed. Nevertheless, some measures, including soil class, soil pH at 30 cm depth and nitrogen concentration at 5 cm depth were significant predictors of abundance and diversity. Our qualitative observations suggest that the highest diversity occurs in small undisturbed patches of remnant coachwood in Coastal Sandstone Gallery Rainforest (OEH 2016), and particularly along creek-lines in southwest facing valleys.

Managing and conserving fungal diversity will become increasingly important if we want to restore degraded landscapes and ensure their resilience in the face of global change. Simply replanting the original vegetation is not necessarily accompanied by recovery of the soil microbiota required for successful reestablishment of pre-disturbance plant communities (Hart et al. 2019). Monitoring the fungal diversity in soil using DNA based methods could be a valuable tool to aid our understanding and management of restoration efforts (Van Der Heyde et al. 2020).

Any such DNA monitoring will require a much better understanding of the interactions of fungi with their plant hosts. The first step in this process involves identifying what species are present. The preliminary work we have described here will form the basis of a platform for more detailed description and characterization of the Australian club and coral fungi.

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