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Environmental DNA analysis of airborne poaceae (grass) pollen reveals taxonomic diversity across seasons and climate zones

Shanice Van Haeften [a,#], Bradley C. Campbell [a,#], Andelija Milic [a], Elizabeth Addison-Smith [a], Jane Al Kouba [c,1], Alfredo Huete [b], Paul J. Beggs [c], Janet M. Davies [a,*]

[a] School of Biomedical Sciences, Centre Immunology and Infection Control and Centre for Environment, Queensland University of Technology, Brisbane, Queensland, Australia
[b] School of Life Sciences, University of Technology Sydney, Sydney, New South Wales, Australia
[c] School of Natural Sciences, Faculty of Science and Engineering, Macquarie University, Sydney, New South Wales, Australia

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ABSTRACT

Background: Grasses populate most biogeographical zones, and their diversity influences allergic sensitisation to pollen. Previously, the contribution of different Poaceae subfamilies to airborne pollen has mostly been inferred from historical herbarium records. We recently applied environmental (e)DNA metabarcoding at one subtropical site revealing that successive airborne grass pollen peaks were derived from repeated flowering of Chloridoid and Panicoid grasses over a season. This study aimed to compare spatiotemporal patterns in grass pollen exposure across seasons and climate zones.

Methods: Airborne pollen concentrations across two austral pollen seasons spanning 2017–2019 at subtropical (Mutdapilly and Rocklea, Queensland) and temperate (Macquarie Park and Richmond, New South Wales) sites, were determined with a routine volumetric impaction sampler and counting by light microscopy. Poaceae rbcL metabarcode sequences amplified from daily pollen samples collected once per week were assigned to subfamily and genus using a ribosomal classifier and compared with Atlas of Living Australia sighting records.

Results: eDNA analysis revealed distinct dominance patterns of grass pollen at various sites: Panicoid grasses prevailed in both subtropical Mutdapilly and temperate Macquarie Park, whilst Chloridoid grasses dominated the subtropical Rocklea site. Overall, subtropical sites showed significantly higher proportion of pollen from Chloridoid grasses than temperate sites, whereas the temperate sites showed a significantly higher proportion of pollen from Pooideae grasses than subtropical sites. Timing of airborne Pooid (spring), Panicoid and Chloridoid (late spring to autumn), and Arundinoid (autumn) pollen were significantly related to number of days from mid-winter. Proportions of eDNA for subfamilies correlated with distributions grass sighting records between climate zones.

Conclusions: eDNA analysis enabled finer taxonomic discernment of Poaceae pollen records across seasons and climate zones with implications for understanding adaptation of grasslands to climate change, and the complexity of pollen exposure for patients with allergic respiratory diseases.

1. Introduction

Allergic rhinitis (AR) is a disease that impacts over 500 million people worldwide and can aggravate comorbid conditions such as sinusitis and asthma (Guerra et al., 2002; Bousquet et al., 2019). AR adversely impacts an individual's health, quality of life and wellbeing, and has a considerable socio-economic burden (Colas et al., 2017; Borchers-Arriagada et al., 2021). Grass pollen is recognised as a clinically important outdoor allergen source and is a major trigger for AR globally (Garcia-Mozo, 2017). With over 10,000 species of grasses across the globe, multiple subfamilies occupy diverse ecological niches. In geographically large countries with various climatic zones, airborne grass pollen arises from both temperate (Pooideae) and subtropical (e.g. Panicoideae and Chloridoideae) grasses, which differ in allergen

* Corresponding author. Queensland University of Technology, Q Block, 60 Musk Avenue, Kelvin Grove, QLD 4006, Australia.
E-mail address: j36.davies@qut.edu.au (J.M. Davies).
Centre for Crop Science, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland; Brisbane, Queensland, Australia.
1 School of Chemistry and Molecular Bioscience, Faculty of Science, Medicine and Health, University of Wollongong, Wollongong, New South Wales, Australia.

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composition and immune recognition (Davies, 2014; Nony et al., 2015; Kailaivasan et al., 2020). Providing current local airborne pollen information to the community allows patients suffering from AR to develop and implement strategies to self-manage symptoms of themselves and others (Medek et al., 2019; Bastl et al., 2018). Information on pollen exposure is also valuable for healthcare professionals for clinical management of patients with moderate and severe AR (Matricardi et al., 2020).

As the effects of climate change manifest with increasing pollen production in some taxa, lengthening pollen seasons, and altering the distribution of many species, it is predicted that the prevalence and severity of AR will continue to increase (Beggs et al., 2021; Ziska et al., 2019). However, large-scale northern hemisphere longitudinal studies of recent airborne pollen records in both Europe and the USA failed to detect clear impacts of climate change on the timing of pollen seasons for Poaceae (Anderegg et al., 2021; Ziello et al., 2012). Its tempting to speculate this may be attributable to latitudinal gradients in distributions and timing of pollination of subtropical and temperate grasses, which have been demonstrated in the continents (Davies et al., 2022) of Australia (Medek et al., 2016), Europe (Frenguelli et al., 2010), and North America (Lo et al., 2019), and consequent differences in responses of temperate and subtropical grasses to climate drivers (Preston and Fjellheim, 2020; Xie et al., 2022). An ability to verify sources of airborne Poaceae pollen to subfamily and genus level resolution is therefore necessary to understand complex responses of grasslands to shifts in climate drivers.

Currently, local airborne pollen diversity and abundance has mostly been monitored by collecting bioaerosols using volumetric impaction samplers, and identifying and quantifying the pollen grains (10–150 μm) by light microscopy. The manual process is highly labour-intensive and costly and requires trained personnel to visually identify the different pollen taxa (Bianchi et al., 2020a; Milic et al., 2020). Many pollen, and particularly pollen within the Poaceae family, which are in the order of 40 μm, cannot be discriminated to the subfamily, genus or species level by light microscopy because grasses share morphologically indistinguishable pollen (Garcia-Mozo, 2017; Ong et al., 1995), making pollen records taxonomically coarse. However, being able to identify and track Poaceae pollen sources on a finer taxonomic scale is important for understanding the biogeographical differences in grass phenology, as well as responses of grasses to climate change and exposure of patients allergic to pollen of separate grass subfamilies (Kailaivasan et al., 2020; Minami et al., 2019).

Recent advances in environmental (e)DNA metabarcoding have provided new possibilities to analyse the diversity and abundance of taxa present within airborne pollen samples (Bell et al., 2016). However, this technique has hitherto only been successfully applied in a limited number of aerobiology studies of the composition of environmental air samples (Kraaijeveld et al., 2014; Korpelainen and Pietiläinen, 2017; Brennan et al., 2019; Campbell et al., 2020, 2023). Notably, only our studies have been conducted in the Southern Hemisphere (Campbell et al., 2020, 2023), where less is known of aerobiological trends and the response of anemophilous plants to climate change (Addison-Smith et al., 2021; Ramon et al., 2020). It was found, using standard pollen monitoring equipment, across a complete pollen season in an urban location in South-East Queensland, Australia, that the eDNA sequences clustered to the months of the year as well as the season, which demonstrates the ability to discriminate pollen diversity across the airborne pollen season (Campbell et al., 2020). We dissected the Poaceae composition over a full pollen season and found that consecutive Poaceae pollen peaks during summer and autumn were the result of repeated flowering of key taxa, rather than successive flowering of different species, as was found in temperate sites of the United Kingdom by Brennan et al. (2019). However, no previous research has applied eDNA methods to compare Poaceae pollen diversity between seasons at the same sampling location, nor between subtropical and temperate climate zones.

The present study focuses on use of eDNA metabarcoding to analyse airborne Poaceae pollen records across biogeographical regions and over multiple pollen seasons. Our hypotheses were that there would be differences in the representation of pollen from different Poaceae subfamilies between sites consistent with distinct biogeographical and climatic zones, and that within one site there would be consistency between seasons. The objective of this study is to apply eDNA metabarcoding to investigate on a finer scale than pollen concentration data, the diversity and seasonality of airborne Poaceae pollen between subtropical and temperate sites over multiple seasons.

2. Methods

2.1. Sampling sites

Aerobiological samples were collected from four sites in Australia; two subtropical sites in Queensland and two temperate sites in New South Wales (NSW) which have been described previously (Campbell et al., 2023). In each state, one site was peri-urban; Mutdapilly (Queensland) and Richmond (NSW), and one site was urban; Rocklea (Queensland) and Macquarie Park (NSW), although both these monitoring sites were adjacent to an open grass paddocks/fields (Figure S1, supporting information). These sites were part of the AusPollen Aerobiology Collaboration Network and monitored pollen according to the 'Australian Airborne Pollen and Spore Monitoring Network Interim Standard and Protocols' (Beggs et al., 2018). Samples were collected at each site with a Seven-day Hirst-type Volumetric Pollen and Spore trap (Burkard Manufacturing, United Kingdom).

2.2. Sample collection, identification, quantification, and eDNA methods

Pollen was sampled over two consecutive Southern Hemisphere pollen seasons at Rocklea and Macquarie Park: 2017–18 and 2018-19 by standard pollen monitoring methods (Preston and Fjellheim, 2020). Sampling at the Mutdapilly and Richmond sites only occurred in the 2018–19 and 2017-18 seasons respectively. Sampling was conducted from the 1st of October to the 31st of May. Pollen identification and quantification, DNA extraction, rbcL amplification, Illumina library preparation and sequencing were previously detailed in Campbell et al. (2023). All sequencing data has been uploaded to the National Centre for Biotechnology Information, https://www.ncbi.nlm.nih.gov/with the corresponding BioProject ID number PRJNA848427 (Campbell and Davies, 2022).

2.3. Taxonomic assignment for poaceae

Partial operational taxonomic unit (OTU) sequences that were less than 350bp in length were removed from the dataset. The taxonomy of the remaining OTUs was assigned using the ribosomal database project (RDP) classifier with a rbcL reference training set generated from the NCBI database (https://github.com/terrimporter/rbcLClassifier). The RDP classifier uses the naïve Bayes classification algorithm to provide the best matching taxa to the sequence as well as a bootstrap value score to provide a level of confidence to the assignment (Wang et al., 2007). As per Keller et al. (2014), assignments that had a bootstrap value < 0.8 were considered unassignable. All OTUs with a Poaceae family assignment of <0.8 and those that had a total read value of <20 reads were removed from the data. OTUs with a bootstrapping genus assignment value between 0.50 and 0.79 were treated as individual taxa. Those OTUs with the same genus assignment at a bootstrapping score of >0.8 were combined. OTUs with an assignment to genus with a bootstrapping score of <0.5 were assigned to only the subfamily level.

2.4. Phylogenetic and distribution analyses

Multiple sequence alignments for OTU from each location were

undertaken using MUSCLE (Edgar, 2004) with default parameters in Geneious Pro v. 7.1.9 (https://www.geneious.com). Sequence alignments were performed to generate phylogenetic trees displayed with Juke Cantor tree builder (no resampling applied).

2.5. Heatmap construction

Heatmaps for each site-year set were constructed using the processed OTU read number data. For each dataset, colour intensity was scaled logarithmically. Each set is visually divided into Poaceae subfamily groups, but the same data-range-to-colour-intensity mapping was applied to all groups within each set.

2.6. Atlas of living Australia data extraction

All sighting observation records for Poaceae within 50 km of each pollen sampling site were downloaded from the Atlas of Living Australia (ALA) database (https://www.ala.org.au). Each record was assigned a subfamily using genus, and records without subfamily classification discarded. The remaining data were divided into two states based on area of interest with Rocklea and Mutdapilly (Queensland) in one zone, Macquarie Park and Richmond (NSW) in the other. The area covered by each state was divided into a grid with cells of size 0.01° (approximately 1 km). The most frequently observed subfamily was found for each cell. Cells were assigned a subfamily colour if their most frequently observed Poaceae subfamily fell in the group (Pooideae, Arundoideae, Chloridoideae, Panicoideae), i.e., the group of subfamilies identified for analysis from the DNA data.

2.7. Statistical analyses

Data distributions of eDNA read number per sample, or proportion of eDNA reads per subfamily per sample, were tested for normality by Kolmogorov-Smirnov and Shapiro-Wilk tests. The overall proportions of OTU read counts and ALA sighting records aggregated for the gridded area of interest in each state were assessed by Fishers exact test for difference in proportions. For normally distributed aggregated seasonal proportions data for ALA sightings and eDNA reads, a Pearson's correlation test was used, whereas for non-parametrically distributed daily proportions of eDNA reads by subfamily the Spearman's correlation coefficient for relationship with days post July 1 was determined. A simple linear regression model with 95% confidence intervals was determined for the relationship between the overall ALA sighting proportions and eDNA read count proportions by subfamily. Differences in median daily proportions of eDNA reads by subfamily for data aggregated from all sites, were examined by Kruskal-Wallis test with Dunn's multiple comparisons.

3. Results

3.1. Seasonal daily airborne poaceae pollen concentration distributions by light microscopy

During the Poaceae pollen season (October to May), the temporal distribution of pollen load differed between sites. The subtropical Rocklea site showed multiple peaks in both seasons, beginning in December, and extending through the pollen season (Fig. 1). The 2018-19 season had more distinct periods of lower daily pollen concentrations (troughs). The single year of Mutdapilly pollen data (2018–19) showed lower levels of Poaceae pollen than the Rocklea site, with increased airborne Poaceae pollen concentrations during late spring-early summer (November–December), and then a second and higher Poaceae pollen peak from late March to the end of the pollen monitoring period. For both seasons, Macquarie Park showed only one major peak, occurring in autumn (March to April). The single year of data (2017–18) from Richmond showed a spring peak (from the beginning of monitoring (October 8) until the end of November) as well as a smaller autumn peak (March to April).

The mean, median, sum and maximum daily Poaceae pollen concentrations for each site and pollen season are presented in Table 1. The

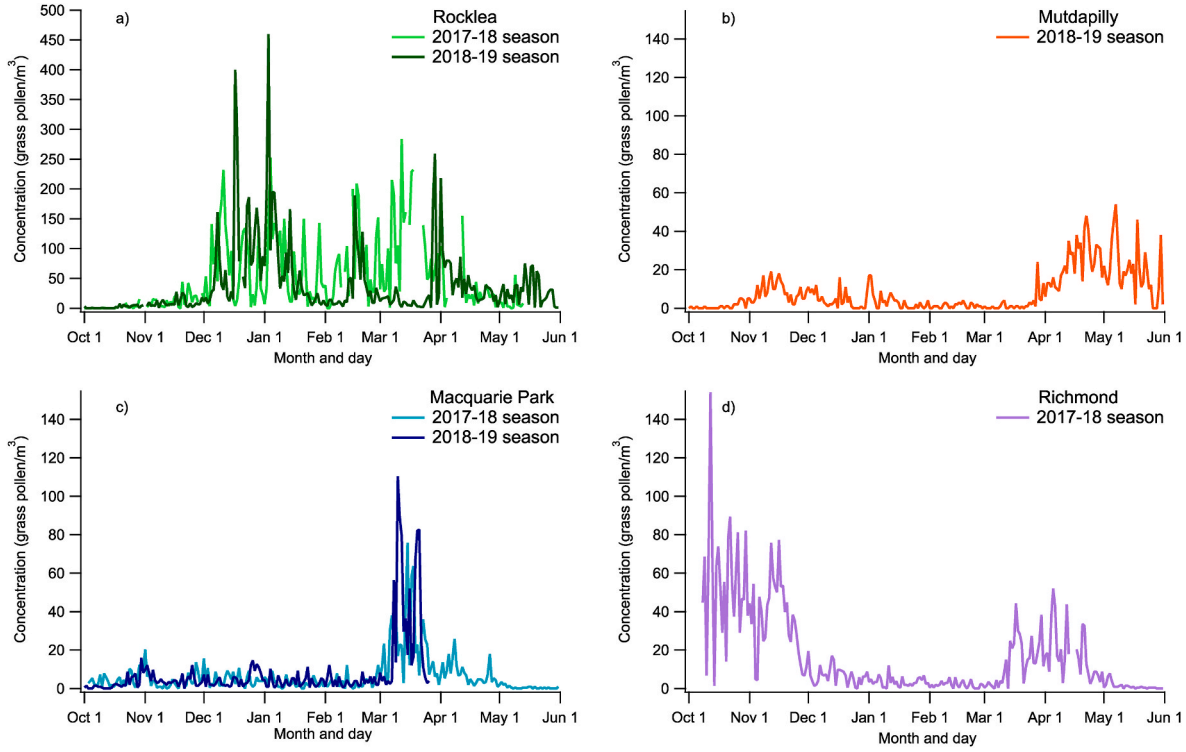

Fig. 1. Average daily airborne grass pollen concentrations (pollen grains/m^3) counted by light microscopy during the 2017–18 and 2018–19 pollen seasons (October–May) in Rocklea, Queensland, and Macquarie Park, NSW, and for one pollen season in Mutdapilly, Queensland, and Richmond, NSW.

Table 1
Summary statistics for average daily airborne grass pollen concentration determine by microscopy. Concentrations are in pollen grains per cubic metre. Missing days were excluded from calculations.

Site (Season)	Mean	Median	Max	Sum	N days[a]	Low (<20)	Moderate (20-49	High (50-99	Extreme (≥100)
Rocklea (2017–2018)	51	27	284	10,578	206	87 (42%)	47 (23%)	27 (13%)	45 (22%)
Rocklea (2018–2019)	38	16	460	9117	241	133 (55%)	57 (24%)	31 (13%)	20 (8%)
Mutdapilly (2018–2019)	8	3	54	1942	243	214 (88%)	28 (12%)	1 (0%)	0 (0%)
Macquarie Park (2017–2018)	6	3	76	1555	241	223 (93%)	15 (6%)	3 (1%)	0 (0%)
Macquarie Park (2018–2019)	8	3	110	1402	177	166 (94%)	3 (2%)	7 (4%)	1 (0%)
Richmond (2017–2018)	16	6	154	3753	233	171 (73%)	39 (17%)	22 (9%)	1 (1%)

[a] Maximum possible duration of study season was 243 days.

number of days between October and May that showed low, moderate, high and extreme Poaceae pollen concentrations were also tabulated (Table 1). Rocklea had 2.4–7.5 fold higher Poaceae pollen loads than the other sites, and it experienced 72 (35%) and 51 (21%) high or extreme Poaceae pollen days in 2017–2018 and 2018–2019, respectively. In contrast, Macquarie Park had only 3 (1%) and 8 (4%) days with high or extreme Poaceae pollen levels during these periods of monitoring. Similarly, Mutdapilly had only one high and no extreme Poaceae pollen days, and Richmond had 22 (9%) high and one extreme Poaceae pollen days during the monitoring period.

3.2. eDNA sequencing output

Between 309 (Richmond, 2017–2018) and 511 (Rocklea, 2018–2019) different OTUs were identified as Poaceae (Table 2). The number of eDNA read counts per OTU were highly skewed, evident by large differences in mean and median number of reads per OTU, indicating both wide diversity and dominance of some Poaceae taxa. Whilst between 35.9% and 80.3% of Poaceae OTUs could be assigned to a subfamily with a bootstrap value score of >0.8, this encompassed a large proportion (92.8–99.3%) of the Poaceae OTU reads observed per site (Table 2). Of those taxa assigned to subfamily, the number of OTUs that could be designated to genus level classification with a bootstrap value greater than 0.5 ranged from 17.6% (Rocklea) to 96.1% (Richmond). At Rocklea the proportion of OTU that could be designated to genus of those assigned to subfamily was low; 17.6% for 2017–2018 and 21.7% for 2018–2019, whereas at Richmond and Mutdapilly the proportion of OTUs designated to genus of those assigned to subfamily was high; 96.1% and 79.3%.

3.3. Poaceae pollen diversity (metabarcoding) between sites

It is evident from phylogenetic trees of all Poaceae OTUs that were assigned to subfamily that pollen of the subtropical Poaceae subfamilies were highly diverse in both states and at all four locations, with multiple discrete clades of Panicoid and Chloridoid grasses (Fig. 2).

There were substantial differences in proportion of eDNA reads from pollen of Poaceae subfamilies found across monitoring periods and locations (Fig. 3a). At Rocklea across both monitoring periods, the majority of OTUs were assigned to the Chloridoideae, followed by Panicoideae, with only a small portion of OTUs assigned to Pooideae (Fig. 3a and b). At this site, the 2018-19 season included Arundinoideae (0.7%) and other families; Danthoniodeae and Oryzoideae made up only 0.6% of the total OTU. In the 2018-19 monitoring period in Mutdapilly, Panicoideae was the predominant assignment observed, followed by Chloridoideae (Fig. 3a). Additionally, Aristidoideae (0.9%), and other subfamilies; Pooideae, Arundinoideae, and Oryzoideae collectively (0.5%), made up only small portions of total assignment. In the Macquarie Park NSW site, the majority of the OTUs were assigned to the Panicoideae subfamily. At Richmond NSW, Chloridoideae and Panicoideae were similarly high (38.5% and 36.7%). Pooideae represented between 7.6% and 23.3% of Poaceae eDNA reads at these sites. Macquarie Park had a marked proportion of OTUs assigned to Arundinoideae in both monitoring periods.

Together, the subtropical Queensland sites showed a significantly higher proportion of the aggregated eDNA read counts from Chloridoid (38.2%) subfamily than the temperate NSW sites (19.5%, $p = 0.0045$, Fig. 3c–Table S1, supporting information). In contrast, the combined data for NSW sites, showed a significantly higher proportion of eDNA reads for Pooideae (12.6%, $p = 0.0101$) and Arundoideae (7.8%, $p = 0.014$) grasses than Queensland sites (2.1% and 0.6%, respectively, Fig. 3c–Table S1, supporting information). Overall, there was similarly

Table 2
Summary of number of Poaceae OTUs and eDNA read number assigned to subfamily and genus for airborne pollen samples from Macquarie Park (NSW), Richmond (NSW), Rocklea (Queensland), and Mutdapilly (Queensland) in 2017-18 and 2018–19.

	2017–18						2018–19					
	Poaceae output (no filter)		Subfamily assignment (% of initial)		Genus assignment (%)[a]		Poaceae output (no filter)		Subfamily assignment (% of initial)		Genus assignment (%)[a]	
	OTUs	**Reads** (mean/ median: IQR)[b]	**OTUs**	**Reads**	**OTUs**	**Reads**	**OTUs**	**Reads** (mean/, median: IQR)	**OTUs**	**Reads**	**OTUs**	**Reads**
Macquarie Park	375	138,684 (370/ 38: 21-81)	270 (72.0)	135,894 (98.0)	92 (34.1)	78,382 (57.7)	345	53,540 (155/20: 5-45)	154 (44.6)	49,676 (92.8)	65 (42.2)	23,403 (47.1)
Richmond	309	60,055 (194/12: 2-45)	111 (35.9)	58,323 (97.1)	85 (76.6)	56,023 (96.1)						
Rocklea	320	191,045 (597/ 20: 6–44)	155 (48.3)	189,695 (99.3)	40 (25.8)	33,424 (17.6)	511	191,383 (374/ 38: 23-90)	405 (79.3)	189,369 (98.9)	139 (34.3)	41,099 (21.7)
Mutdapilly							442	405,781 (918/ 42: 24-108)	355 (80.3)	400,640 (98.7)	75 (21.1)	317,727 (79.3)

[a] Percentage of subfamily assignment.
[b] The mean, median and interquartile range (IQR) of read numbers per OTU.

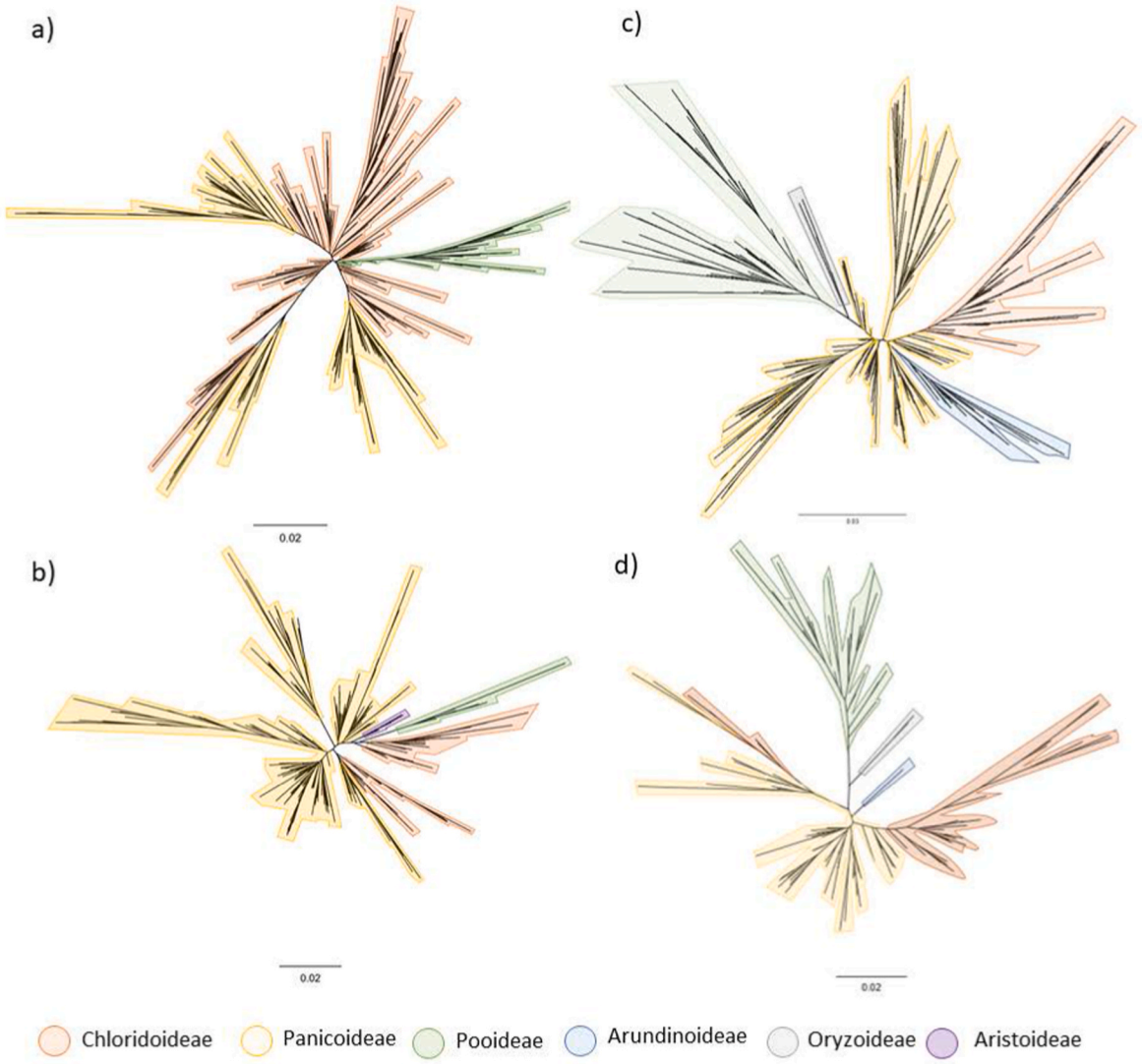

Fig. 2. Phylogenetic trees of grass subfamilies identified from metabarcoding for a) Rocklea, b) Mutdapilly, c) Macquarie Park, and d) Richmond. Pooled data were used for locations with multiple pollen seasons. Trees were produced using muscle alignment, and branches of commonly represented subfamilies are shaded.

high daily proportions of pollen from Panicoideae in both states with 58.8% and 55.7% for Queensland and NSW respectively (Fig. 3c–Table S1, supplementary material).

3.4. Analysis of poaceae pollen diversity across the season

At a subfamily level, the presence of airborne Panicoid and Chloridoid eDNA reads extended throughout the monitoring period from spring to summer and into autumn at all the sites and seasons, showing a weak significant increase across the season (Fig. 3b, Figure S2b, supplementary material). Arundinoid grasses were observed at Macquarie Park in 2018-19 from February but tended to be present in autumn months whereas the Pooideae pollen showed a high proportion of eDNA reads during spring, particularly for Macquarie Park and Richmond. Overall, there was a significant weak negative correlation between the proportion of eDNA reads for Pooideae pollen and the number of days since mid-winter ($r = -0.364$, $p < 0.001$), whilst the proportion of Arundinoideae pollen showed a weak positive correlation with time over the season ($r = 0.409$, $p < 0.001$; Fig. 3b, Figure S2b, supplementary information).

Within the subtropical regions of Rocklea and Mutdapilly, the Poaceae pollen season showed multiple peaks in eDNA records, with summer/early autumn representing peak times, regularly extending beyond spring (Fig. 4a and b). Consistent with the pollen concentration data, the eDNA data showed multiple peaks in the same Poaceae genera over the season with approximately 30–50% of Poaceae genera displaying this pattern of repeated pollination in Mutdapilly and Rocklea, respectively. The heatmap data showed that Chloridoideae genera such as *Chloris*, *Cynodon*, *Sporobolus*, *Eragrostis*; Panicoideae genera such as *Paspalum, Heteropogon*; as well as Pooideae genera such as *Triticum* showed peaks of airborne Poaceae pollen across the season. However, there were also examples of several genera peaking at distinct times such as *Digitaria* (Panicoideae; Rocklea, 2017–18), *Eremochla* sp. (Panicoideae; Mutdapilly, 2018–19), *Phleum* (Pooideae; Rocklea, 2017–18) and *Zoysia* sp. (Chloridoideae; Mutdapilly, 2018–19) (Fig. 4a–c). When comparing between the years in Rocklea, many of the same Poaceae genera were detected, however, some genera occurred in only one season, such as Arundinoideae *Phragmites*. Heatmap analysis also showed that for some genera, there is a distinct pollen season for one year but multiple pollen peaks in the other, e.g., Panicoideae *Digitaria* sp. and Pooideae *Poa* sp.

At the NSW sites there were more genera with distinct pollinating times, than with multiple peaks (Fig. 4d and e). Prime examples of genera with distinct pollinating times include Pooideae; *Anthoxanthum, Holcus* and *Festuca* (Macquarie Park, 2017–18), Pooideae *Arctagrostis* (Macquarie Park, 2018–19) and Panicoideae *Echinochloa* (Richmond, 2017–18) (Fig. 4d–f). Examples of temperate Pooideae Poaceae species

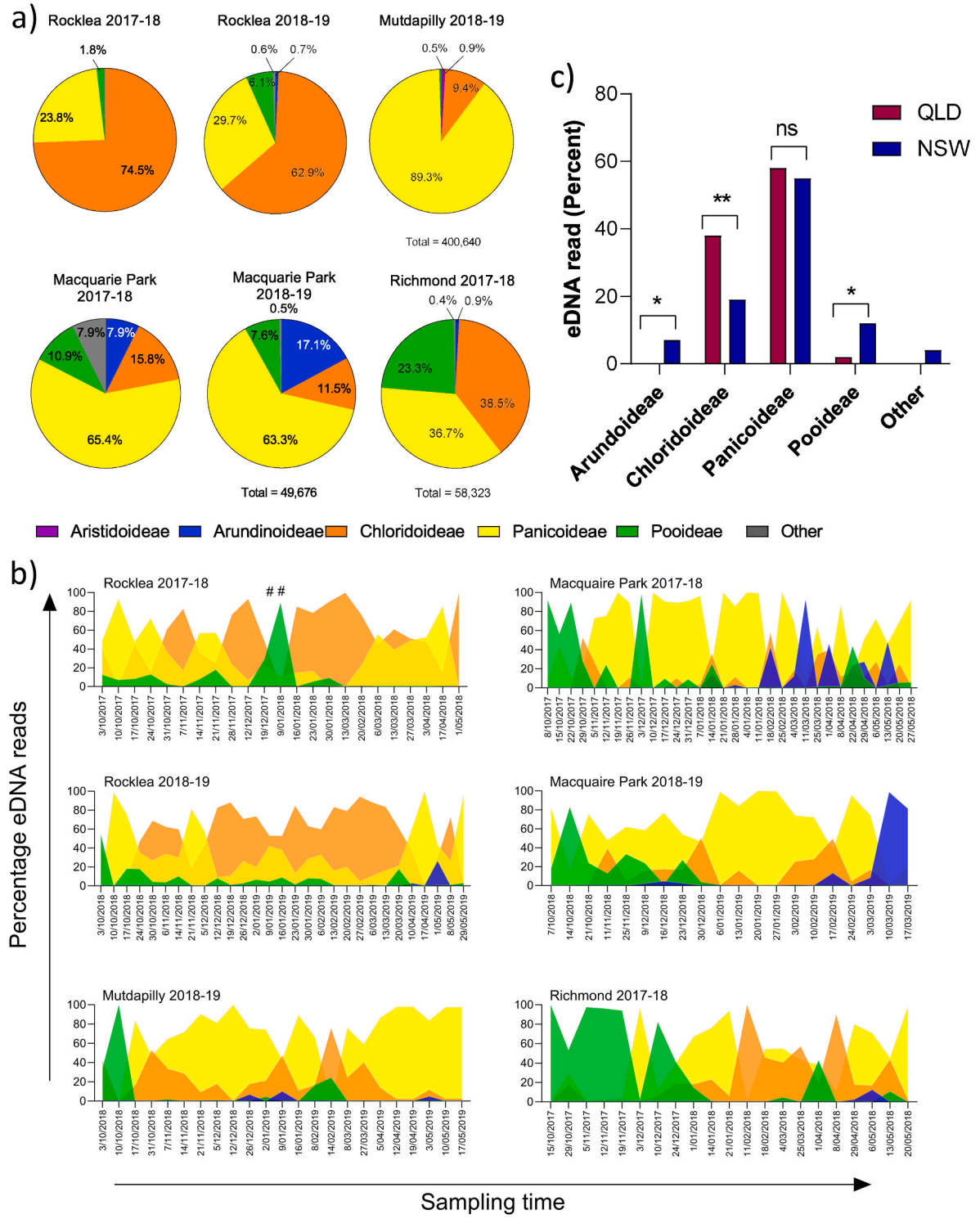

Fig. 3. The a) overall proportion and b) daily proportion timeseries per monitoring period at each site of eDNA read counts from operational taxonomic units (OTUs) assigned to Aristidoideae, Arundinoideae, Chloridoideae, Panicoideae, Pooideae and other grass subfamilies. (# days with very low grass pollen concentrations.) c) Comparison of proportions of eDNA read count by subfamily between combined Queensland (QLD) and New South Wales (NSW) data (Fisher exact test Table S1, supplementary information). *p < 0.05; **p < 0.01; ns: not significant.

observed include *Lolium* and *Poa,* which both pollinated in late spring/early summer. Poaceae genera displaying multiple peaks across most of the pollen season included the same prominent subfamilies already described in subtropical regions, such as Chloridoideae; *Chloris* sp., *Cynodon*, and Panicoideae; *Paspalum* sp. and *Cenchrus* (Fig. 4d–f). At Richmond, several peaks were observed for Chloridoideae; *Cynodon* and *Chloris* as well as Panicoideae; *Paspalum* and *Axonopus*. When comparing heat map signatures between years for Macquarie Park, many of the key Poaceae genera were the same, but as with Rocklea in subtropical Queensland, there were the odd differences in composition reflecting the dynamic nature of the aerobiome and possibly the effects of localised climatic factors for the year in question. At Richmond, the site that showed a spring pollen peak (Fig. 1d), the grasses that contributed to eDNA at that time were mostly Pooideae; *Lolium*, *Poa*, *Aegilops*, and

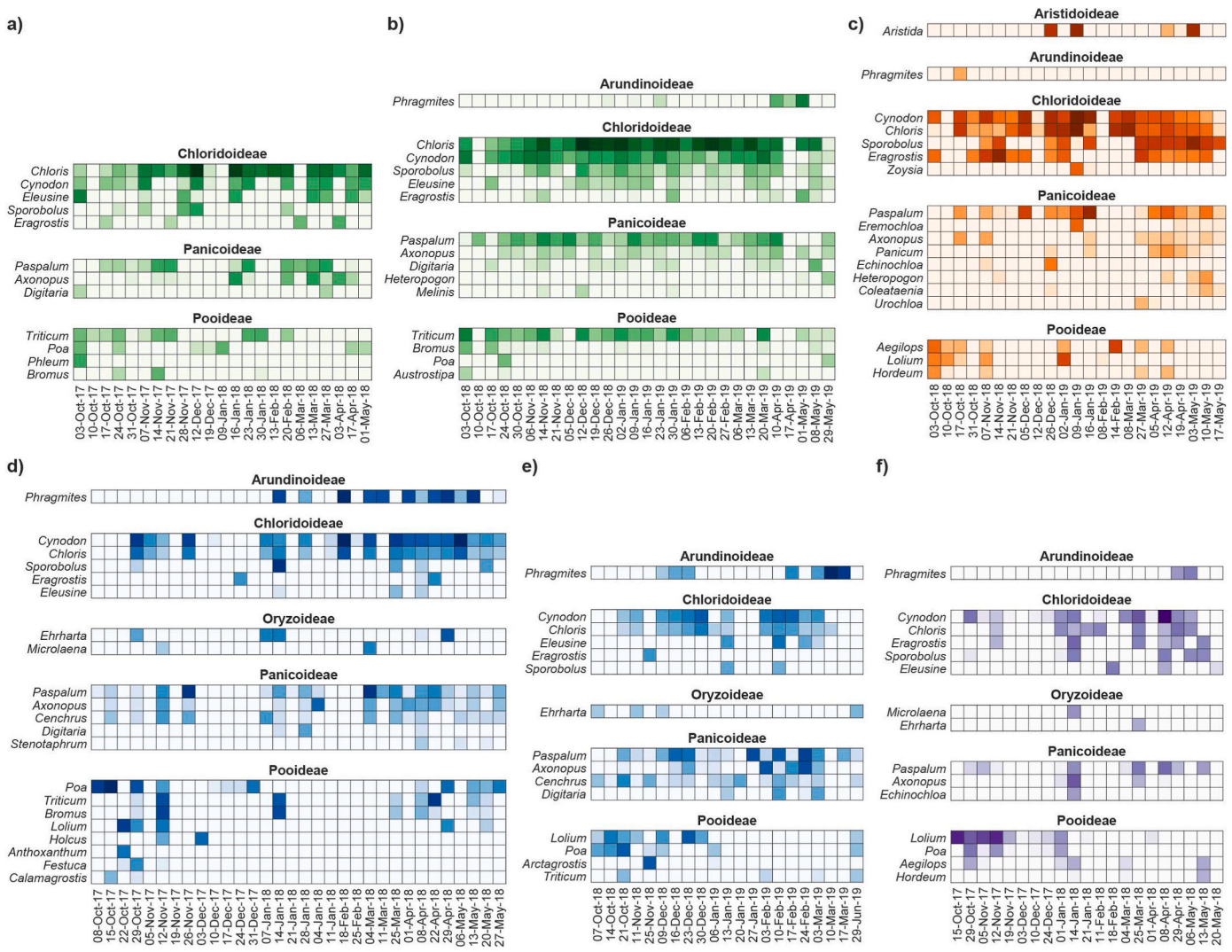

Fig. 4. Heat maps of eDNA read counts for prominent grass genus level classifications at each location across the periods of monitoring a) Rocklea 2017–18, b) Rocklea 2018–19, c) Mutdapilly 2018–19, d) Macquarie Park 2017–18, e) Macquarie Park 2018–19, and f) Richmond 2017–18. Genera have been separated by subfamily and displayed with colour intensity depicting abundance (read counts) and peak timing of each genera relative to others at that site.

Hordeum (Fig. 4f).

3.5. Comparison of eDNA with herbarium sighting records

The compound area of interest for which herbarium records of Poaceae plant sightings were compiled was approximately 18 percent larger in Queensland (10,500 km^2) than in NSW (8900 km^2). There were, however, approximately 10 times more ALA Poaceae observations in NSW than Queensland (115,416 and 11,453 respectively), probably due to the larger population in NSW area, and a tendency for more observations in populated areas. Within the area of interest, the difference in number of Poaceae observations between states was less pronounced, with observations in 4245 cells (NSW) and 1845 cells (Queensland) (Fig. 5).

For both the whole state and the area of interest, Panicoideae was the most frequently observed Poaceae subfamily, and the proportion of sightings of Panicoid grasses in areas of interest for Queensland (70.0%) and NSW (70.6%) did not differ (Fig. 5a., Figure S3; Table S2, supporting information). In NSW, the next two most frequently sighted subfamilies were Pooideae, followed by Chloridoideae, whereas in Queensland Chloridoideae was the second most frequent, followed by Pooideae. In both states, the fourth subfamily of interest based on the eDNA results, Arundoideae, was both less frequently observed. Within the local area of the Macquarie Park site there is a stand of *Phragmites* reeds, and adjacent to the Rocklea site there is a paddock where *Chloris* is cultivated as fodder, contributing to local Poaceae pollen sources at the respective sites (Figs. 2–4). Within the NSW compound area of interest, Pooideae subfamily sightings dominated over other subfamilies in the western mountainous area (Blue Mountains). Within the areas of interest, there was a higher proportion of sightings for Chloridoideae grasses in Queensland (13.1%) than in NSW (6.8%, $p < 0.0001$), whilst there was a higher proportion of Pooideae sightings for NSW (15.0%) than in Queensland (6.9%, $p < 0.0001$) (Fig. 5a., Figure S3 and Table S2, supporting information). Overall, the proportion of eDNA reads for each subfamily was correlated with the proportion ALA Poaceae sightings for each subfamily in the areas of interest ($R^2 = 0.815$, $p = 0.0021$, Fig. 5b).

4. Discussion

Surprisingly little is known globally about contribution of Poaceae of diverse subfamilies to airborne pollen records, particularly for biogeographically diverse environments. With climate change it is predicted that the burden of AR will increase and that subtropical climate zones will continue to expand (Seidel et al., 2008; Turton, 2017), thus increasing the contribution of subtropical grasses to allergic disease (Beggs and Bennett, 2011), meaning from an environmental health

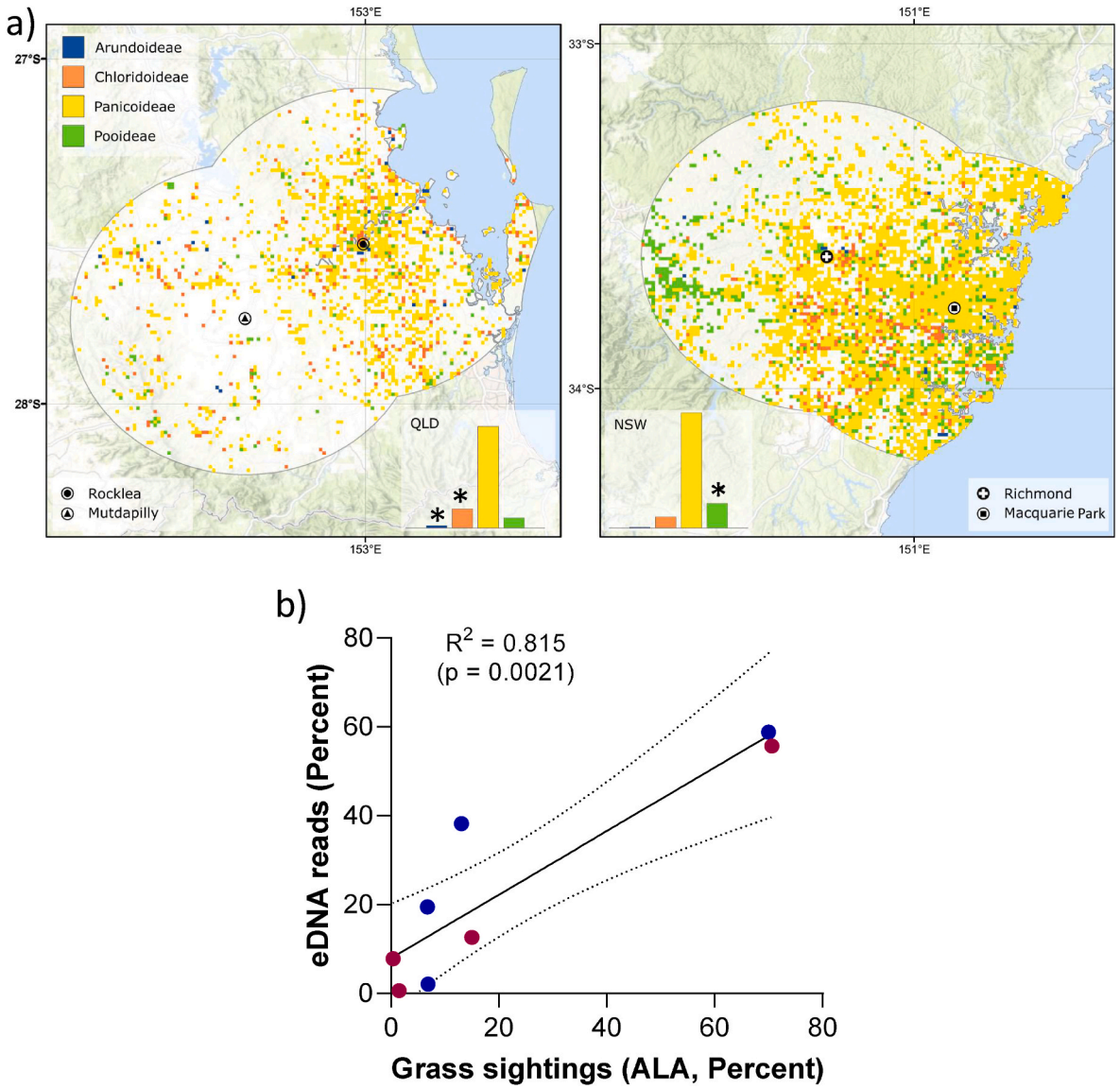

Fig. 5. Herbarium records of grass sightings classified to subfamily level within 50 km radii of pollen monitoring sites for the states of Queensland (Queensland; QLD left; Rocklea and Mutdapilly), and New South Wales (NSW right; Richmond and Macquarie Park). The inset histograms indicate sighting number of each subfamily grid cells within the area of interest of the Queensland and NSW sites (significant differences (*; $p < 0.001$) in proportion of sightings by subfamily for the areas of interest between states, Table S2, supplementary information). b) Correlation between aggregated data for proportions of Poaceae eDNA read counts and proportions of ALA grass sightings by subfamily for the areas of interest within each state (Queensland maroon, NSW blue). The Pearson's correlation coefficient and linear regression with 95% confidence intervals are shown.

perspective the capacity to discern airborne Poaceae pollen sources from different subfamilies will become increasingly important. With the exception of Campbell et al. (2023), previous eDNA research has not compared pollen diversity between seasons at the same sampling location (Brennan et al., 2019; Campbell et al., 2020; Bianchi et al., 2020b). Here we used the chloroplast *rbcL* barcode marker to provide a detailed analysis of the different Poaceae taxa found in airborne pollen samples, using routine pollen monitoring equipment, across two pollen seasons and locations in the subtropical region of South-East Queensland, and temperate region of NSW, Australia.

We note that other metabarcode targets proposed for eDNA analysis of plant sources and may provide different or superior assignment of Poaceae diversity than rbcL, e.g., ITS (Brennan et al., 2019), which suggests further methodological development could improve future analyses. The Rocklea site, with the highest proportion of Chloridoideae pollen but the lowest assignment to genus, suggests that broader representation of sequences for genera and species within this subfamily in particular is needed within the rbcL classifier database. Nonetheless, with the rbcL marker used herein, we could observe a difference between Poaceae pollen diversity and seasonal distributions between climate zones. The overall trends might reflect fundamental differences between temperate grasses which use a three-carbon (C3) intermediate, and subtropical grasses which use a four carbon (C4) pathway, using more carbon and loosing less water during photosynthesis.

There was an initial expectation that a greater proportion, especially in spring, of Pooideae grasses would contribute to airborne pollen in the more temperate regions of NSW. There was certainly more Pooideae diversity, and a greater proportion of Pooideae pollen, in NSW compared to Queensland, but not an overall dominance of Pooideae pollen at either temperate site. However, the eDNA proportions for Pooideae matched the plant sighting records for Pooideae. We note that this monitoring period occurred during a drought period, which may have decreased the magnitude of the spring pollen peak expected at the NSW sites (Katelaris and Burke, 2003). During this study, Richmond was the

only site that showed the spring Poaceae pollen peak, and the highest Pooideae representation. Macquarie Park, showed almost no spring Poaceae pollen during this study period consistent with drought impacting pollen levels at another NSW site during this current study Davies et al. (2022).

Interestingly, at the genus level, there were some differences in composition of airborne pollen between years for Rocklea and Macquarie Park, reflecting the dynamic nature of the aerobiome, and possibly the effects of localised climatic factors each year. In Campbell et al. (2020), which dissected the 2016-17 season at Rocklea using eDNA, several genera released pollen at distinct times such as *Sporobolus elongatus* (Chloridoideae). Interestingly in this current survey, we observed a difference in pollinating behaviour between *Sporobolus* sp., in the subtropical locations of Rocklea and Mutdapilly, which displayed a subtropical pollen release pattern of multiple pollen peaks versus the same genera in Macquarie Park or Richmond, where the timing of pollen release was more delineated.

Whilst Pooideae tended to contribute to pollen in springtime, the original assumption that different Poaceae genera contribute to the airborne pollen load at different times across the season, as proposed by Davies et al. (2015), did not entirely hold true across in sites where C4 grasses predominated. Multiple Panicoid and Chloridoid genera repeatedly flowered from late spring to autumn in all sites. This contrasts with Brennan et al. (2019) in the United Kingdom, where temperate Pooideae species exhibited distinct, temporally constrained peaks of pollen release across the season.

The multiple airborne grass pollen peaks over the season occurred mostly in Queensland for the subtropical (C4) grasses, which take their flowering cue from rainfall and temperature rather than photoperiod (Tarumoto et al., 2005). It is not possible to discern whether successive airborne pollen peaks from the same genera at a site arise from exactly the same plant. However, from an ecological perspective, it is important to consider that whilst climate change may drive shifts in C4 grass distributions (Xie et al., 2022), alterations in day length are not anticipated with climate change. Thus, depending on the relative importance of photoperiod, and other factors; CO_2 levels and vernalisation, the timing and magnitude of pollination of C3 and C4 grasses may be differentially affected by climate change. Indeed, in the USA, whilst tree pollen and weed pollen showed significant season lengthening and increases in seasonal pollen integrals with time (Anderegg et al., 2021), the Poaceae seasonal pollen integrals did not change, suggesting a complexity for Poaceae pollen records across diverse sites, that may mask local medium-term trends when data from sites in diverse climate zone are aggregated for analysis. The biogeographical influence on Poaceae pollen season timing and magnitude was not clear in that study (Anderegg et al., 2021), but Lo et al. (2019) showed latitudinal gradients in Poaceae pollen season timing in the USA, with longer seasons occurring at more southern sites, which we suggest may be related to compositional diversity of C3 and C4 grasses being influenced by local climatic and environmental factors.

Anthropogenic climate change could impact respiratory health, through temperature- and CO_2-driven increases in airborne pollen, but the long-term continental pollen trends and role of climate change in pollen patterns are not well-understood (Anderegg et al., 2021; Tong et al., 2022). Recently, our study by Xie et al. (2022), mapped using herbarium sighting data and satellite-observed shifts in C3/C4 abundance in grasslands in eastern Australia, over a 15-year period, indicating a long-term shift in grass community composition. That C4 grasses can peak multiple times per season based on eDNA analysis, challenges an assumption that the late peak is due to subtropical C4 grasses and the early spring peak is C3 temperate grasses. eDNA analysis of diversity of multiple Poaceae peaks might elucidate and guide review of land surface phenology models and algorithms (Devadas et al., 2018; Zeng et al., 2022), where it is currently assumed that landscapes only exhibit one or two phenology peaks per year.

5. Conclusions

eDNA analysis revealed distinct subfamily dominance patterns of grass pollen across seasons and climate zones. Leveraging capacity for eDNA metabarcoding of airborne pollen diversity now enables more accurate epidemiological studies to link specific grass pollens with health outcomes such as hospital attendance for asthma (Simunovic et al., 2020, 2023; Idrose et al., 2020). This might be particularly valuable for analysis of thunderstorm asthma events (Thien et al., 2018). This research is also crucial to monitoring and evaluating the impacts of pollen exposure on aeroallergen sensitisation (Minami et al., 2019; Matricardi et al., 2016).

The outcomes presented deepen our understanding of novel environmental profiling techniques globally including, intentionally and otherwise, human eDNA in air quality sampling (Littlefair et al., 2023; Whitmore et al., 2023). The study contributes data and insights giving more nuanced knowledge of aerobiology in diverse biogeographical zones, and in particular Southern Hemisphere locations (Addison-Smith et al., 2021). These insights are critical for monitoring environmental threats to respiratory health, and Poaceae response to climate change, to address ongoing challenges faced by humanity.

CRediT authorship contribution statement

Shanice Van Haeften: Formal analysis, Investigation, Writing – original draft. **Bradley C. Campbell:** Investigation, Writing – original draft. **Andelija Milic:** Formal analysis, Writing – review & editing. **Elizabeth Addison-Smith:** Formal analysis, Writing – review & editing. **Jane Al Kouba:** Investigation. **Alfredo Huete:** Funding acquisition, Writing – review & editing. **Paul J. Beggs:** Funding acquisition, Investigation, Writing – review & editing. **Janet M. Davies:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

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Data availability

We have shared the link to all data in the body of the manuscript and cited the source in the references

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2023.117983.

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