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Decrypting seasonal patterns of key pollen taxa in cool temperate Australia: A multi-barcode metabarcoding analysis

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ABSTRACT

Pollen allergies pose a considerable global public health concern. Allergy risk can vary significantly within plant families, yet some key pollen allergens can only be identified to family level by current optical methods. Pollen information with greater taxonomic resolution is therefore required to best support allergy prevention and self-management. We used environmental DNA (eDNA) metabarcoding to deepen taxonomic insights into the seasonal composition of airborne pollen in cool temperate Australia, a region with high rates of allergic respiratory disease. In Hobart, Tasmania, we collected routine weekly air samples from December 2018 until October 2020 and sequenced the internal transcribed spacer 2 (ITS2) and chloroplastic tRNA-Leucine tRNA-Phenylalanine intergenic spacer (*trnL-trnF*) regions in order to address the following questions: a) What is the genus-level diversity of known and potential aeroallergens in Hobart, in particular, in the families Poaceae, Cupressaceae and Myrtaceae? b) How do the atmospheric concentrations of these taxa change over time, and c) Does *trnL-trnF* enhance resolution of biodiversity when used in addition to ITS2? Our results suggest that individuals in the region are exposed to temperate grasses including *Poa* and *Bromus* in the peak grass pollen season, however low levels of exposure to the subtropical grass *Cynodon* may occur in autumn and winter. Within Cupressaceae, both metabarcodes showed that exposure is predominantly to pollen from the introduced genera *Cupressus* and *Juniperus*. Only ITS2 detected the native genus, *Callitris*. Both metabarcodes detected *Eucalyptus* as the major Myrtaceae genus, with *trnL-trnF* exhibiting primer bias for this family. These findings help refine our understanding of allergy triggers in Tasmania and highlight the utility of multiple metabarcodes in aerobiome studies.

1. Introduction

Allergic respiratory diseases, such as allergic rhinitis (AR, commonly called hay fever) and allergic asthma, pose a substantial burden on the health of individuals and the global economy (Masoli et al., 2004; Pawankar et al., 2013; Nunes et al., 2017; Kulthanan et al., 2018). Pollen exposure is known to be a key contributor to the allergic respiratory disease burden, with elevated levels of atmospheric pollen correlated with outcomes such as antihistamine pharmaceutical sales (Johnston et al., 2009), self-reported AR symptoms (K. Bastl et al., 2018; P. J. Jones et al., 2020; Silver et al., 2020), increased asthma hospital admissions (Schäppi et al., 1999; Erbas et al., 2012; Osborne et al., 2017; Guilbert et al., 2018; Kitinoja et al., 2020) and epidemic thunderstorm asthma (Thien et al., 2018; Idrose et al., 2020). In a changing climate, increasing atmospheric carbon dioxide, and the increasing geographic range of allergenic plants, are anticipated to increase the pollen-related burden of disease (Ziska et al., 2019; Anderegg et al., 2021; Manangan et al., 2021).

Airborne pollen monitoring is commonly utilised as a mitigating strategy: by providing information about when different pollen types are

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abundant, monitoring programs seek to support clinical diagnosis and individual management and thereby mitigate the burden of pollen-related disease (K. Bastl et al., 2014; Kmenta et al., 2014; K. Bastl et al., 2018; Johnston et al., 2018; M. Bastl et al., 2021; Silver et al., 2018; Damialis et al., 2019; P. J. Jones et al., 2020; Silver et al., 2020). While valuable, pollen monitoring currently relies on microscopy-based pollen identification which, in some situations, lacks the taxonomic resolution for optimal diagnosis and management. For example, grass pollen can only be identified to family level, when genus level would be more suitable in some situations. For example, Rowney et al. (2021) recently demonstrated that some grass genera (*Cynosurus cristatus* L. and *Phleum pratense* L.) correlated more strongly with asthma symptoms than others in the United Kingdom. Furthermore, Davies et al. (2012) showed that subtropical grasses (e.g., *Paspalum notatum* Flüggé) elicit stronger immune responses than temperate grasses (e.g., *Lolium perenne* L.) in one population of AR sufferers in subtropical Australia. These findings suggest methods with greater taxonomic resolution are required to elucidate allergic respiratory symptom triggers with greater precision.

Metabarcoding now offers a potential solution, capable of identifying all aerobiological material in a sample to high taxonomic resolution, including plant bioaerosols such as pollen (Kraaijeveld et al., 2015; Bell et al., 2016; Bell et al., 2017; Tegart, 2017; Brennan et al., 2019; Nilsson et al., 2019; Campbell et al., 2020; Banchi et al., 2020; Johnson et al., 2021; Polling et al., 2021; Uetake et al., 2021; Campbell et al., 2022; Fragola et al., 2022). In this study, we utilise metabarcoding, combined with traditional microscopic pollen monitoring, to provide high taxonomic resolution insights into the distribution of pollen taxa in cool temperate Australia. We specifically explored this in Hobart, Tasmania, a region that typifies many of the geographical and ecological characteristics of the cool temperate Australian bioregion. Research is a high priority in this region due to higher rates of asthma and AR by global standards: in Tasmania reaching 24% and 12.9% respectively, with similar rates across much of the Australian cool temperate bioregion (Health and Welfare, 2020, 2023). Addressing the issue, however, remains hampered by low taxonomic resolution understanding of key pollen allergy triggers (P. J. Jones et al., 2020).

To address these gaps, we collected week-long airborne samples for two years, investigating the diversity and composition of airborne pollen via metabarcoding, and compared this to routine morphological pollen counts. We sequenced the internal transcribed spacer 2 (ITS2) region; a nuclear barcode commonly used to assess plant biodiversity (Bell et al., 2018; Suchan et al., 2019); and trnL-trnF, a less commonly used chloroplast region (Omelchenko et al., 2022) to investigate its capacity to enhance biodiversity insights. We focus particularly on the Poaceae (grass), and Cupressaceae (cypress) and Myrtaceae (including eucalypts and tea trees) families - these taxa have been identified as key allergy triggers in Tasmania by P. J. Jones et al. (2020) and have been associated with allergies and asthma elsewhere (Hanigan and Johnston, 2007; Burbach et al., 2009; Johnston et al., 2009; Wang et al., 2013; Davies et al., 2015; Gibbs, 2015; Medek et al., 2016), yet cannot be identified beyond the family using microscopy-based methods.

We aimed to answer the questions: a) What is the genus-level diversity of known and potential aeroallergens in Hobart, in particular, in the families Poaceae, Cupressaceae and Myrtaceae? b) How do the atmospheric concentrations of these taxa change over time, and c) Does *trnL-trnF* enhance detection and/or resolution of biodiversity when used in addition to ITS2?

2. Methods

2.1. Sample collection

Ambient air samples were collected using a Hirst-type spore trap (Hirst, 1952) (Burkard Manufacturing) located approximately 13 m above ground on the Natural Sciences building roof at the University of Tasmania's Sandy Bay campus (42°54′17″S 147°19′29″E, Fig. 1). The trap was calibrated to sample air at 10 L per minute and operated for consecutive 168 h periods (7 days). Airborne particles were deposited on Melinex tape (Burkard Manufacturing Co. Ltd, Rickmansworth, Hertfordshire, UK) coated with either a silicon-based adhesive Sylgard (Lanzoni, Bologna, Italy) or petroleum jelly (vaseline), attached to the rotating drum inside the trap (Supplementary File 2; Rojo et al., 2019). On collection, tapes were immediately transferred for storage in a Biopur ® tube (Eppendorf, Hamburg, Germany) at −80 °C.

The sampler used for DNA sample collection was co-located (within 2 m) with a second Hirst-type trap used to collect daily samples for standard microscope-based pollen analysis. Both traps were identical except for the use of a daily vs 7-day head; the daily head collects pollen onto a microscope slide, whereas the 7-day head collects onto a longer tape. The flow rate and other operational aspects are otherwise identical; pollen counts from the two types of head are considered directly comparable. Daily standard microscope-based pollen counts were collected from the latter sampler and pollen taxa were counted following protocols described in Johnston et al. (2018). Mean weekly values were calculated from daily pollen concentrations for 28 routinely collected taxa. Routine pollen counting windows began 4 h after the weekly tape samples began, thus there is a 4-h period that is not co-sampled between weekly tapes and combined daily morphological pollen counts **(Supplementary Fig. 5).** Although the results of morphological pollen counts are calculated and displayed as atmospheric pollen concentrations, the term 'pollen counts' will be used throughout this paper for simplicity. As the relative abundance of sequence counts can be problematic to interpret (Gloor et al., 2017), in this paper we use morphological pollen counts as the baseline measure of abundance to which sequence relative abundances can be compared.

2.2. DNA extraction

DNA material was extracted under sterile conditions. Silicone adhesive coated-tape samples were unfurled and transferred into a new microfuge tube for DNA extraction. For cold season tape samples, Vaseline was removed with a sterile blade and transferred into separate microcentrifuge tube. To disrupt the pollen grains, a stainless-steel bead was added to each tube, samples were frozen, lypophilised and processed on a TissueLyser II (Qiagen). Then 1 mL of CTAB buffer (2% CTAB. 1.4M NaCl, 0.2% β-mercaptoethanol, 20 mM EDTA 100 mM Tris-HCl, pH8) was added to each tube and DNA extractions were performed as described in Tegart (2017) following the CTAB method by Doyle and Doyle (1987). Blank extraction controls were also processed following the same protocol to serve as negative controls. DNA concentration was estimated with Nanodrop (Thermo Fisher Scientific), each extraction over 10 ng/μL was normalised to 10 ng/μL for subsequent PCRs. Reactions that were <10 ng/μL were undiluted.

2.3. Metabarcode amplification and sequencing

The internal transcribed spacer 2 (ITS2) was selected as the primary metabarcode because of its frequent use as a universal eukaryotic survey region (Bell et al., 2018; Suchan et al., 2019). While ITS2 may be used on its own, *trnL-trnF* was used as an accessory metabarcode to provide the potential for better taxonomic resolution in some situations. *trnL-trnF* is less commonly used than other metabarcodes (such as a *trnL* or *rbcL*) but it has been used successfully in another study (Tegart, 2017) and assessed against ITS2 comprehensively in an artificial mixture experiment by Omelchenko et al. (2022). However, this latter study only compared the two in artificial mixtures, this study aimed to compare *trnL-trnF* against ITS2 in a natural system.

A 'Dual-index barcoding' approach was used in this study (Clarke et al., 2017; Suchan et al., 2019). Both metabarcodes were amplified from each DNA extraction in an initial round of PCR. Supplementary Table 1 lists the primer sequences used and Supplementary Tables 2 and

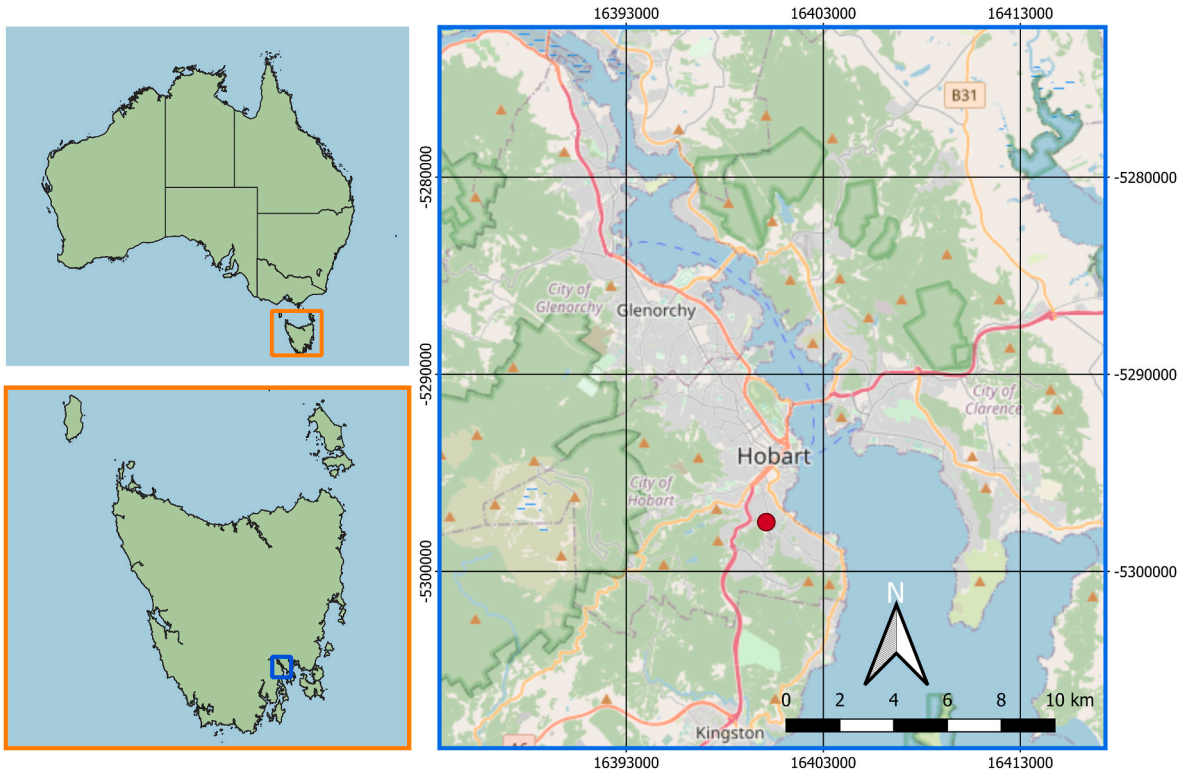

Fig. 1. Geographic context of Hobart, Tasmania and the sampling location. (A) Australia, with the state of Tasmania inset with an orange border. (B) The state of Tasmania with the greater Hobart region inset in blue. (C) The greater Hobart region with the sampling location, the University of Tasmania Sandy Bay campus, indicated with the red circle.

3 provide details on mixtures and cycling conditions that were used. Reactions were completed in duplicate. Successful reactions were determined by SYBR green fluorescence. Of the 100 samples that were tested, 84 samples had at least one successful ITS2 duplicate, and 99 samples had at least one successful *trnL-trnF* duplicate (Supplementary Table 4). Successful reactions were then diluted 1:10 and each sample was pooled by duplicate and amplicon. Each sample pool then underwent second round amplification, again in duplicate, following primer sequences listed in Supplementary Table 1 and PCR details in Supplementary Table 2 and **3** Successful reactions (98 out of 100) from this round were pooled together in equal volumes and purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA). A QIAquick Gel Extraction (QIAGEN) was used to remove any residual products larger than 600 bp or less than 150. Purified libraries were quantified using the Qubit dsDNA BR assay (Life Technologies, Carlsbad, CA, USA). The library was diluted to 4 nM and paired-end sequencing reads generated on a MiSeq (Illumina, San Diego, CA, USA) using MiSeq Reagent Kit v3 (2 × 300 bp paired-end, loaded at 12 pM concentration). Illumina software (v2) was used to demultiplex barcodes and sort reads into their corresponding samples.

2.4. Bioinformatic analysis

Demultiplexed (per sample) FASTQ files were further separated into amplicon specific files per sample using CutAdapt V2.9 (Martin, 2011) to find sequences with exact matches to reaction specific barcodes (**Supplementary File 3**). Sequences without exact matches to these barcodes were discarded.

For each amplicon (ITS2 and *trnL-trnF*), paired reads were merged with Flash2 v2.2.0 (Magoc and Salzberg, 2011). Primer sequences were removed and sequences without exact matches to both forward and reverse amplicon specific primers were discarded (CutAdapt v2.9). Finally, resulting merged sequences with long homopolymer runs (>12) or ambiguous bases were removed.

For each amplicon, remaining sequences were denoised to zero radius operational taxonomic units (zOTU) with USEARCH v11.0.667 (Edgar, 2010) by dereplicating sequences (-fastx_uniques), followed by implementation of the UNOISE3 algorithm (Edgar, 2016) (-unoise3 -minsize 4). Finally, abundance tables were built for each amplicon using the USEARCH -otutab command with default arguments.

ITS2 zOTUs were classified to the lowest taxonomic level possible against the UNITE FASTA database v8.3 (Abarenkov et al., 2021) for all eukaryotes, with the *assignTaxonomy* function in DADA2 (Callahan et al., 2016), using default arguments. As there was no readily available, curated *trnL-trnF database,* the CRABS program was used to create a curated database (Jeunen et al., 2022). Full details of this process are available at https://github.com/mrgambero/trn-Ltrn-F_database. To reduce the computational burden, zOTUs shorter than 180 bp were removed, and the remaining zOTUs were classified to *trnL-trnF* with the new database with the *assignTaxonomy* function. The results of this classification were manually curated with BLAST alignments against NR to ensure classifications were biologically plausible, any misidentified ASVs were corrected post-hoc. After bioinformatic analysis, for the ITS2 region, 34018 reads (classified as all eukaryotes) from 29 samples and 234 zOTUs passed quality filtering, while for *trnL-trnF* 38740 reads, from 33 samples (290 zOTUs) passed (Supplementary Table 4).

2.5. Data analysis

All data analysis and visualisation was performed in R (R Development Core Team, 2022) with the use of the packages dplyr (Wickham et al., 2022), tibble (Wickham and Müller, 2022), lubridate (Grolemund and Wickham, 2011) and reshape (Wickham, 2007).

For both ITS2 and *trnL-trnF*, only OTUs classified to Kingdom Viridaeplantae were kept. To ensure only deeply sequenced samples were included, samples with fewer than 100 Viridaeplantae sequences were

then discarded. The relative abundance of sequence counts for *trnL-trnF,* ITS2 and microscopy-based pollen concentrations were compared using Spearman correlations. This analysis focused on families in common between each method. To assess patterns in greater detail, accounting for differences in sequencing depth between samples, Bray-Curtis dissimilarities were then calculated by averaging the results of multiple subsampling iterations with the function "avg.dist" in the vegan package (100 iterations at sampling depth 100 (Oksanen et al., 2022),). Permutation Multivariate ANOVA (PERMANOVA) was used on these Bray Curtis dissimilarities to test for differences in community composition across Australian meteorological seasons (referring to: Summer (December, January, February), Autumn (March, April, May), Winter (June, July, August), Spring (September, October, November)) (Anderson, 2001). Nonmetric dimensional scaling (NMDS) plots based on the Bray-Curtis dissimilarities were used for visualisation. Correlations between dissimilarity matrices from each method were calculated with Mantel tests using sample pairs shared by methods.

3. Results

3.1. Summary of overall biodiversity and temporal trends

The routine morphological pollen counts, shown in Supplementary Fig. 1, provide a robust baseline metric of airborne pollen abundance for the monitored taxa. These counts reveal Cupressaceae, Myrtaceae, Poaceae, *Dodonaea* (Sapindaceae), *Betula* (Betulaceae) and *Allocasuarina* as the dominant taxa over the study period. With respect to seasonality, Cupressaceae is abundant for several months, peaking from late winter to spring; Myrtaceae peaks across much of spring and summer, while Poaceae peaks from November to January (summer). *Dodonaea* and *Betula* show brief defined peaks in December and September respectively, while *Allocasuarina* is present year-round. Other taxa with notable seasonal peaks include the European trees Oleaceae (olive family), *Pinus* and *Salix* (willow).

Thirteen plant families were detected in common between all three methods (that is, optical counts, ITS2 and *trnL-trnF*). These include the

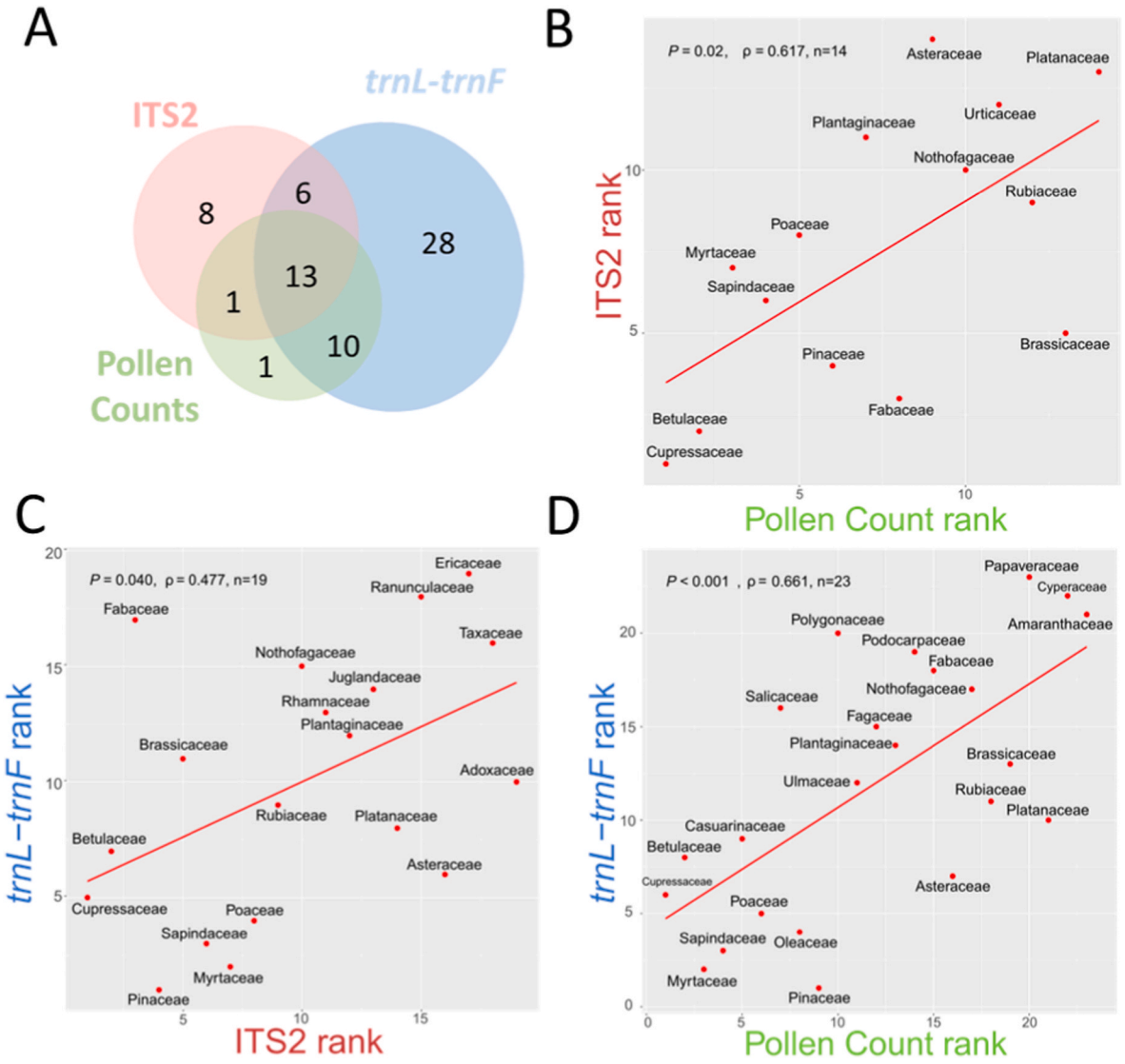

Fig. 2. Visualisations comparing results from the three methods of aerial pollen detection used in this study, which were morphological pollen counts, ITS2 metabarcoding and trnL-trnF metabarcoding. Panel (A) shows a venn diagram of the numbers of botanical land plant (Kingdom Viridiplantae) families detected by each method and in common between them. Panels (B,C,D) show pairwise correlations between the rank order abundance of families detected. These correlations were based on total sequence counts for ITS2 and trnL-trnF and summed atmospheric concentrations for pollen counts, using Spearman's correlation. Each pairwise correlation uses only the families that were detected by both methods. With ρ, p-value, number of families by each pairwise comparison (n=) and with a linear model trend line through each plot.

three families of interest in this study - Poaceae, Myrtaceae and Cupressaceae - as well as other key allergenic pollen families. Metabarcoding enabled the detection of an additional 42 plant families beyond those detected by the pollen counting method alone (Fig. 2A, details in Supplementary Table 4).

With the caveat that sequence counts can only be considered semi-quantitative, we found a significant correlation between the rank order of pollen counts and ITS2 sequence counts (Fig. 2B $\rho = 0.617$, $p = 0.02$, $n = 14$) as well as between pollen counts and *trnL-trnF* sequence counts (Fig. 2D, $\rho = 0.661$, $p < 0.001$ $n = 19$). The correlation between the rank order of ITS2 and *trnL-trnF* sequence counts respectively was also significant although slightly weaker (Fig. 2C, $\rho = 0.477$, $p = 0.040$, $n = 23$), partly due to a large discrepancy in the relative abundance of Fabaceae (including the Australian native genus *Acacia*, a large and heavy pollen grain). Mantel tests based on Bray-Curtis dissimilarities found the strongest relationship between ITS2 and pollen counts ($\rho = 0.55$, $p < 0.001$), followed by *trnL-trnF* and pollen counts (pollen counts vs *trnL-trnF*, $\rho = 0.41$, $p < 0.001$; *trnL-trnF* vs ITS2, $\rho = 0.39$, $p < 0.001$) (Supplementary Fig. 2). Morphological pollen counts showed the strongest seasonal variation in composition, although significant seasonal differences in composition were detected across all methods (Supplementary Fig. 2).

Fig. 3A and **B** highlight substantial differences in seasonal composition across methods, visualised as the relative abundance of both families and genera. For example, pollen counts detected Poaceae in considerable amounts in summer, but in smaller amounts for the rest of the year. In contrast, *trnL-trnF* detected Poaceae with relative abundance greatest in autumn (noting only one successfully sequenced sample was available for summer), and ITS2 did not detect Poaceae in sufficient amounts for inclusion in the top ten most abundant families. ITS2 also failed to detect Myrtaceae (the Eucalypt family) within the top ten; whereas both pollen counts and *trnL-trnF* detected Myrtaceae in substantial amounts. Conversely, Cupressaceae is substantially more dominant in ITS2 sequence counts compared to the other methods, across winter, autumn and spring. *Dodonaea*, the fourth most common pollen count taxa, is detected almost entirely in the summer by pollen counts and ITS2, whereas *trnL-trnF* results show substantial representation across summer, autumn, and spring.

Further substantial differences are evident when the data are further broken down into different seasons of different years. As shown in Supplementary Fig. 3A and **3B**, the compositional profile of pollen counts is reasonably consistent within seasons between years, with some exceptions such as the lower relative abundance of *Dodonaea* (Sapindaceae) in summer 2019-20. In contrast, metabarcoding results show major discrepancies in the relative abundance of multiple taxa across the same seasons of different years, noting the low sample sizes for many individual seasons. For example, Pinaceae was detected by ITS2 in winter 2019, but not in winter 2020. Autumn 2019 for ITS2 is entirely dominated by the Fabaceae taxon *Genista*, in contrast in Autumn (2020) the Cupressaceae genera *Cupressus* and *Juniperus* were the main taxa detected. For *trnL-trnF*, there are also multiple major discrepancies, including in the relative abundance of Sapindaceae (*Dodonaea*) between springs.

3.2. Seasonal trends of genera of key families

This section provides a more detailed analysis of trends in Cupressaceae, Myrtaceae and Poaceae, as the dominant taxa known to be associated with allergies in Tasmania (P. J. Jones et al., 2020), and for which metabarcoding has potential to enrich taxonomic insights. Both ITS2 and *trnL-trnF* metabarcodes identified these three families when amplification was successful.

Morphological pollen counts show the peak Poaceae season in Hobart ranges from late spring (November) to early-mid summer (January). Results from the ITS2 metabarcode suggest that the spring component of the Poaceae season was likely dominated by genera such as *Poa* L. and *Bromus* Scop. (Fig. 4). Summer appears to be dominated by *Holcus* L.*;* noting that for ITS2, sampling coverage was restricted to one spring and one summer season (2018 and 2019 respectively, see Fig. 4). In autumn and winter, when pollen counts are low, genera such as *Cynodon* (L.) Pers. and *Microlaena* R. Br. appear more abundant. In contrast, *trnL-trnF* identified *Ehrharta* Thunb as dominant in the spring/summer peak, and genera including *Poa and Lolium* in the autumn/winter months. At the subfamily level, these patterns translate into ITS2 revealing a spring/summer peak season dominated by Pooideae with the cooler months dominated by Chloridoideae and Oryzoideae. In contrast, *trnL-trnF* suggests ubiquitous Oryzoideae, and minimal Pooideae during the main spring/summer season, and very little Chloridoideae at any point (Fig. 4).

Pollen counts show Cupressaceae peaking from winter into early spring (Fig. 5). Both metabarcodes suggest that pollen during this main winter/spring season was primarily comprised of introduced cypresses, such as *Cupressus* L, *Callitropsis* Oerst. and *Hesperocyparis* Bartel & R. A. Price. *Juniper* L., another introduced tree, features prominently in ITS2 sequences, especially in the latter portion of the season (spring), however this taxon is less abundant and only present in winter according to *trnL-trnF*. The native *Callitris* was only detected by ITS2 and only in low levels only in spring.

Myrtaceae pollen has a peak season extending from spring into early autumn. Myrtaceae sequences were detected in all seasons by *trnL-trnF;* but ITS2 only detected it in winter and spring (Supplementary Figure 1 and Fig. 5). We note that the *trnL-trnF* amplicon size for Myrtaceae is approximately 150bp smaller than other families (Supplementary Fig. 4). Both metabarcodes suggest composition is dominated by *Eucalyptus*. ITS2 also detected *Melaleuca*, in spring 2019 and winter 2020. *trnL-trnF* also detected *Callistemon* and *Syzygium* in very low amounts, and *Metrosideros, Kunzea, Arillastrum* and *Melaleuca* with relative abundance <1%.

4. Discussion

This study used ITS2 and *trnL-trnF* metabarcoding to enrich our taxonomic insights into the composition of airborne pollen in Hobart, Tasmania, and to identify key contributing taxa within the allergenic Poaceae, Cupressaceae and Myrtaceae families. With the caveat that sequencing coverage was poor in some seasons, our results highlight the taxonomic richness of the southern Australian aerobiome, revealing 42 more families than detected by optical methods and providing a snapshot of biodiversity to serve ongoing ecological monitoring. Our data also deepen resolution with respect to allergen exposure, demonstrating the peak Poaceae season was predominantly composed of the temperate grass subfamily Pooideae, with key allergens from subtropical subfamilies including *Cynodon* only appearing off-season in the autumn and winter. The sequencing methods suggest the main contributors of Hobart's Cupressaceae pollen burden were introduced taxa such as *Cupressus* and *Juniper*, rather than native taxa, and supported that Myrtaceae pollen exposure is predominantly *Eucalpytus*. *trnL-trnF* proved to be a useful complement to ITS2 in identifying some ecologically important taxa, reinforcing the benefits of investing in multi-metabarcoding for aerobiome research.

4.1. Taxonomic composition and temporal dynamics of key pollen families

4.1.1. Poaceae

The morphological homology of grass pollen means that substantial gaps remain in our understanding of which species of grass pollen are in the air when, and where, and which of those are most likely to trigger allergy (Frisk et al., 2023). This study contributes to a deeper understanding of the biogeography of Australian grass pollen. We show that grass pollen exposure in Hobart, a cool temperate region, is dominated by Pooideae, with smaller amounts of Oryzoideae and very little

subtropical grass, in a distinct pattern compared with subtropical and warm temperate Australia (Campbell et al., 2020).

Our observations suggest that the Poaceae pollen peak-season is comprised primarily of temperate grass genera such as *Holcus, Poa, Bromus, Anthoxanthum* L. and *Lolium*. Limitations in sampling coverage mean we are not able to disentangle the temporal dynamics of these genera within the peak-season; further research would be beneficial to resolve whether grass genera in this region exhibit single discrete peaks as observed in the UK (Brennan et al., 2019), or multiple peaks as observed in subtropical Australia (Campbell et al., 2020).

The Pooideae genera observed across the peak grass pollen season share the group 5 allergen, making them allergenically cross-reactive (Matricardi et al., 2016). However, there is growing evidence that allergy risk is not equal across Pooideae members, for example, Rowney et al. (2021) showed that some Pooideae species were more closely correlated to indicators of disease than others. This may relate to variations in allergen content of pollen grains and/or the degree to which allergen is dispersed into the environment (Buters et al., 2015; Tegart et al., 2021); this hypothesis is supported by evidence of varying levels of group 5 allergen in fifteen grass cultivars Jung et al. (2018). Notably, Ryegrass (*Lolium*), a genus in Pooideae that grows Australia-wide and is commonly invoked as the primary cause of hay fever symptoms and epidemic thunderstorm asthma in temperate Australia (Erbas et al., 2012, 2018; Idrose et al., 2020), was not recognised as a major contributor to pollen load in this study. This may relate to the Hobart sampling environment: Hobart is flanked to the west (the direction of the prevailing winds) by large areas of native forest, with areas of pasture (likely to contain ryegrass) confined to areas >20 km to the north and south. Hobart's relatively low grass pollen load (see Supplementary Material) may therefore largely derive from exotic garden grasses, native forest grasses and smaller agricultural grass contributions.

Our data suggest grass pollen exposure in Hobart's autumn and winter months includes small amounts of the subtropical grass *Cynodon* (commonly called Bermuda grass), and the genera *Ehrharta* and *Microlaena* in the subfamily Oryzoideae. Our findings align with reported flowering times for *Cynodon dactylon* in Tasmania (ala.org.au). The subtropical grasses have a distinct group 1 allergen, attributed as the major source of sensitisation in subtropical regions of Australia (Davies et al., 2012, 2015; Simunovic et al., 2020). The clinical impact of subtropical grass allergy is less understood in temperate regions of Australia, such as Hobart; our findings suggest exposure is minimal but potentially of seasonal significance for sensitised individuals. Less is known about the allergenicity of the Oryzoideae family, although *Oryza sativa*, also a member of Oryzoideae, likely bears an allergen that is likely cross reactive with other grass pollen (Sen et al., 2003).

4.1.2. Cupressaceae

Australia has a distinctive overall flora that includes unique Cupressaceae genera. These include *Callitris* Vent., found in all Australian states, and the Tasmanian endemics *Diselma* Hook. f. and *Athrotaxis* D. Don. Our data did not detect either of the Tasmanian endemic species with either metabarcode and detected *Callitris* minimally, we note that these taxa were present in the taxonomic database used for classification, therefore they could have been identified if present. This suggests native taxa are not major contributors to the Hobart allergy burden, which instead appears driven largely by introduced taxa such as *Juniperus* and *Cupressus,* both of which are recognised globally as significant aeroallergens (Uetake et al., 2021; De Linares et al., 2021). The poor representation of native Cupressaceae likely relates to biogeographical distribution, with the endemics restricted to cool, wet, alpine regions and coastal *Callitris* pollen less likely to be transported to Hobart by the prevailing winds. Despite this finding, there remains a need for further research to determine the allergenicity of native Cupresscaeae taxa as potentially important local sources of exposure, for example, Campbell et al. (2022) found *Callitris* pollen to be an important contributor to Cupressaceae pollen exposure in some areas of subtropical and rural warm temperate Australia.

4.1.3. Myrtaceae

Myrtaceae pollen has been linked to allergic respiratory symptoms in Tasmania by P. J. Jones et al. (2020), and elsewhere (Gibbs, 2015; Phillips et al., 2010) however Myrtaceae pollen allergy has been comparatively little studied and very little is known about the potential for differential allergenicity across Myrtaceae species and genera. Here, both markers identified *Eucalyptus* across all seasons where Myrtaceae sequences were identified. This is consistent with *Eucalyptus* phenology, with flowering from different species spread across seasons (Birtchnell and Gibson, 2006; R. C. Jones et al., 2011; Rawal et al., 2015). ITS2 detected a more diverse range of genera, including *Melaleuca* - also invoked as an allergen in some contexts (Phillips et al., 2010). However, as noted with respect to Fig. 3, *trnL-trnF* detected Myrtaceae at higher relative abundance and in many more samples compared with ITS2. The shorter length of the Myrtaceae *trnL-trnF* amplicon may have influenced this by causing length-based amplification and sequencing bias (Supplementary Fig. 4), (Fig. 2B and C), which can occur due to shorter amplicons (here such as Myrtaceae), tending to finish elongation before longer amplicons, resulting in an increased abundance of the shorter reads. Future studies blending metabarcoding, morphological pollen counts and phenology could elucidate in further detail the relationship between *Eucalyptus* species and allergic respiratory disease.

4.2. Utility of trnL-trnF as an accessory marker

In order for eDNA metabarcoding to become fully established as an aerobiological tool, ongoing research is required to optimise the metabarcode target/s to maximise reliable detection of pollen to genus, and where possible, species level. ITS2 is frequently used as a versatile plant metabarcoding marker, including in aerobiome studies (Brennan et al., 2019; Núñez et al., 2019; Polling et al., 2021; Sánchez-Parra et al., 2021; Bell et al., 2022), our study provides a basis for assessing *trnL-trnF* as a complementary marker. While (Omelchenko et al., 2022) assessed *trnL-trnF* in the context of artificial grass pollen mixtures, to our knowledge *trnL-trnF* has not previously been assessed as a complementary metabarcode in the context of mixed, routinely collected environmental samples.

Here, *trnL-trnF* clearly increased biodiversity detection in ways that were important to capturing a holistic understanding of local biodiversity. Many of the families detected by *trnL-trnF*, and not ITS2, are important components of the airborne pollen load, including Salicaceae, Oleaceae, Casuarinaceae, and Ulmaceae (full list in Supp. Table 5). *trnL-trnF* also detected ferns, which although not considered allergenic, can be important biogeographical indicators Campbell et al. (2022). Both the fern and major pollen families noted above have been detected in other using plastome markers (rbcL and *trnL*) (Campbell et al., 2022; Uetake et al., 2021). The ITS2 database contains many representative sequences of Salicaceae, Oleaceae and Ulmanceae and therefore should hold the capacity to identify them; the failure of ITS2 to detect them in this study may therefore reflect gaps in sequencing coverage. However, Casuarinaceae and the fern families, important components of the Australian aerobiome, are poorly represented in the ITS2 database; here *trnL-trnF* is highly likely to offer greater opportunity for detection and resolution. We acknowledge that this enhanced detection capacity is not exclusive to *trnL-trnF* but there is clear benefit in investing in multiple meta-barcodes to ensure aerobiome biodiversity surveys are comprehensive and robust. We suggest the choice of complementary metabarcodes is informed by the target gene's capacity to increase resolution in key target taxa. A key point highlighted by this study is that no metabarcode will provide a complete or precise quantification of the diversity of the sample, therefore this work highlights the importance of having more than one point of reference. It is important to note that biological differences between barcodes, for example, copy number

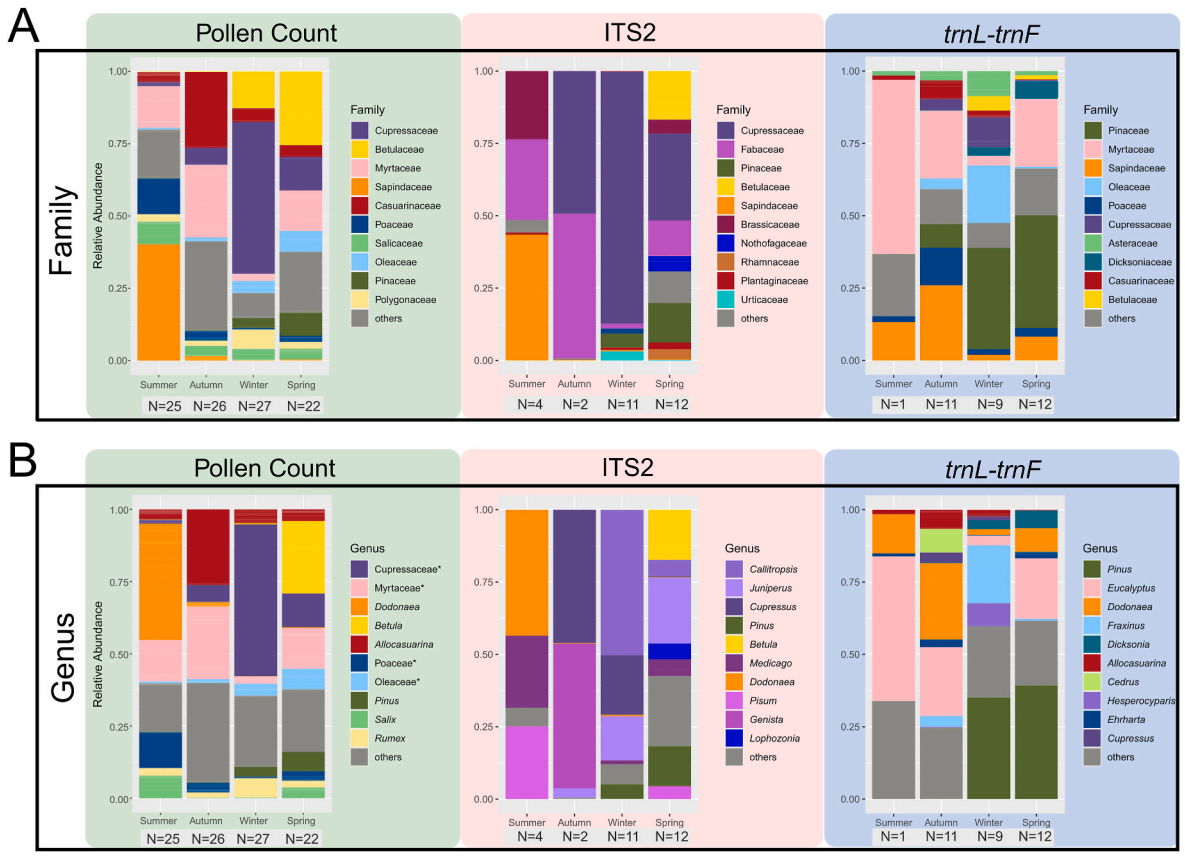

Fig. 3. Family and genus composition of pollen counts, ITS2 and *trnL-trnF* for each season. A. The top ten most abundant families for each detection method, listed in the legend in descending order. B. The top ten most abundant genera for each detection method. For both sections, relative abundance is defined for pollen count to the proportion of total pollen grains per total grains for that season, and for ITS2 and *trnL-trnF* it refers to the proportion of sequence counts of the total counts for that season. Each season by detection method has the number of weekly samples that make up each season (N =). 'other' refers to the sum of the other families and genera not in the top ten. Note for pollen count, some families are listed in place of genera (indicated with a *) because these pollen taxa are not distinguishable past family level, but are listed with equivalence to family level.

variation (Alvarez and Wendel, 2003; Nieto Feliner and Rossello, 2007), and tissue-related differences between nuclear and plastome markers remain mostly unexplored in metabarcoding literature (Bell et al., 2016). Thus, choosing the best metabarcode (or selection of metabarcodes) remains an open area of research (Hollingsworth et al., 2009; Bell et al., 2018; Baksay et al., 2020; Kolter and Gemeinholzer, 2020; Omelchenko et al., 2022). We argue that wherever cost permits, multi-metabarcoding should be utilised wherever possible; in addition to increasing biodiversity detection, the clear differences in relative abundance and taxonomic structure between metabarcodes also ensures relative abundances of sequence counts are not over-interpreted.

4.3. Strengths and limitations

This study represents the first multi-metabarcode study of aerial pollen in Australia and provides new insights into the temporal dynamics of taxa within three key allergenic plant families, in addition to total plant biodiversity in an understudied cool temperate bioregion. An important limitation was the number of undersampled or failed samples. This complicates the interpretation of the metabarcoding results; in particular, poorly or entirely unsampled seasons limit interpretations of temporal dynamics, and precluded temporal analysis at a weekly or monthly level.

The number of undersampled and unsequenced samples were the result of technical difficulties, which may be related to any stage of the experimental pipeline including sample collection, DNA extraction, amplification and sequencing. However, the moderately high DNA concentrations (Supplementary File 2) would suggest that the sample collection and DNA extraction methods yielded sufficient biomass to facilitate successful amplification. Similarly, many studies have used similar sample collection (Leontidou et al., 2017; Campbell et al., 2020, 2022; De Groot et al., 2021) and DNA extraction methods (Johnson et al., 2021; Campbell et al., 2020, 2022; Omelchenko et al., 2022) to those used in this study, yet none report a degree of sample failure similar to ours. Furthermore, our samples successfully amplifed in the first and second round with high success rates (Supplementary File 2). We therefore suspect the high level of sample sequencing failure is more likely related to barcode selection and sequencing library preparation. The ITS2 primers we selected amplified the ITS2 from all eukaryotes, not just plants. This may have resulted in dilution of amplified plant DNA with DNA from other more abundant bioaerosols, such as fungi. Including fewer samples from low biomass aerial samples in a sequencing run may also be a prudent measure of maximising successful samples in the same run.

More broadly, there are well recognised limitations in interpreting relative abundances when from sequencing data (Gloor et al., 2017; Bell et al., 2018; Lamb et al., 2019). Sequencing removes the assumption of true independence of entities in a sample, the relative abundance of sequences in a given sample therefore cannot be interpreted as a representation of the true abundance of material from that taxon in the original sample (Gloor et al., 2017). The distinct differences in the relative abundances of taxa observed in our study across pollen counts and metabarcodes, at least in part reflect this sequencing artefact. We addressed this issue by transforming samples prior to beta diversity analysis, and by taking a very conservative approach with respect to interpreting relative abundance results. qPCR methods are more

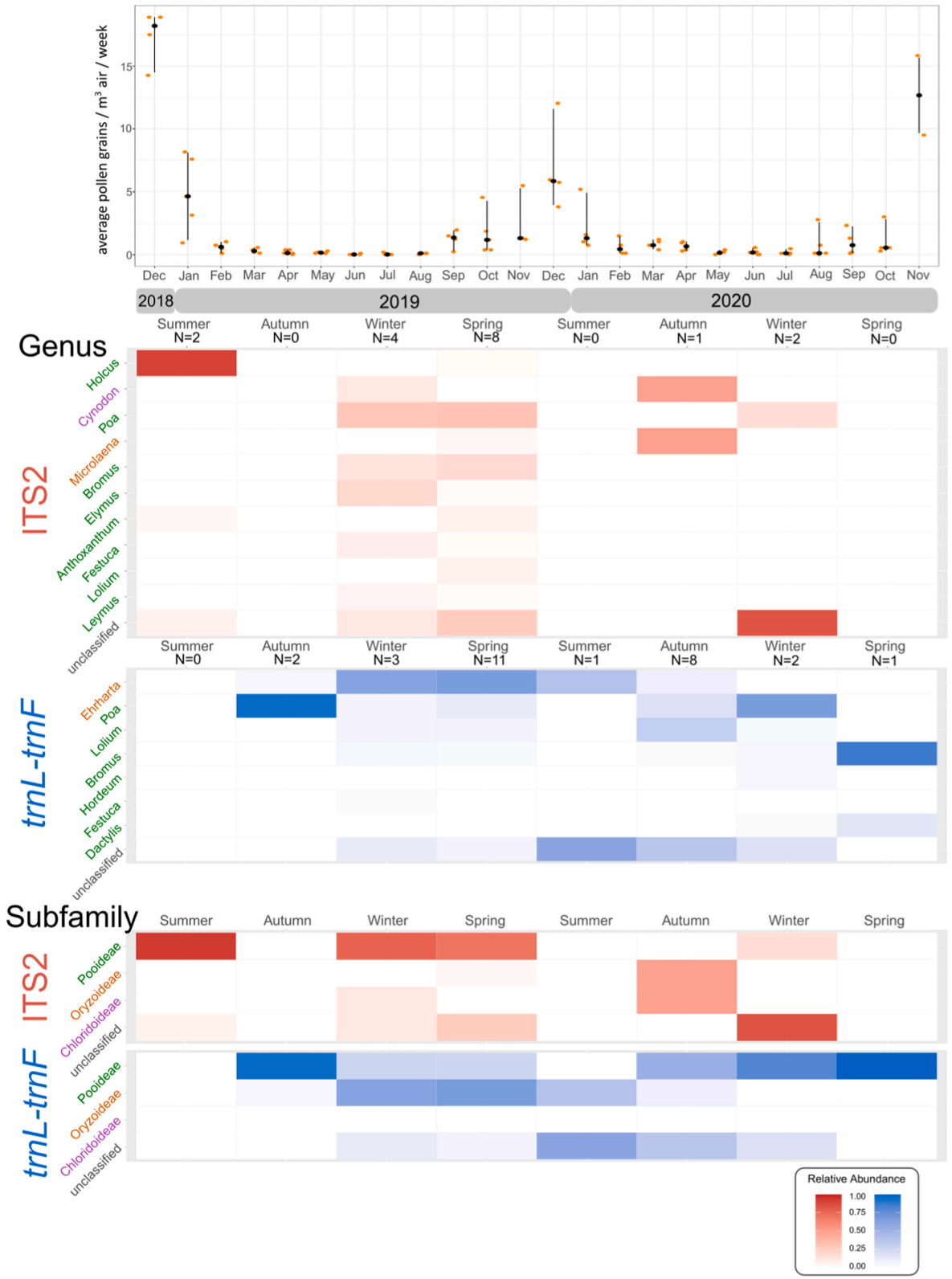

Fig. 4. Genus and subfamily breakdown of Poaceae using relative abundance of ITS2 and *trnL-trnF* metabarcoding in heat maps alongside morphological pollen counts. Morphological pollen counts are grouped by month and displayed as points (in orange), with black lines representing the range of points and black points representing the median point for that month. Pollen counts are measured in grains/cubic metre of air/week. Below, heatmaps of relative abundance of each genus present in at least 1% relative abundance, by season, ranked by descending abundance, by metabarcoding region (ITS2 and *trnL-trnF)*. In this context, relative abundance refers to the proportion of sequence read counts of the total read counts for that season. ASVs that were not classified to genus level within each family are displayed below genera as 'unclassified'. Poaceae genera *Cynodon*, *Tetrarrhena* and *Podagrostis* were all detected with relative abundance less than 1% by *trnL-trnF*. The subfamily level breakdown by relative abundance is below genus. Each genus is coloured the same as its respective subfamily. N = denotes the number of samples that detected Poaceae in each metabarcoding season.

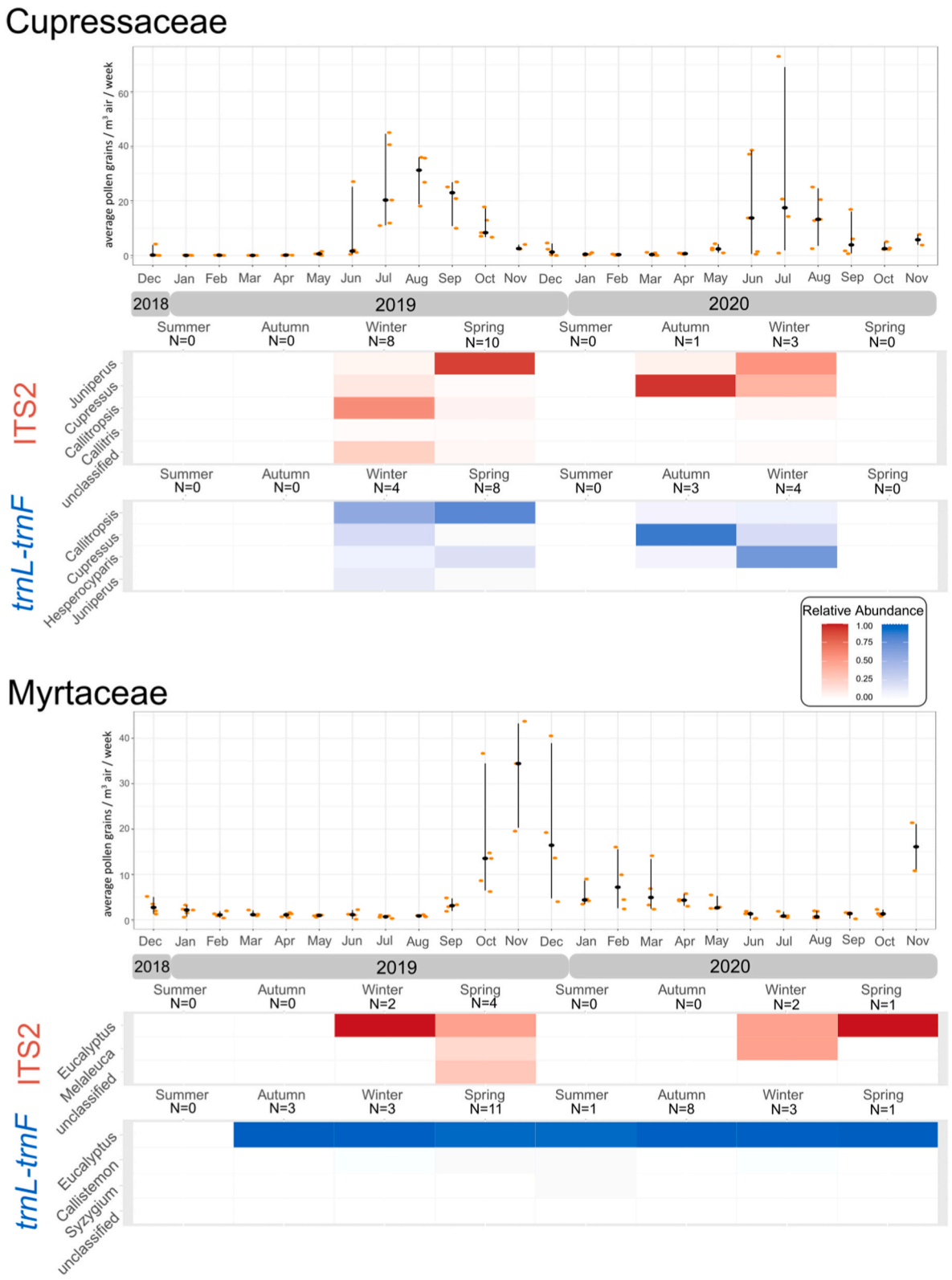

Fig. 5. Genus breakdown of Cupressaceae and Myrtaceae using relative abundance of ITS2 and *trnL-trnF* metabarcoding sequences. Morphological pollen counts are grouped by month and displayed as boxplots of average grains/cubic metre of air/week. Below, heatmaps show relative abundance of each genus present at >1% relative abundance, by season, ranked by descending abundance, by metabarcoding region (ITS2 and *trnL*-trnF). In this context, relative abundance refers to the proportion of sequence read counts of the total read counts for that season. ASVs that were not classified to genus level within each family are displayed as 'unclassified'. Myrtaceae genera *Metrosideros*, *Kunzea*, *Arillastrum* and *Melaleuca* are all detected with relative abundance <1% by *trnL-trnF*. N = indicates the number of weekly samples that detected that family in each season.

appropriate and show clear promise for obtaining robust quantitative insights into the temporal dynamics of selected species or genera (Rowney et al., 2021), noting the time, cost and methodological intensity of qPCR renders it inappropriate for whole of aerobiome surveys such as ours.

It is also important to acknowledge some differences in detection methods between the morphological and eDNA methods. The metabarcodes are based on bulk air samples and include other plant bioaerosols such as trichomes, leaf fragments or seed, while morphological pollen counts are limited to pollen alone. Furthermore, Supplementary Fig. 5, illustrates that there is a 4-h gap for each metabarcoding sample that is not covered by the daily pollen counts, thus, there is a very small window that might lead to some taxa missed in the metabarcoding sample. Despite these differences, our sampling of eDNA and pollen counts was in all other aspects identical, and the strength of correlations between the metabarcodes and pollen counts reinforces the broad comparability of air sampling methods.

4.4. Implications

Our findings provide an enriched perspective on airborne biodiversity which shapes the understandings of allergy in cool temperate Australia and provide clear directions for future research. With respect to pollen allergies, our results of this study identified that the Hobart peak grass pollen season is predominantly temperate grass pollen, but that there may be low level exposure to subtropical grass pollen in autumn and winter. Targeted qPCR studies would be of benefit in further elucidating the temporal dynamics of key grass species and genera, providing capacity to obtain highly granular information on relationships with health. Further research is warranted into the allergenicity of Oryzoideae grasses, including *Ehrharta* and *Microlaena*; although rice, a member of this subfamily, has sometimes been invoked as an allergen (Sen et al., 2003), very little is known about their allergenic potential. Our study suggests that native Cupressaceae taxa such as *Callitris* are unlikely to be important contributors to the allergy burden in Hobart, but reinforces the importance of considering *Eucalyptus* as a potentially important allergen for public health consideration. Overall, our study contributes the first published eDNA investigation into the pollen of the cool temperate Australian bioregion, which complements similar surveys of subtropical and warm temperate Australia. Further studies to increase the spatial granularity and temporal continuity of aerobiome surveys, where possible linked to measures of symptoms frequency and severity, will be important to further deepen our resolution on the aerobiome for both ecological monitoring and public health benefit.

Credit author statement

LJT (Conceptualisation, Methodology, Formal Analysis, Investigation, Writing – original draft, Visualisation), GS (Methodology, Formal Analysis, Investigation, Writing – original draft, Visualisation), JLD (Conceptualisation, Methodology, Resources, Supervision, Writing – review & editing), BJG (Conceptualisation, Writing - review & editing), A. Barberán (Methodology, Data Curation, Supervision, Writing - review & editing), JRM (Methodology, Resources, Writing - review & editing), A. Bissett (Data Curation, Supervision, Writing - review & editing), FHJ (Conceptualisation, Methodology, Funding acquisition, Resources, Supervision, Writing - review & editing), PJJ (Conceptualisation, Methodology, Investigation, Resources, Writing – original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition).

Code availability

The instructions to curate the *trnL-trnF* database via CRABS can be found https://github.com/mrgambero/trn-Ltrn-F_database. The rest of the code used to complete these analysis can be found at: https://github.com/treegart/Hobart-Pollen-Metabarcoding.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Fay H Johnston reports financial support was provided by Tasmania Department of Health. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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