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Airborne Environmental DNA Analysis as a Tool to Monitor Live Bird Trade

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ABSTRACT

The global trade in live birds is extensive, involving both international exports and large-scale domestic trade. Despite international regulations, trade in some CITES-listed species remains largely undocumented and illegal. Songbird markets are difficult to monitor, and conventional surveys are constrained by limited taxonomic expertise and deliberate concealment of illegally traded species. Here, we explore airborne environmental DNA (eDNA) analysis as a novel tool to detect avian species and assess its potential for monitoring bird trade. We collected airborne samples in both enclosed and open market environments, accompanied by visual surveys, and processed them using DNA extraction and metabarcoding to identify avian taxa. In enclosed settings, three of six present species were consistently detected across all sampling days, with the remaining three likely detected but assigned to closely related species, demonstrating high accuracy under controlled conditions. In open markets, eDNA analysis captured approximately 20% of visually observed species with available reference sequences, and detection probability increased with species abundance. A key limitation was the large proportion of amplicon sequence variants (ASVs) that could not be assigned to species, emphasizing the need for expanded reference databases and the use of multiple primers to improve detection. Despite these challenges, our results indicate that airborne eDNA analysis can complement conventional monitoring, particularly in enclosed trade contexts such as cargo containers. While open market applications face methodological constraints, further refinement and integration with conventional surveys could enable scalable, non-invasive monitoring of traded bird biodiversity.

1 | Introduction

Encompassing both organismal and extra-organismal DNA isolated from the environment, Environmental DNA (eDNA) analysis serves as a valuable tool for inferring taxon presence through PCR or sequence capture (Rodriguez-Ezpeleta et al. 2021). As such, eDNA techniques have emerged within a rapidly expanding ecological toolkit, revolutionizing biological monitoring in recent years. The number of publications utilizing eDNA analysis for studying the aquatic environment has shown a significant increase, rising from 4 in 2012 to 124 in 2021 (Takahashi et al. 2023). Airborne macrobial eDNA studies have likewise grown in number, increasing from 8 publications in 2020 to 39 by mid-2024 (Johnson and Barnes 2024). Lynggaard et al. (2022) and Clare et al. (2022) were among the first to successfully collect and detect vertebrate species using

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airborne eDNA in outdoor environments. Building on this foundation, Tournayre et al. (2025) and Polling et al. (2024) further advanced the field by exploring the use of airborne eDNA in biodiversity monitoring through large-scale comparisons with conventional survey data. Motivated by this exciting trend, our study aimed to explore the potential of airborne eDNA analysis as a tool for monitoring wildlife trade, with a specific focus on the live bird trade. This idea was inspired by a previous study that employed eDNA analysis to survey urban fish markets with water samples collected, which successfully revealed a substantial number of species missed and/or misinterpreted by visual surveys (Richards et al. 2022).

Live birds, the most species-rich class in the pet trade, saw around half a million exports yearly in the 2010s (Bush et al. 2014; Chan et al. 2021; CITES 2020). In East and Southeast Asia alone, tens of millions of wild-caught animals are traded domestically each year (Karesh et al. 2007), threatening native wildlife populations. However, the international trade of some CITES-listed species remains undocumented and illegal (Shepherd et al. 2020; Nijman et al. 2018). In countries like Indonesia, where there is high demand for songbirds and rich local biodiversity, domestic bird trade is challenging to trace, making the impact on wild bird populations largely unknown (Harris et al. 2015; Indraswari et al. 2020). To monitor songbird trade, literature commonly suggests continued bird market surveys (Aloysius et al. 2020; Indraswari et al. 2020; Shepherd et al. 2020), but the lack of taxonomic expertise among customs officials poses a significant obstacle. Survey frequency may also decline during public health events, as seen by the lack of bird market surveys across China following the COVID-19 pandemic (Zhang et al. 2025); similar disruptions could occur during avian influenza outbreaks.

Conducting comprehensive bird surveys to understand trade dynamics in the market requires significant expertise to identify the range of bird species on sale from around the world and can be time-consuming to confirm the identity of every individual. Some bird families contain many species that are visually similar and can be difficult for inexperienced observers to separate, especially if plumage is damaged during captivity. In Hong Kong, the Yuen Po Bird Garden is the primary location for the trade of live birds, particularly songbirds, with a substantial volume of bird trade. On average, a single survey in the garden during 2018–2019 records the presence of 1109 individuals (Dingle et al. 2026) and more than 200 species have been observed for sale in the market (Chan 2006; Dingle et al. 2026). Another major challenge for customs officers occurs when endangered bird species are intentionally concealed, or when large numbers of birds are transported in multiple cargo containers, as is often the case in major transport hubs like Hong Kong (ADMCF 2018) and Singapore (Aloysius et al. 2020).

Hence, this study serves as a pioneering effort to achieve two main objectives: (1) demonstrate the use of airborne eDNA analysis to detect avian species in both enclosed and open environments, and (2) discuss the practical challenges, potential applications, and possible solutions for incorporating this method into bird trade monitoring.

2 | Methods

2.1 | Study Site, Sample Collection, and Collection Materials

Airborne eDNA was collected using an Airmetrics MiniVol TAS air sampler, at a flow rate of 5 L/min. To ensure optimal sampling, we utilized the recommended Polyethersulphone (PES) filter membrane (47 mm in diameter, with a pore size of 0.2 μm; Luhung et al. 2015). Strict measures were implemented to prevent contamination during the process of changing and recollecting filter membranes. All equipment used, including gloves, scissors, and forceps, underwent sterilization by washing with 10% bleach, followed by 75% alcohol and exposure to UV light for 15 min. Subsequently, the sterilized equipment was sealed in airtight bags for further use. The collected filter membranes were placed into Whirl-Pak Sterile Sampling Bags (water-airtight) for immediate transportation back to the laboratory. Upon arrival, the samples were stored at −20 degrees Celsius to halt DNA degradation until further DNA extraction.

To test the efficacy of the sampler in an enclosed and controlled environment, we first placed one air sampler in a parrot holding room (50 m^2, Figure S2b in Appendix I) within the zoological facility of Ocean Park Hong Kong (hereafter, "parrot room"). The sampler was operated for 12 h per day from June 1 to 3, 2020. During the sampling period, the parrot room housed 1–2 individuals of each of six species (Table S1 in Appendix I): five identified *Ara* spp. (macaw species) and *Amazona ochrocephala* (yellow-crowned Amazon). The bird species list was directly obtained from Ocean Park Hong Kong staff. To maintain the integrity of the samples, the filter membranes were changed daily. The parrot room is cleaned every morning, with disinfectant (Virkon) that effectively removes DNA from surfaces (Radgen-Morvant et al. 2024).

For the open environment, two air samplers were deployed in Yuen Po Bird Garden (hereafter, "bird market") from January 18th to 20th, 2020, aiming to capture representative airborne conditions within this moderate-sized market. The air samplers were positioned at designated locations around noon on the first day and operated continuously for 8 and 12 h overnight (Figures S1 and S2a in Appendix 1). The filter membranes were also changed daily. The floors and surfaces of the bird market are thoroughly rinsed with bleach daily, before dawn, potentially serving as a reset point for eDNA source material within the market.

2.2 | Visual Bird Survey

To determine the effectiveness of sampling species using the eDNA technique, we compared the number of bird species identified through eDNA analysis with species counted through conventional visual bird surveys. To conduct the visual market surveys, one observer (JAA) conducted a comprehensive visual bird survey on each of the three days of the sample collection. The Yuen Po Bird Garden is an open-air street-like environment covering 3000 m^2 and containing 70 open-fronted shops (Figure 1a,b). Unlike the parrot room,

FIGURE 1 | (a–b) Photographs illustrating how vendors display birds in the market. Photos were taken in October 2025; conditions remained similar to those during the sampling period.

air from the shops at the bird garden is not confined within a fixed volume, being able to circulate freely around the market and into the surrounding environment. About 20 shops within the bird garden sell live birds (the exact number varies as some shops do not sell birds every day), with others selling bird food, cages, or other aviculture equipment and some shops unoccupied. Birds for sale are displayed either at the entrance to the shop or in the roofless paved area in front of the shop, sometimes covered with a tarpaulin to provide shade and shelter from rain. Most birds are kept at the market overnight and locked within the shop.

During each survey, the observer visited every open shop in the market and recorded the number of individuals of each bird species on display. Surveys lasted for 1–1.5 h per visit and were conducted in late morning or early afternoon, between 11:00 and 14:00. Previous surveys have found that most bird shops at the market are open at this time, whereas some may be closed during earlier or later visits. As well as birds for sale at the market, some bird owners bring pet birds to the market for social gatherings in open areas away from the shops. Because DNA from these birds may be shed within the market, any additional birds seen in cages away from the stores were also recorded during the visual surveys and included in the list of species recorded on that day. There remains a possibility that some birds were brought into the market on the day of the sampling but outside the hours covered by the survey, although the number of missing species is likely to be relatively small. Being an open-air environment, the market is accessible to wild birds from the surrounding area. Feral Pigeons *Columba livia* and Eurasian Tree Sparrows *Passer montanus* were observed within the market during our visual surveys, but we did not conduct surveys of the surrounding area for the presence of any other wild birds that might visit the market outside the survey times.

2.3 | DNA Extraction

Filter-based airborne eDNA contains low amounts of particulate matter as well as DNA material. We utilized an extraction method to release material attached to the filter membranes as this was among best practices at the time (Luhung et al. 2015). We used the DNeasy PowerSoil Pro Kit for eDNA extraction with some minor amendments before processing as outlined below to improve the DNA yield.

Once placed into the PowerBead Pro Tube each sample was placed in Sonicator for 30 min at 65 degrees Celsius to facilitate the release of DNA from the filter membrane (Luhung et al. 2015). A TissueLyser was then applied for 5 min to further break the filter membrane through mechanical force and increase DNA yield. After centrifuging samples for 1 min at 15000 g, the supernatant was collected while impurities and beads were discarded. The samples were then processed following the DNeasy PowerSoil Pro Kit for DNA extraction (Detail shown in Appendix II—DNeasy PowerSoil Pro Kit Handbook) with an addition of 5 min incubation time in a 65°C oven before elution to increase DNA yield. A NanoDrop Spectrophotometer (Thermo Scientific 2000) was used to quantify the DNA concentration. Samples were stored at −20°C until amplification after confirming the DNA concentration.

2.4 | Amplification, Library Preparation Sequencing, Taxonomic Assignment and Bioinformatics

Previous studies have suggested that sequence-to-sample misidentifications exist in metabarcoding studies; false tag combinations comprised 2.6% of the resulting sequences in a leech diet

study (Schnell et al. 2015). To detect and account for potential false-positive results, three PCR replicates, using different tag combinations, were conducted for each of the samples as well as an extraction negative control. Only reads found in two out of the three replicates were considered to be valid. There are 12 samples in total: three collected on consecutive days from the parrot room, six from the bird market using two samplers over three days, and three from an outdoor aviary in Ocean Park Hong Kong. The aviary samples were later excluded from the study due to low detection, likely caused by rainfall and misplacement of the air sampler.

A 171 bp of the bird 12S region of mtDNA was targeted using the primers MiBird-U-F/MiBird-U-R (Ushio et al. 2018). PCR was carried out with 40 cycles of a 20 μL reaction volume containing 12 μL sterile distilled H2O, 5 μL 4X AllTaq Master Mix (Qiagen, USA), 0.5 μL of each primer (10 μM), and 2 μL template. The thermal cycle profile after an initial 1 min denaturation at 94°C was as follows: denaturation at 98°C for 10 s; annealing at 63°C for 10 s; and extension at 68°C for 10 s, with a final extension at the same temperature for 7 min. Amplification products were checked by a 1.5% agarose gel. All samples were submitted for sequencing and outputs were analyzed.

PCR products with different tags were pooled into three tubes, each containing one replicate of each of the samples and an extraction negative control. The samples were then submitted to the University of Hong Kong Centre for PanorOmic Sciences, Genomics Core, for 251-bp paired-end sequencing on the Illumina MiSeq platform. Library preparation was performed based on the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina). Purified amplicons with partial Nextera adapter sequences were used to generate libraries for validation and quality control using Qubit and qPCR.

To ensure data quality and minimize false positives, we included both PCR negative controls and extraction controls in our eDNA processing. No amplification bands were observed in the PCR negative controls, indicating no contamination during PCR. However, faint bands were detected in the extraction control, suggesting low-level contamination during the DNA extraction process. Consequently, we excluded two Avian ASVs detected in the extraction controls, *Columba livia* and *Agapornis roseicollis*, from the final eDNA species list to avoid potential false positives. No additional unassigned avian ASVs were present.

2.5 | Data Analysis

The resulting NGS data (Chung et al. 2026) were processed through a pipeline designed for tagged and pooled sample replicates: Briefly, AdapterRemovalv2 (Schubert et al. 2016) and Sickle (Joshi and Fass 2011) were used for adapter trimming and removing low-quality reads, respectively. Spades was used for error correction and PandaSeq for read merging (Masella et al. 2012; Prjibelski et al. 2020). The resulting merged reads were then processed through Begum to remove those reads not present in at least 2 of the 3 PCR replicate pools and keep only those with a minimal occurrence of 5 (Yang et al. 2021). The filtered reads were clustered using SumaClust (−*t* 0.98 −*r* 0.85) (Mercier et al. 2013). The resulting ASVs were compared to the best matches in the NCBI GenBank database using the BLAST algorithm. ASVs were considered identified to species level if they showed a 100% match to a record.

Since a high number of ASVs in the bird market samples were taxonomically unidentified, we performed the following analysis to assess their phylogenetic relatedness. For each ASV, we downloaded the top two best matches based on Bit-Score. We also searched the NCBI Genbank database for the targeted 12S loci of all bird species identified in the visual survey. If no representative sequence for the species was available, we included available representatives of the genus. All sequences were then aligned using CLUSTAL Omega and trimmed down to the target region. A Neighbor-Joining phylogenetic tree was constructed using the Geneious Prime Tree Builder with a Tamura-Nei distance model and no outgroup. The resulting tree was imported into iTOL for further annotations.

To quantify temporal consistency in species detection for each method, we calculated the Jaccard similarity index between all pairs of sampling days, which captures the proportion of shared species relative to the total observed. To assess whether abundance (number of individuals of each species) relates to detection of that species using eDNA analysis, we fitted a series of binomial generalized linear models (GLMs) using a dataset of species presence across three consecutive days (See *R* script in Appendix III). The response variable was binary eDNA detection (0 = not detected, 1 = detected). The primary explanatory variable was species abundance, which we tested in both raw and log-transformed forms. To examine potential variation in detection across days, we also included sampling day as a categorical fixed effect in the log-transformed model, with Day 1 set as the reference level. Only species-level entries are included. All models were implemented using base *R*'s glm() function with a logit link and binomial error structure. Model fit was compared using AIC and McFadden's pseudo-R^2.

3 | Results

3.1 | Identification Accuracy Within an Enclosed Area

Across all three sampling days, three out of the six bird species present were consistently and accurately detected using the eDNA method, all of which belonged to the genus *Ara*. Three species known to be present in the enclosure were not confidently detected. The first and second cases involved *Ara ambiguus* (great green macaw) and *Ara macao* (scarlet macaw), which were the only *Ara* species present that did not appear in the BLAST results. However, one ASV matching *Ara macao* with 99.40% sequence identity (GenBank Accession: NC_045076) was detected, differing by only a single base pair. On the other hand, an ASV matching *Ara militaris* (military macaw) with 100% sequence identity (GenBank Accession: NC_027839) was detected, despite *A. militaris* not being present in the room. A comparison of published 12S gene sequences shows that *A. ambiguus* and *A. militaris* differ by only a single base pair in the targeted region, suggesting that this detection may reflect a misassignment of *A. ambiguus* due to

limited taxonomic resolution in the marker. The third case involved *Amazona ochrocephala*, which was also not directly detected. One ASV, however, showed 99.40% pairwise identity with *Amazona barbadensis* (yellow-shouldered amazon) which clustered closely with *A. ochrocephala* from Colombia and Venezuela (Urantówka et al. 2014), again suggesting a likely misclassification. The three true positives and the three suspected matches listed above were observed across all three days. The remaining ASVs assigned to Aves could not be identified to species level ($n = 10$), all with GenBank identity scores $< 97.20\%$.

3.2 | Identification Accuracy Within the Open Market

A total of 124 bird species were recorded through visual surveys, with 5 individuals identified only to genus level (see visual_tax.xlsx, Appendix III). Of the 124 species, 70.97% belonged to the order Passeriformes (songbirds) and 27.42% to Psittaciformes (parrots). In building a reference genetic database, we found that 38 of those lacked sequence data for the region targeted by the MiBird primer on NCBI on species level, and 4 species lacked any sequences on a genus level.

A total of 53 ASVs were recorded using eDNA analysis, which, in a naïve numerical comparison, corresponds to 42.74% of the number of species recorded by visual survey. 22 ASVs were identified to species level with a 100% match to NCBI reference sequences (Figure 2; see Appendix IV for the complete list), while the remaining ASVs showed sequence similarity ranging from 87.00% to 99.40%. Taxonomically unidentified ASVs were included in the Neighbor-Joining phylogenetic tree to illustrate their relatedness (Figure 3). While most were scattered across different families, 10 ASVs clustered within Columbidae. 17 bird species were detected by both visual and eDNA surveys, representing 19.76% of the visually observed species with available reference sequences. However, without estimates of detection probability for either method, the true recovery of species present in the market by eDNA analysis cannot be quantitatively constrained. Note that ASV31 was counted as detected by both methods because it matched *Agapornis lilianae* (Lillian's lovebird) which was observed in the visual survey. However, the exact sequence also matches *Agapornis pullarius* (red-headed lovebird), a species not recorded in the visual survey.

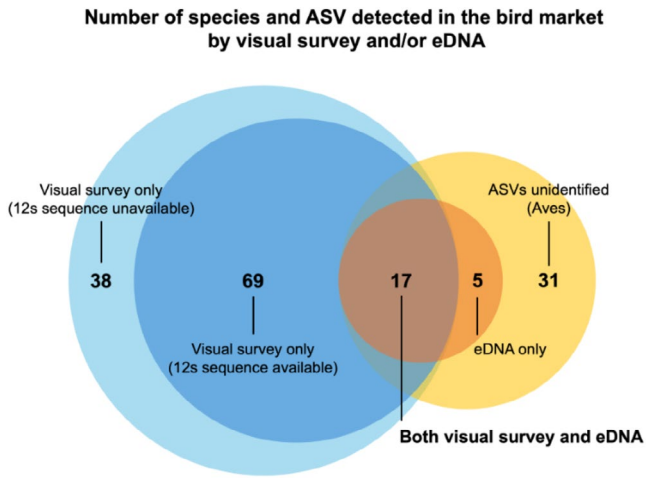

FIGURE 2 | The Venn diagram illustrates the overall number of species and ASVs detected in the bird market through visual survey and/or eDNA analysis over the course of three sampling days. The blue color represents species identified through the visual survey, with the lighter shade indicating species for which sequence region targeted by the MiBird primer is unavailable. The orange color represents the species/ASVs identified through eDNA analysis, with the lighter shade indicating ASVs that cannot be specifically identified to a species but belong to the Aves class.

Five bird species were exclusively captured through eDNA analysis and not observed during the visual survey. Two of these species are not typically associated with the live bird trade, but are commonly found in Hong Kong. One of the species was *Streptopelia chinensis* (spotted dove), which is a common wild bird found in Hong Kong and often observed in the market environment (JAA and CD personal observation). The second species was *Gallus gallus* (domestic chicken) commonly sold as a food product for consumption. The other species included three parrot species: *Pionus chalcopterus*, *Cacatua moluccensis*, and *Ara severus*. While *Cacatua moluccensis* is known to be sold or kept as pets at the market, *Pionus chalcopterus* and *Ara severus* have not been directly observed there (JAA and CD personal observation). However, other species within the genera *Pionus* and *Ara* are regularly traded, and thus these ASVs may correspond to closely related or hybrid species.

Three endangered species listed under CITES were recorded through visual surveys: *Cacatua alba* and *Lonchura oryzivora* (both CITES Appendix II), and *Psittacus erithacus* (CITES Appendix I). Of these, only *Cacatua alba* and *Lonchura oryzivora* were detected using eDNA analysis, while *Psittacus erithacus* was not. Including the aforementioned CITES Appendix II species, a total of 34 CITES-listed species were observed visually, of which 5 were detected by eDNA analysis.

3.3 | Probability and Consistency of Detection

Consistency of detection was higher for visual surveys (mean within-method Jaccard similarity = 0.83) than for eDNA analysis (0.33). The sample size was insufficient to conduct a permutation test to assess whether temporal similarity differed significantly between methods. Nevertheless, the observed difference in mean Jaccard similarity (0.50) is very large on a 0–1 scale. Using Day 3 as an example, only 3 of the 119 species detected by visual survey were unique to that day, whereas half of the 38 species detected by eDNA on Day 3 were unique.

The abundance of individuals of a given species present in the market was positively associated with eDNA detection across all models. In the model with raw abundance, each additional individual increased the log-odds of detection by 0.01 ($z = 3.98$, $p < 0.001$), with a pseudo-R^2 of 0.074 and AIC of 258.83. The log-transformed model provided a better fit, with a pseudo-R^2 of 0.098 and AIC of 252.35. This model indicated that a tenfold increase in abundance was associated with an increase of log-odds of detection by 0.58 ($z = 5.18$, $p < 0.001$). The result suggests

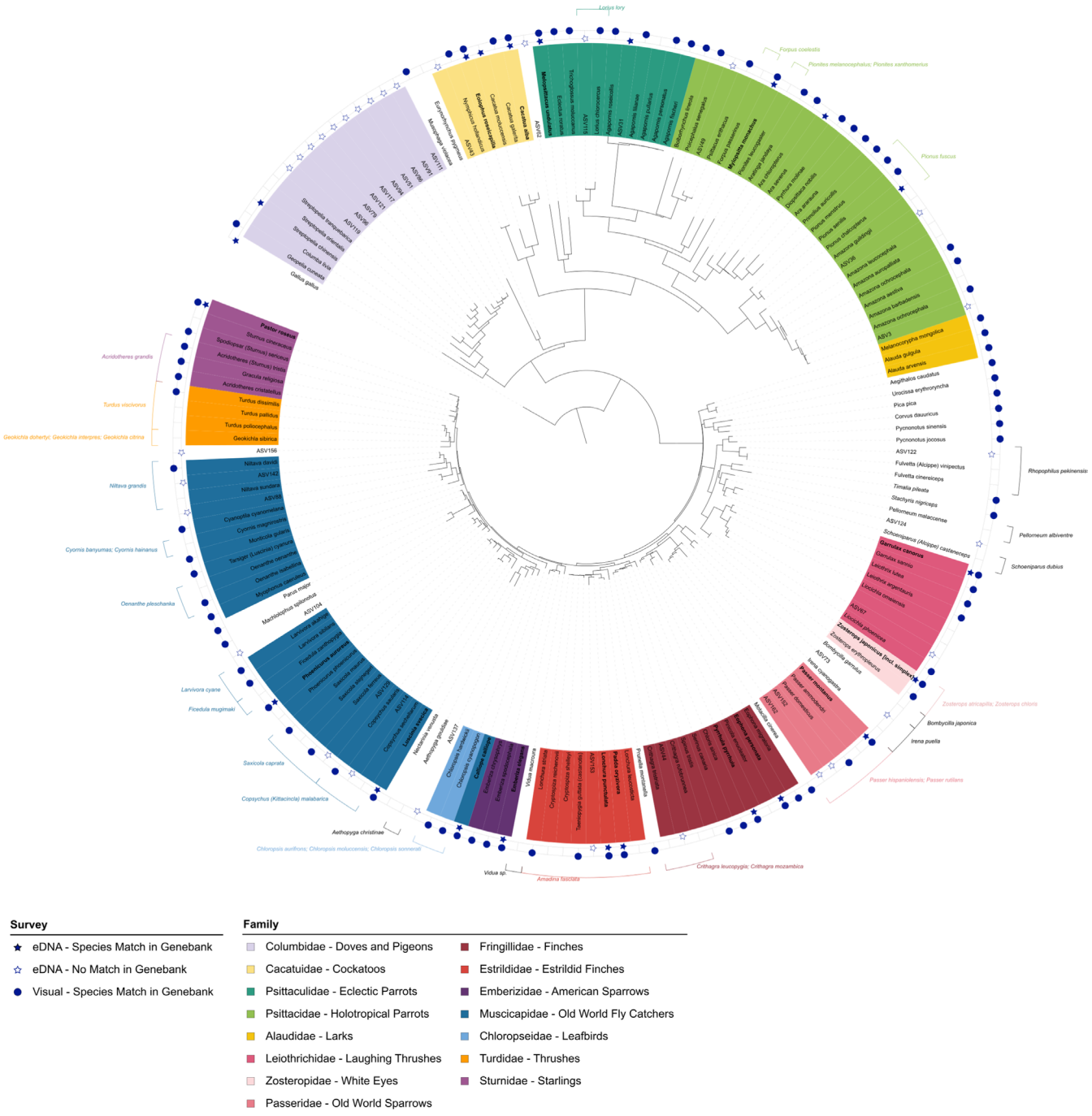

FIGURE 3 | Phylogenetic tree of ASVs and sequences of species detected in the visual survey, obtained from GenBank. Filled circles indicate species observed in the visual survey and with available sequence data. Species names listed outside the tree represent species detected visually but lacking sequence data for the targeted region. Stars represent species detected by eDNA analysis: Filled stars indicate sequence data are available; empty stars indicate no sequence is available. Duplicated species names indicate the presence of sequence variation within the targeted region. *Brachypteryx leucophris*, *Myiomela leucura*, *Polytelis swainsonii*, and *Psephotus haematonotus* were observed visually but had no available sequences for their respective genera and are therefore not shown in the figure. ASV31 is shown as a filled star because it matches both *Agapornis lilianae* and *Agapornis pullarius*.

a saturating relationship, where increased abundance boosts detection probability non-linearly, but with diminishing effect (Figure 4).

Adding sampling day as a fixed effect to the log-transformed model had little impact on the overall fit (AIC = 256.35; pseudo-$R^2 = 0.098$). Compared to Day 1, the odds of detection were about 0.035 higher on Day 2 and 0.026 higher on Day 3, but these differences were small and not statistically significant ($p > 0.9$). This suggests that detection patterns were consistent across days, and that the strong effect of abundance on eDNA detection holds regardless of sampling day.

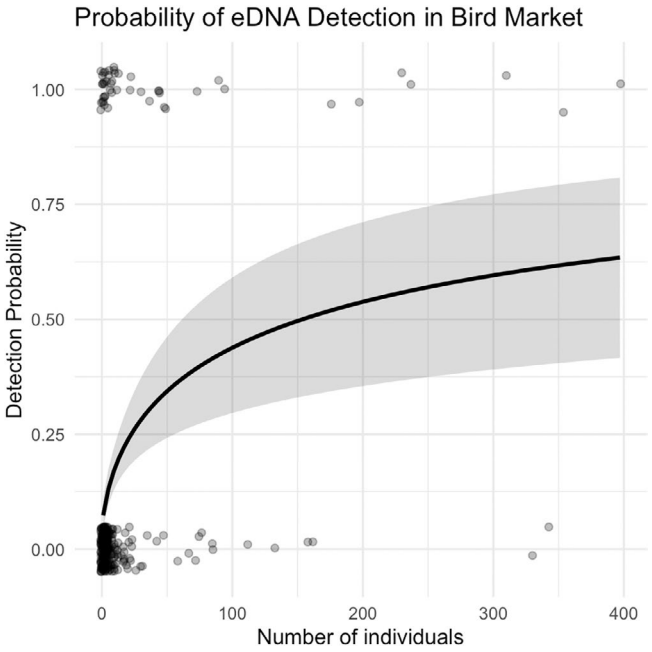

FIGURE 4 | Relationship between species abundance (number of individuals) and the probability of eDNA detection. Points represent individual samples jittered to show distribution. The line shows the predicted detection probability from the log-transformed abundance logistic regression model, with the shaded ribbon indicating the 95% confidence interval around the prediction. Detection probability increases with abundance, reflecting higher likelihood of detecting eDNA as more individuals are present.

4 | Discussion

This study demonstrates the potential of air samplers to help monitor wildlife trade through the detection of avian species in both enclosed and open environments. Under controlled, enclosed conditions, four of six species were consistently detected across all sampling days. In open market settings, 19.76% of visually observed species with reference sequences were identified through eDNA analysis, and detection likelihood rose with increasing abundance of that species in the market. In the following discussion, we explore the limitations and challenges in detail and suggest practical strategies to improve the reliability and resolution of airborne eDNA monitoring in avian trade.

4.1 | Factors Influencing the eDNA Capture

While many factors can influence the effectiveness of airborne eDNA capture, our study highlights two particularly important ones: the spatial configuration of the environment and the biomass or number of animals present. In our study, the differences in air sampler-to-area ratio and avian species composition between the two sampling settings limited our ability to statistically compare eDNA monitoring effectiveness across them. Still, the enclosed parrot room consistently showed higher eDNA recovery across all three sampling days.

Spatial configuration played a critical role in this outcome. In contrast to the challenges faced in the larger bird market, the placement of the air sampler in the parrot room was more advantageous. The sampler was positioned at the center of the room, allowing it to face all the cages where parrots were housed. The room was enclosed and not heavily ventilated, which are conditions that likely promoted the accumulation of airborne DNA. The relatively small size of the parrot room also meant that the distance between the air sampler and the birds was short, further facilitating efficient DNA capture. These structural factors likely contributed to the improved performance of eDNA monitoring in the parrot room. In contrast, the bird market's complex structure posed limitations. The market's long and narrow layout prevents the placement of a single air sampler that can simultaneously capture airborne material from all stores. Although the stores are generally open fronted with birds on display, they are physically separated by walls, and some cages are partially covered with cloth or other materials. Air does not circulate in a centralized manner, and unlike the previous study in a wet market (Richards et al. 2022), where water samples could be collected from predictable drainage convergence points, airborne eDNA lacks such centralized accumulation. These spatial constraints likely contribute to the uneven distribution and patchy detection of eDNA. The fact that it is open to the environment outside the market can also impact the air flow and accumulation of eDNA, affecting the detection of species.

The second critical factor is biomass or abundance. Lynggaard et al. (2022) revealed an increased probability of detecting airborne eDNA from animals with higher biomass and at shorter distances from the sampler. Parrots are medium to large-sized birds, and their proximity to the air sampler in the parrot room likely enhanced DNA recovery. Additionally, despite spatial limitations in the bird market, our results still showed a positive relationship between detection likelihood and the number of individuals present, suggesting that abundance remains an important factor in shaping airborne eDNA signals.

There are additional environmental, technical and biological factors that can shape airborne eDNA detection, especially in outdoor settings, which were not evaluated in this study. For instance, weather conditions such as wind and rainfall can disperse or dilute airborne particles, potentially reducing detection rates (Ke et al. 2025). It was shown that in aquatic settings, high ambient temperatures can also accelerate the natural degradation of eDNA, shortening the window of detectability (Yu et al. 2022). Moreover, the type and performance of air samplers can influence results, as shown by recent findings (Polling et al. 2024) indicating that different devices vary in their efficiency of capturing airborne biological material. Biologically, airborne eDNA detection might be higher for certain birds or under specific conditions. For example, in species with powder down feathers such as pigeons and parrots, during molt, or in birds with feather damage or plucking. These factors could increase the release of feather particles and other biological material into the air.

These considerations highlight the complexity of airborne eDNA monitoring and the need for further research to determine optimal sampling conditions for outdoor settings such as bird markets. Nonetheless, our findings demonstrate that applying airborne eDNA methods in enclosed, indoor settings where

spatial structure and proximity to animals are more controlled is a promising approach for species detection.

4.2 | Limitations of Study Design

We acknowledge two important limitations of this study, the first being the absence of field negative controls. Consequently, despite thorough bleaching of the apparatus and sampler and the inclusion of extraction and PCR controls, our study design was not fully protected against false positives, as potential contamination arising during field sampling and handling was not explicitly controlled. Johnson and Barnes (2024) provide a useful analogy for the nature of airborne eDNA sampling, describing it as "pipetting liquids underwater," which highlights the inherent difficulty of defining an appropriate field negative for airborne eDNA studies. This study was designed and conducted in 2020, before the emergence of the first outdoor macrobial airborne eDNA studies, at a time when methodological references were limited. Recent studies have proposed various approaches, including the use of sterilized, sealed, unused filters transported to and from the field to assess background airborne contamination before and after sampling (Johnson and Barnes 2024), as well as the inclusion of spare sampler components present at the sampling location (Polling et al. 2024). Notably, these practices could vary depending on sampler type, and field negative controls are not uniformly implemented across recent airborne eDNA studies (Appendix V). Whether field negatives are strictly required, and if so, whether they should be exposed to ambient air at the sampling location, remains an open methodological question that requires further evaluation in future eDNA review and methodological studies.

The second limitation of this study is limited eDNA sampling in the bird market, both in terms of the total volume of air sampled and the number of sampling days. This limitation is reflected in the low overlap between airborne eDNA detections and visual surveys, as well as in the limited consistency of detections across eDNA sampling days. Such variability may reflect the patchy and transient nature of airborne eDNA, as previously reported in field studies where all 71 pairs of replicate air samples did not recover identical community compositions (Lynggaard et al. 2024). However, the total air volume sampled, 18 m^3 (6 m^3 per day for three days), lies at the lower end of the range reported in the airborne eDNA literature. Although a comprehensive methodological meta-analysis is beyond the scope of this study, comparison across seven selected studies (Appendix V) indicates substantial variation in sampling volume, duration, and area. This variation is associated with sampler choice, which broadly ranges from factory-manufactured volumetric samplers to custom-built fan-based samplers. Studies using custom samplers frequently report higher calculated air volumes (e.g., Garrett et al. 2023; Lynggaard et al. 2022; Lynggaard et al. 2024), in some cases reaching several hundred cubic metres (e.g., ~240 m^3 per sample for a 24 V sampler in Lynggaard et al. 2022). However, the extent to which fan-derived airflow and volume estimates are comparable to those obtained using factory-manufactured samplers with known calibration remains to be assessed. Importantly, the lower cost of custom-made samplers enables increased replication, which in turn facilitates methodological comparisons. The studies using custom-built samplers have reported no significant differences in DNA concentration, read counts, or detected biodiversity among different sampler designs (Garrett et al. 2023; Lynggaard et al. 2022), although higher airflow has been associated with increased overlap between technical replicates, suggesting improved consistency (Lynggaard et al. 2024). Given the differing principles of airflow measurement, filter characteristics, and flow regulation between custom-built samplers and factory-manufactured volumetric machines, direct quantitative comparisons of sampling effort across sampler types remain challenging.

In the present study, the bird market was likely under-sampled by eDNA analysis, but a considerable proportion of the community was recovered, with naïve ASV richness corresponding to 42.74% of that detected by visual surveys. The detection of multiple taxa under these constrained conditions demonstrates the potential of airborne eDNA as a complementary tool for biodiversity assessment. Increasing the number of samplers would likely improve spatial coverage; however, financial limitations may necessitate a shift toward custom-built sampling devices in future studies. More broadly, the wide range of reported air volumes and spatial sampling designs across studies highlights the need for clearer and more standardized reporting of airflow rates, total sampled volume, and spatial sampling context to support standardization of sampling effort and cross-study comparisons.

4.3 | Choice of Primers

Approximately 20% of the species visually identified in the market with reference sequences were detected with eDNA analysis, and eDNA analysis detected some additional species that visual surveys missed. For example, three parrots were detected using eDNA analysis only. If these species were being sold in the market at the time, they most likely would have been detected by the visual surveys as these are easily identifiable species. However, the sampling period coincided with the weekend when parrot owners often gather in the park for socializing, and whether all the birds were displayed by shop owners is also unclear. Therefore, it is plausible that the eDNA detected accurately represents the presence of these species, although the residence time of airborne eDNA is also unknown and some signals may reflect birds that were present earlier rather than during sampling. A substantial proportion (80.24%) of visually recorded species went undetected in the eDNA samples.

One potential explanation for the high non-detection rate is the limited availability of reference sequences for the 12S rRNA region. We noticed this issue during the process of collecting 12S sequences to construct phylogenetic trees for the ambiguous BLAST results. In our study, 59.26% of the ASVs detected in the bird market could not be identified to the species level. This is partly because other genetic markers, such as the NADH dehydrogenase subunit 2 (ND2) gene and the mitochondrial cytochrome oxidase subunit 1 (COI) gene, have been more widely used in avian genetic research (Eberhard and Bermingham 2004; Joseph et al. 2014; Lijtmaer et al. 2012; Luttrell et al. 2020; Sorenson 2003). A comparison of available

sequences for species on our visual survey list highlights this disparity: 25.00% lacked any 12S sequence data, compared to just 8.06% for COI, 5.65% for cytochrome b (cytb), and 2.42% for ND2 (based on a GenBank search using the R package "rentrez" on 26 May 2025). We remain optimistic about the use of 12S primers, as the same search conducted on 15 August 2023 showed that 48.39% of species on the visual survey list lacked 12S sequences, suggesting that the avian gene reference library is expanding rapidly. This issue also highlights the importance of shifting toward whole mitochondrial genome sequencing, which can help mitigate primer-associated biases and reduce dependence on single-marker reference completeness.

The availability of reference sequences is likely to be particularly limited for Asian and African species, which is relevant because most species sold in the market are from these regions (Davies et al. 2026; Dingle et al. 2026). Global analyses of avian diversity reveal pronounced hemispheric differences in endemism, with tropical regions in the Southern Hemisphere and parts of Asia harboring high levels of unique species (Dehling and Chown 2025). However, these regions are typically underrepresented in reference databases, where most reference specimens originate from the Global North, as shown in studies on plant barcodes and marine biota (e.g., Kartzinel et al. 2025; Marques et al. 2021). Given the high diversity, endemism, and vulnerability of these species to trade, we strongly encourage research programs in these regions to address and reduce potential biases in genetic reference databases.

Nevertheless, even with an expanded library, a third challenge remains: insufficient inter-specific variation in the target region, which may limit the ability of the primer to discriminate among closely related species. The primer used in this study was the MiBird primer, which targets the mitochondrial 12S region. 12S primers are the most commonly used in eDNA studies to date for detecting avian species (Ke et al. 2025). The MiBird primer was also robustly tested in silico with a database of 410 species, including both avian samples and eDNA samples from a pond (Ushio et al. 2018). However, for certain bird genera investigated in this study, the region targeted by the MiBird primers exhibited insufficient inter-specific differences. This includes Psittaciformes (the two *Ara* species and three *Agapornis* species mentioned earlier) and Passeriformes (African finches in the genus *Crithagra* and bullfinches in the genus *Pyrrhula*).

The pitfalls related to the target regions were briefly addressed in Ushio et al. (2018), who recommended further validation of the MiBird primers. Our study supports this view: while ample avian eDNA can be detected through air sampling, relying solely on the MiBird primer may be insufficient for studies aiming to detect a wide range of avian taxa. Although 12S remains a popular choice for avian eDNA analysis, we highly recommend the use of complementary primers. Polling et al. (2024) provide a strong example by combining the 16S '16Smam' primers (Taylor 1996), originally designed for mammals, with cytochrome b (cytb) primers L15411F and H15546R (Galan et al. 2012), originally developed for rodents, to detect 106 bird species. We are optimistic that the accuracy and effectiveness of airborne avian eDNA analyses can be improved by selecting more appropriate target regions and incorporating multiple primer sets.

5 | Conclusion

In conclusion, the use of eDNA monitoring has demonstrated its effectiveness, particularly in enclosed conditions such as the parrot room. This method shows great potential as a tool for monitoring bird trade, especially when a large number of birds are transported using cargo containers. However, it is important to acknowledge that eDNA monitoring is not free of biases and requires further standardization in the future. This is especially crucial when considering the challenges associated with measuring the volume of air compared to the volume of water in aquatic eDNA studies.

Author Contributions

Haze W.Y. Chung and John L. Richards conceived the original idea, which was further developed with input from all co-authors. Haze W.Y. Chung and John L. Richards collected and extracted the eDNA data, and John A. Allcock conducted the visual survey. Data analyses were performed by Haze W.Y. Chung, John L. Richards, and Shelby E. McIlroy. Haze W.Y. Chung led the writing of the manuscript, with methodological sections contributed by John L. Richards and Shelby E. McIlroy. Caroline Dingle and John A. Allcock provided expertise on bird species and the bird trade. All authors contributed critically to the revisions and approved the final manuscript for publication.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in Zenado at https://doi.org/10.5281/zenodo.17225114.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** List of bird species in the parrot room. **Figure S1:** Satellite map of Yuen Po Street Bird Garden. **Figure S2:** Placement of air samplers in the bird market and the parrot room. **Appendix II** DNeasy PowerSoil Pro Kit Handbook. **Appendix III** R script for the GLM of abundance and detection, and Excel files of the visual survey species list by day and taxa, with the latter indicating the availability of each species for each sequencing region (COI, CytB, ND2, 12S). **Appendix IV** BLASTN results of the ASVs, alongside reasons for excluding entries. **Appendix V** Comparison of air eDNA sampling methods for vertebrates across seven selected studies (e. g., flow rate, total air volume).