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Omnivorous Diets of Sympatric Duck Species in a Subtropical East Asia Wetland Unveiled by Multi-Marker DNA Metabarcoding

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ABSTRACT

The East Asian–Australasian Flyway (EAAF) is experiencing notable population declines in its migratory waterbird species. Understanding the foraging ecology of these waterbirds, including ducks, is crucial for monitoring and safeguarding their food sources and wetland habitats. Here, we used a DNA metabarcoding approach to analyze fecal DNA from duck species to elucidate their dietary composition during the wintering period in a subtropical East Asian wetland. By employing multiple markers (*18S*, *COI*, and *trnL*) targeting different taxonomic groups and levels, we offered a comprehensive dietary analysis for omnivores that consume both plants and animals. We revealed the dietary compositions of common migratory duck species and their intraspecific and interspecific dietary variations. While ducks are generally known to be omnivorous, *Anas crecca* (green-winged teal) had a more specialized diet and was primarily herbivorous throughout winter. In contrast, the sympatric *Mareca penelope* (Eurasian wigeon) and *Spatula clypeata* (northern shoveler) exhibited more omnivorous foraging behaviors. Moreover, *A. crecca* displayed less dietary variation among samples, while samples of *M. penelope* and *S. clypeata* were highly variable in their compositions. Comparing our results with those of studies conducted in different regions, we found that the dietary compositions of these duck species varied to different degrees across geographic locations. This variation underscores the flexibility of these duck species in their diets and their adaptable foraging strategies. Our findings also indicate that grasslands rich in herbaceous plants and aquatic environments abundant with small aquatic invertebrates are vital foraging habitats for duck species during their winter period.

1 | Introduction

Ducks are a diverse group of waterfowl that belong to the family Anatidae, which also includes geese and swans. They are not a monophyletic group and are divided into several subfamilies based on their genetic and physical characteristics (Johnson and Sorenson 1999). These mostly aquatic birds inhabit both freshwater and saltwater environments, and some duck species, particularly those breeding in the temperate and Arctic regions of the Northern Hemisphere, are known for their long-distance migrations (Arzel et al. 2006). In contrast, ducks living in tropical areas tend to be nonmigratory.

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Migratory ducks follow through "flyways" annually, with the East Asian–Australasian Flyway (EAAF) being one of the most notable, spanning 22 countries and territories. Waterbirds that use the EAAF predominantly breed in far eastern Russia, Alaska, the Mongolian Plateau, and the Amur River basin, and winter in East Asia, Southeast Asia, Australia, and New Zealand (EAAFP 2024). During migration, waterbirds depend on highly productive wetlands to rest and feed, building up sufficient energy to fuel the next phase of their journey. Ducks, in particular, consume a variety of plants and animals found in wetlands, including grasses, aquatic plants, crustaceans, fish, insects, small amphibians, mollusks, and many other invertebrates (Hitchcock Jr. et al. 2021). The abundance, quality, and diversity of these food resources provided by wetlands directly impact the reproductive success and survival of duck species (Holopainen et al. 2015). However, wetlands are increasingly threatened by global changes, and migratory birds in the EAAF are among the world's most vulnerable to these pressures, given Asia's large population and booming economies. The EAAF is experiencing significant declines in its migratory waterbirds populations (Zhang et al. 2023). Addressing this issue requires a better understanding of the foraging ecology of ducks and other waterbirds, which is essential for better monitoring and protecting their wetland habitats for future generations.

Over the last century, considerable effort has been devoted to studying the diets of common duck species in North America and the Western Palearctic (Dessborn et al. 2011; Callicutt et al. 2011). Notably, extensive studies have been carried out on the diets of several duck species, such as *Anas acuta*, *Mareca penelope*, *Anas platyrhynchos*, and *Anas crecca* in the Western Palearctic. However, specific knowledge gaps exist in these studies, with geographical and temporal biases identified as key areas of concern (Dessborn et al. 2011). In addition, compared to North America and the Western Palearctic, very few dietary studies on ducks have been conducted in the regions within EAAF. Only China, Japan, South Korea, New Zealand, and Pakistan have conducted such studies, which cover various species, including *Anas* (*A. acuta*, *A. crecca*, *A. chlorotis*, *A. platyrhynchos*, and *A. zonorhyncha*), *Spatula* (*S. querquedula* and *S. clypeata*), *Mareca* (*M. strepera*, *M. penelope*, and *M. falcata*), *Aythya* (*A. fuligula*, *A. ferina*, and *A. nyroca*), and *Hymenolaimus* (*H. malacorhynchos*) (Ando et al. 2023; Shin et al. 2016; Collier 1991; Raza et al. 2023; Luo et al. 2024). Studies have shown that food compositions consumed by the same duck species using different flyways varied considerably in relation to the availability of food resources in different parts of their migratory range (Dessborn et al. 2011). Moreover, the dietary variation among conspecifics within the same foraging ground is not known. Therefore, it is crucial to conduct more dietary studies on ducks at important breeding and non-breeding locations along the EAAF. By doing so, we can better understand the food requirements during different life cycle stages of migratory ducks at both species and individual levels and provide valuable data to protect their critical food sources and habitats.

Hong Kong, a highly developed coastal city in the central part of the EAAF, provides essential wetland habitats for migratory waterbirds (Huang et al. 2021). The northwestern region of Hong Kong is a wetland complex that includes natural, seminatural, and artificial habitats. One vital area of this region is the Mai Po Inner Deep Bay Ramsar Site, which covers approximately 1,500 ha (Huang et al. 2022). Ducks are among the most abundant waterbird groups wintering in the Deep Bay area, with at least thirty species recorded at the site. These include *S. clypeata*, *A. crecca*, *M. penelope*, and *A. acuta*, all of which represent more than 0.25% of the EAAF population (WWFHK 2024). These four duck species are widely distributed across both the Old and New Worlds, except for *M. penelope*, which is primarily found in the Palearctic range (Kulikova et al. 2019). All these species forage by water dabbling. *Spatula clypeata* is particularly notable for its specialized spatulate bill, uniquely adapted for filtering tiny organisms from the water (Kooloos et al. 1989). However, other species may occasionally engage in similar feeding behaviors. *Mareca penelope* sometimes grazes on aquatic vegetation or, like *A. crecca* or *A. acuta*, tips forward to reach submerged food sources (Ramírez-Albores et al. 2021). Although *S. clypeata*, *M. penelope*, *A. crecca*, and *A. acuta* are classified as "Least Concern" in the International Union for Conservation of Nature (IUCN) Red List (IUCN 2025), some of their wintering populations have exhibited an obvious decline. For example, *M. penelope*, *A. crecca*, and *A. acuta* populations declined significantly between 1998 and 2017 (Sung et al. 2021). Their populations are predicted to decline further in the future due to various threats to these duck species, such as wetland habitat loss, avian diseases, and hunting activities (Madsen and Fox 1995; Duan et al. 2021; Patil et al. 2021). Despite the presence of hydrological management in the water ponds in the Ramsar Site (WWFHK 2023), there is a lack of even fundamental information on food utilization within the managed habitat.

In the past, the primary method used to study the diets of ducks worldwide was through sacrificing them to collect the contents of their esophagus/proventriculus, gizzard, or gut for microscopic examination (Miller et al. 2009; Jamieson et al. 2001). These studies have revealed that ducks are omnivores and consume animal and plant matter (Barboza and Jorde 2001). However, this method is not suitable for species of conservation concern. Direct examination of digested contents also poses many challenges. Firstly, variations in digestion rates among different food types often result in reduced taxonomic resolution of the digested material, increasing the likelihood of bias and errors. Accurate identification requires a high level of taxonomic expertise (Nielsen et al. 2018). Secondly, the most common conventional method for quantifying food items in dietary studies is using the frequency of occurrence and counting the number of items of different taxa. However, relying solely on food item counts may provide little insight into the relative importance of different taxa in terms of nutrition or energy intake (Dessborn et al. 2011). With the recent advent of DNA metabarcoding, it is now possible to collect fecal samples from ducks noninvasively and analyze the composition of fecal DNA with high taxonomic resolution using genetic markers. While it is true that DNA metabarcoding is not a bias-free approach, it has been shown that using relative read abundance information often provides a more accurate view of population-level diet, even with moderate recovery biases incorporated (Deagle et al. 2019). Furthermore, multiple markers can be used in DNA metabarcoding to reveal the relative abundance and frequencies of occurrence of each food item and unveil the relative importance of animal and plant matter in the diet of omnivores (Da Silva et al. 2019).

Understanding the foraging ecology of duck species is crucial to their conservation, particularly in regions within EAAF. Therefore, this study addresses important gaps in our understanding of food use by wintering ducks in Mai Po wetland. Specifically, we use DNA metabarcoding with multiple markers to (1) investigate the dietary compositions of four common migratory duck species, including *S. clypeata*, *A. crecca*, *M. penelope*, and *A. acuta*, to gain insights into the specific food resources required by these species wintering in Mai Po wetland, and (2) reveal the intraspecific and interspecific variation in diets among these species. This information will provide critical insights into the foraging strategies of these species and help us better understand how they utilize the wetland habitat during the wintering period.

2 | Materials and Methods

2.1 | Sample Collection and DNA Metabarcoding

The Mai Po Nature Reserve (MPNR; part of the Mai Po Inner Deep Bay Ramsar Site) is a wetland complex comprising five main habitats, including gei wai, freshwater ponds, intertidal mudflats, mangroves, and reedbeds (WWFHK 2025). Gei wai, or gei wai pond, is a traditional shrimp pond system commonly found in coastal areas of southern China and Hong Kong. These ponds are used for aquaculture, particularly for shrimp farming, and are characterized by a series of interconnected shallow ponds with controlled water flow for raising aquatic species (Cha et al. 1997). We obtained permission to enter the MPNR (22°29′20.7″ N, 114°02′09.9″ E) and collected duck fecal samples from the ground around gei wai. Shortly after the ducks departed at dawn, we collected each fresh fecal sample near the ponds using sterilized spatulas and gloved hands, placing each sample into a 1.5 mL tube. Used sterilized spatulas were not reused. Only the upper portions of the feces, which had not contacted the ground, were collected (Huang et al. 2021). We spaced the collection points at least 0.5 m apart to avoid collecting duplicate samples from the same individual. Between January and February 2020, 150 fecal samples were collected. These samples were immediately preserved on dry ice in the field and stored at −80°C until DNA extraction (Appendix S1 and Method S1) (Huang et al. 2022).

To identify host species, we designed DNA barcoding primers and conducted DNA barcoding on each sample (Appendix S1 and Method S1). Specifically, we obtained 57 samples from *M. penelope*, 48 samples from *S. clypeata*, 42 from *A. crecca*, and three from *A. acuta*. Our library preparation included mock communities (Table S1, Appendix S1 and Method S2), negative controls during DNA extraction and PCR to identify potential contamination and false positives. We used the Qubit dsDNA High-Sensitivity (HS) assay on a Qubit 4 fluorometer (Invitrogen, Carlsbad, USA) for DNA quantification. To identify the dietary composition, we employed specific genetic markers from the literature selected based on the omnivorous feeding habits of ducks. The first primer pair used was a universal genetic marker that amplifies the V7 region of the *18S* small subunit of nuclear ribosomal DNA (see Appendix S1 and Methods for all metabarcoding primer information) (McInnes et al. 2017). Additionally, we utilized a *COI* marker that specifically targets the Folmer region of the mitochondrial cytochrome c oxidase I (Shokralla et al. 2015). This *COI* marker is particularly effective in identifying invertebrates. Lastly, a plant-specific marker was employed to amplify a variable region of the P6 loop in the chloroplast *trnL* (UAA) (Taberlet et al. 2007). Fecal DNA samples, mock communities, and all negative controls were used for library preparation through a two-step polymerase chain reaction (PCR) process (Wan et al. 2024; Huang et al. 2021, 2022; Wei et al. 2024) with the *18S*, *COI*, and *trnL* markers (Appendix S1 and Method S3). To create a library multiplex, we combined the individual libraries in an equimolar ratio for each marker, including those libraries prepared from all negative controls and mock communities. Each multiplex was sequenced to a depth of approximately 400 k reads on a NovaSeq instrument (PE 150 bp reads) by the Novogene Corporation (Hong Kong).

2.2 | Bioinformatics

The demultiplexed raw paired-end fastq reads of *18S*, *trnL*, and *COI* markers were preprocessed by paired read merging, adapter trimming, and quality filtering. The paired-end reads were merged using USEARCH v11.0.667 with the -fastq_mergepairs function (Edgar 2010). PCR primer sequences were trimmed using CUTADAPT v2.4 in linked adapter mode (max_error_rate = 0.15) (Martin 2011). Only the merged reads that matched the primer sequences for the *18S*, *trnL*, or *COI* markers were retained. The quality of reads was assessed using FastQC v0.11.8 (Wingett and Andrews 2018) and the -fastq_eestats2 command in VSEARCH (Rognes et al. 2016). We then performed quality trimming to remove low-quality tails from reads with the expected number of errors per read exceeding one (−fastq_maxee 1) using the -fastq_filter function in VSEARCH. The high-quality reads retained fell around the target lengths: *18S* (130–230 bp), *trnL* (10–100 bp), and *COI* (75–85 bp). All preprocessed reads were dereplicated using the VSEARCH -derep_fulllength. From the dereplicated reads, we generated amplicon sequence variants (ASVs) by removing the chimeras and singletons (with abundance < 0.0001% of all reads) using USEARCH -unoise3 (Edgar 2016b). To cluster all the preprocessed reads into ASVs, we used a similarity threshold of 99% for *18S* and *trnL*, and 95% for *COI* (VSEARCH -usearch_global -id 0.99/0.95).

The taxonomic classification process was conducted in two steps to achieve a higher taxonomic resolution. Firstly, each ASV was assigned to the lowest identifiable taxonomic level using the SINTAX algorithm in USEARCH (Edgar 2016a) with a bootstrap cutoff of 0.7. For the *18S* dataset, the ribosomal RNA database SILVA (Glöckner et al. 2017) was utilized. The *trnL* dataset was classified using the CRUX database from the Anacapa Toolkit (Curd et al. 2019), while the *COI* dataset was classified using the mitochondrial database MIDORI (Leray et al. 2022). Secondly, we searched the ASVs from these datasets against the NCBI nt database (nonredundant nucleotide sequences) (Sayers et al. 2022). We extracted the top 1000 BLAST hits that exhibited similarity above 90% and an e-value < 1e−50 for *18S*, above 90% and an e-value < 1e−5 for *trnL*, and above 80% and an e-value < 1e−5 for *COI*. Afterward, we assigned the lowest common taxonomic level shared by 95% of *18S* BLAST hits (≥ 100 bps), 80% of *trnL* BLAST hits (≥ 40 bps), and 80% of *COI* BLAST hits (≥ 50 bps) using BASTA (Kahlke and Ralph 2019) with the lowest common ancestor (LCA) algorithm. The results were then combined to assign ASVs to lower taxonomic ranks.

To minimize false positive reads and contamination, we applied marker-specific abundance thresholds derived from mock community analysis: 0.17% for *18S*, 0.08% for *trnL*, and 0.03% for *COI* (Table S1a–c), and removed negative control-associated ASVs. Non-dietary items (such as Humans, Aves, Bacteria, and Protists) as well as ASVs with low taxonomic resolution, such as Eukaryota, were excluded. Sequencing depth was assessed using rarefaction curves at the ASV level, generated with the quickRareCurve from ecolFudge package (Clark 2020), by plotting raw read counts against ASV richness. Samples that did not reach a plateau were excluded. To improve interpretability in the figures, taxa with low relative read abundance (≤0.1% of total RRA) were grouped into broader taxonomic categories (e.g., order or family level) using a Lowest Common Ancestor (LCA) approach, provided they shared a higher taxonomic classification with more abundant taxa that were at higher taxonomic levels. Taxa not sharing a classification with more abundant taxa were presented individually, regardless of abundance.

Following quality filtering, the final dataset comprised an average of 237,059 reads per sample for *18S*, 227,500 reads for *trnL*, and 97,084 reads for *COI*. The number of raw reads, ASVs, taxa, taxonomic categories, and samples removed at each filtering step are summarized in Table S1d.

2.3 | Data Analysis

We used 132 samples from four duck species for downstream analysis, including *S. clypeata* ($n = 44$ for *18S*, $n = 42$ for *trnL*, and $n = 45$ for *COI*), *M. penelope* ($n = 41$ for *18S* and *COI*; $n = 42$ for *trnL*), *A. crecca* ($n = 41$ for *18S* and *COI*; $n = 42$ for *trnL*), and *A. acuta* ($n = 3$ for *18S* and *trnL*, $n = 2$ for *COI*). For the *18S* dataset, we identified 39 taxonomic categories from 73 taxa represented by 129 ASVs. The *trnL* dataset yielded 47 taxonomic categories from 73 taxa represented by 125 ASVs. The *COI* dataset resulted in 27 taxonomic categories from 132 taxa represented by 514 ASVs. To analyze the data, we calculated three metrics based on the ASVs: (i) relative read abundance (RRA), which represents the percentage of reads assigned to a taxonomic category in a sample; (ii) weighted percentage of occurrence (wPOO), which indicates the proportion of a taxonomic category relative to all detected categories in a sample, and (iii) frequency of occurrence (FOO), which measures the percentage of individuals in which a taxonomic category was detected within a species. The RRA or wPOO at the population level is presented as the mean of RRA or wPOO of all individual samples of an duck species. FOO estimates the frequency of occurrence of a taxon within all samples of an duck species. The results were visualized using the R packages ggplot2 v3.3.5 (Hadley 2016).

2.3.1 | Diversity Analysis on the Diet of Three Duck Species

As *A. acuta* had a small sample size, it was excluded from diversity analyses. We used Hill numbers (Hill 1973) to assess dietary diversity with the R package hilldiv v1.5.1 (Alberdi and Gilbert 2019). Hill diversity was calculated for q values of 0 (richness), 1 (sensitive to both richness and evenness), and 2 (emphasizing dominant ASVs). Alpha diversity (${}^{q}D_{\alpha}$) represents within-sample diversity, gamma diversity (${}^{q}D_{\gamma}$) reflects overall diversity at the species level, and beta diversity (${}^{q}D_{\beta}$) quantifies dissimilarity between samples, calculated as ${}^{q}D_{\beta} = {}^{q}D_{\gamma}/{}^{q}D_{\alpha}$. These metrics were computed using the div_profile function in hilldiv. We conducted pairwise comparisons using the Kruskal–Wallis test with the function div_test in hilldiv to compare the dietary diversity between duck species at the sample level (alpha diversity). We then performed a post hoc Dunn test, using Benjamini-Hochberg correction ($p < 0.05$) with the div_test function of the hilldiv package.

To assess the differences in taxa composition between duck species, we calculated pairwise binary Jaccard dissimilarity distances based on the occurrence of each ASV, as well as pairwise Bray–Curtis dissimilarity distances based on the fourth root-transformed RRA of each ASV. The results were visualized using Principal Coordinates Analysis (PCoA) with the ordinate and plot_ordination functions in the R package phyloseq v1.30.0 (McMurdie and Holmes 2019). We performed hierarchical clustering analysis using Ward's method in the hclust function. Additionally, we conducted a Permutational Multivariate Analysis of Variance (PERMANOVA) test to evaluate the separation of dietary compositions between the duck species. This test evaluated the centroid and dispersion of compositions for individual samples within each group in a measure of space. To ensure the significance of interspecific variation in the PERMANOVA test, we checked for homogeneity of intragroup beta-dispersion ($p > 0.05$) using the adonis function in the vegan v2.5.7 package (Oksanen et al. 2019). We conducted pairwise PERMANOVAs of the duck species using the pairwise.adonis function (Martinez Arbizu 2020) and evaluated their beta-dispersions using the betadisper function in the vegan package. To determine the contribution of individual taxa to the variations between duck species, we calculated the Similarity Percentage (SIMPER). For the SIMPER analysis, we used the simper.pretty function, and the nonparametric Kruskal–Wallis rank-sum test was carried out using kruskal.pretty functions, respectively implemented in the R scripts simper_pretty.R and R_krusk.R provided by Steinberger (2020). Only taxa exhibiting statistically significant variance ($p < 0.05$) were presented. PERMANOVAs and SIMPER analyses based on occurrence data were conducted using binary Jaccard dissimilarity distances, while those based on RRA were performed using Bray–Curtis distances.

3 | Results

3.1 | Dietary Compositions of *A. crecca*, *M. penelope*, *S. clypeata*, and *A. acuta*

Based on the *18S* data, it was found that *A. crecca* exhibited the highest and most frequent consumption of plants in the clade Streptophytes (94% RRA and 66% wPOO) among the four species studied (Figure 1 and Figure S1; Tables S2–S4). Further analysis of the *trnL* data revealed that the streptophytes targeted by *A. crecca* were primarily asters from the Asteraceae family (83% RRA and 40% wPOO among plants), as well as monocotyledonous grasses from the Poaceae family in the

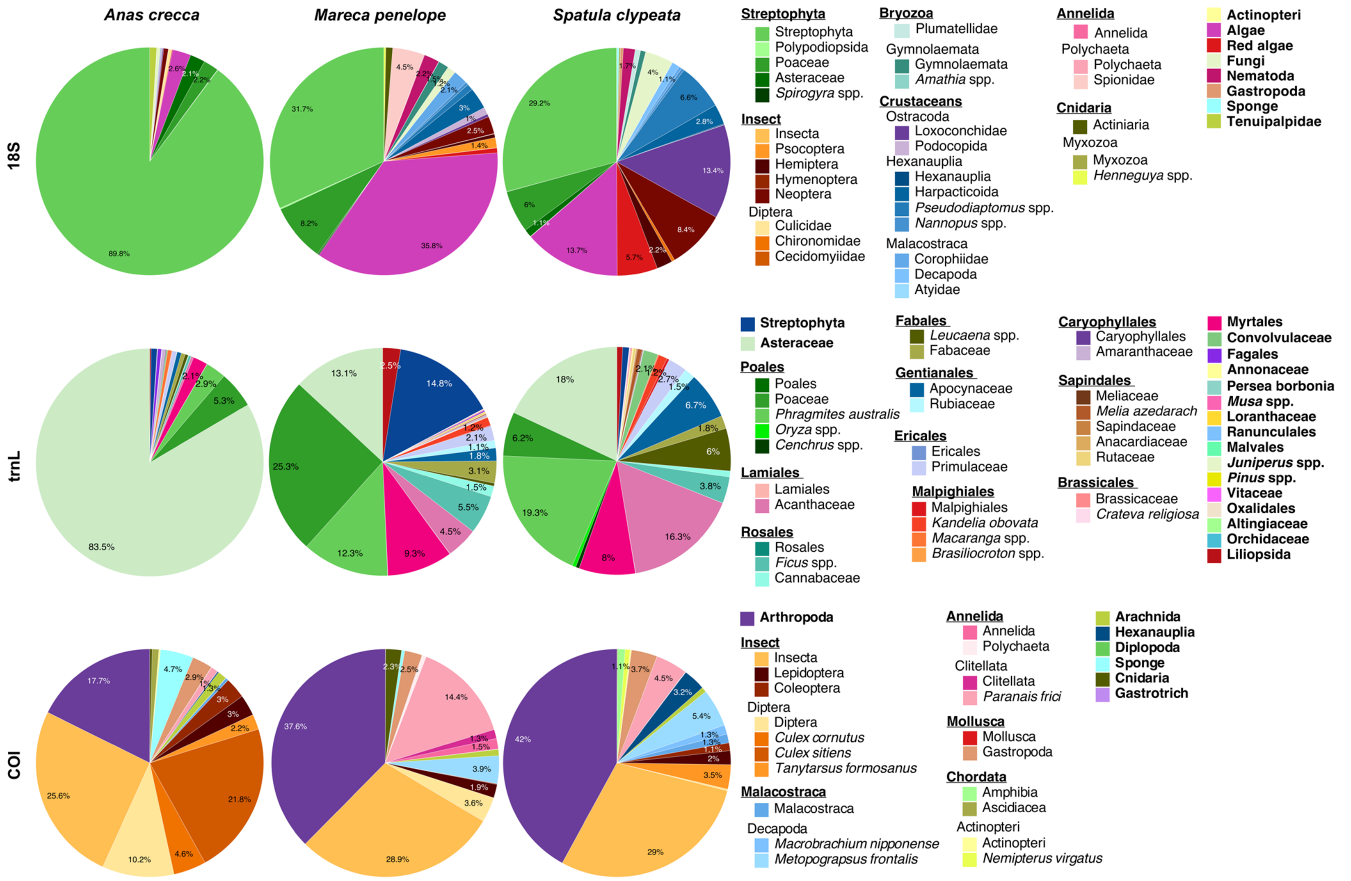

FIGURE 1 | Relative read abundance (RRA) of dietary compositions of four wintering duck species determined using *18S* rDNA, *trnL*, and *COI* markers on fecal DNA. We used samples from *Anas crecca* (*n* = 41 for *18S* and *COI*; *n* = 42 for *trnL*), *Mareca penelope* (*n* = 41 for *18S* and *COI*; *n* = 42 for *trnL*), and *Spatula clypeata* (*n* = 44 for *18S*, *n* = 42 for *trnL*, and *n* = 45 for *COI*). Low-abundance taxa (RRA ≤ 0.1%) were grouped into broader taxonomic categories (e.g., order or family level) if they shared a higher taxonomic classification with abundant taxa that were at higher taxonomic levels. Taxa without shared classifications were presented individually, regardless of their abundance. The RRA of dietary taxonomic categories is shown as color blocks. Only categories with an RRA > 1% are indicated in the figure (For detailed taxonomic categories, see Tables S2, S5 and S8).

order Poales (8% RRA and 42% wPOO among plants), such as *Phragmites australis* (common reed) (Figure 1 and Figure S1; Tables S5–S7). Additionally, the analysis of *COI* data showed that *A. crecca* consumed invertebrates in phylum Arthropoda (90% RRA and 78% wPOO among macroinvertebrates), including those in the order Diptera (e.g., typical mosquitoes in *Culex*), Lepidoptera (butterflies and moths), Coleoptera (beetles), and class Arachnida (Figure 1 and Figure S1; Tables S8–S10).

Regarding *M. penelope*, the analysis of *18S* data indicated that it had a heavy and frequent consumption of streptophytes (40% RRA and 44% wPOO), particularly Poaceae (Figure 1 and Figure S1; Tables S2–S4). Algae also comprised a considerable portion of its diet (36% RRA and 25% wPOO). While arthropods (12% RRA and 13% wPOO), worms in class Polychaeta (bristle worms, e.g., spionids) in the Annelida phylum, Nematoda phylum (roundworms), and moss animals in the Bryozoa phylum (mainly in the class Gymnolaemata) were preyed upon, they constituted smaller proportions of *M. penelope*'s diet. The results from *trnL* data exhibited a similar pattern to the *18S* data for plant consumption. It showed that a high consumption of Poaceae grasses (38% RRA and 24% wPOO among plants), such as *P. australis* (Figure 1 and Figure S1; Tables S5–S7). *Mareca penelope* also ate plant parts from various orders such as Myrtales, Rosales (e.g., *Ficus* figs and Cannabaceae), Lamiales (e.g., Acanthaceae), Fabales, Gentianales (Apocynaceae and Rubiaceae), Ericales (Primulaceae), Malpighiales (e.g., *Kandelia obovata*) orders, among others. Results from *COI* data indicated that arthropods (77% RRA and 70% wPOO among invertebrates) make up the majority of *M. penelope*'s invertebrate diet, with insects (34% RRA and 34% wPOO among invertebrates), such as dipterans and lepidopterans, being the most frequently consumed (Figure 1 and Figure S1; Tables S8–S10). Malacostracans in the order Decapoda, including marsh crabs like *Metopograpsus frontalis*, were also preyed upon. *Mareca penelope* also fed on smaller proportions of annelids (18% RRA and 20% wPOO among invertebrates), mainly clitellate detritus worms like *Paranais frici*, slugs and snails in the class Gastropoda in phylum Mollusca, and invertebrates in the phylum Cnidaria.

The *18S* data revealed that the diet of *S. clypeata* primarily consisted of streptophytes and arthropods (RRA: 36% and 36%; wPOO: 39% and 25%), and the latter was higher than that of the other ducks. There were notable contributions from Poaceae grasses and asters (Figure 1 and Figure S1; Tables S2–S4). The consumption of arthropods in *S. clypeata* included crustaceans, such as those in the class Ostracoda (13% RRA and 5% wPOO, e.g., Loxoconchidae), class Hexanauplia (10% RRA and 12% wPOO, e.g., the order Harpacticoida, *Pseudodiaptomus* spp., and *Nannopus* spp.), and insects (11% RRA and 7% wPOO, e.g., Infraclass Neoptera and order Hemiptera). Other dietary components included algae (14% RRA and 15% wPOO) and red algae (6% RRA and 9% wPOO). Consistent with *18S* data, the *trnL* data showed that *S. clypeata* primarily consumed Poaceae grasses (27% RRA and 18% wPOO, e.g., *P. australis*) and asters (18% RRA and 9% wPOO). Additionally, abundant Lamiales were detected in *trnL* data (16% RRA and 8% wPOO, e.g., Acanthaceae) (Figure 1 and Figure S1; Tables S5–S7). *Spatula clypeata* also fed on plant materials from a diverse range of taxa, including Gentianales (families Apocynaceae and Rubiaceae), Myrtales, Fabaceae, Rosales (e.g., figs *Ficus* in Moraceae), Ericales (e.g., Primulaceae), Malpighiales (such as *K. obovata*), Convolvulaceae (bindweeds), Sapindales, and more. Regarding invertebrates, arthropods (90% RRA and 81% wPOO) formed the main prey for *S. clypeata* (Figure 1 and Figure S1; Tables S8–S10), including insects (36% RRA and 35% wPOO) such as dipterans (e.g., lake flies *Tanytarsus formosanus*), lepidopterans, and coleopterans. They also consumed crustaceans (11% RRA and 11% wPOO) such as malacostraca (e.g., *Macrobrachium nipponense* (oriental river prawn) and *M. frontalis* (marsh crabs)) and species in Hexanauplia. The diet also included small proportions of annelids (e.g., *P. frici*), gastropods, and amphibians.

Anas acuta predominantly fed on streptophytes (62% RRA and 48% wPOO in *18S* data), mainly Poaceae grasses (Tables S2–S4). Additionally, it fed on arthropods (32% RRA and 22% wPOO in *18S* data), especially benthic crustaceans in the order Harpacticoida and *Pseudodiaptomus* of class Copepoda, class Ostracods in the subclass Podocopa, and algae (Figure 1 and Figure S1; Tables S2–S4 and S8–S10). *TrnL* data revealed more plant taxa being consumed, in which asters (30% RRA and 11% wPOO), bindweeds (27% RRA and 11% wPOO), and Poaceae grasses (13% RRA and 23% wPOO) are some of the primary plants (Figure 1 and Figure S1; Tables S5–S7). It also fed on other plants in small amounts, including Acanthaceae, Apocynaceae, and Rubiaceae. Arthropoda, especially insects, were the only invertebrates detected in the diet of *A. acuta* as revealed by the *COI* data.

Our findings identified several food taxa commonly found in the diets of all or most of the duck species studied. These include *asters*, *Poaceae* grasses (such as *P. australis*), as well as members of *Acanthaceae*, *Myrtales*, *Apocynaceae*, algae, *Podocopida* (e.g., *Loxoconchidae*), red algae, fungi, *Nematoda*, and various insects (Figure S2, Tables S2–S10).

3.2 | Intraspecific and Interspecific Dietary Variation in Duck Species

The analysis of the three genetic markers showed relatively low intraspecific dietary variation within *A. crecca* (*A. acuta* was excluded here because of its small sample size). *Mareca penelope* and *S. clypeata* displayed higher levels of intraspecific dietary variation than those observed in *A. crecca* (Figure 2 and Figure S3; Tables S11–S16).

Analysis of the alpha diversities showed that, at the sample level, *S. clypeata* had the highest diversities in its plant (*trnL*) diet, followed by *M. penelope* and *A. crecca* (Figures 3 and 4; Tables S17–S18). Despite having the highest plant diversity at the sample level, the diversities of invertebrate (*COI*) individual samples of *S. clypeata* were the lowest among the duck species based on taxa richness (Figures 3 and 4; Tables S17–S19). *Anas crecca* displayed the highest invertebrate diversities in individual samples. However, we observed decreasing trends in plant and invertebrate diversity values as q values increased in all three duck species, which indicated that consumed items in samples were dominated by several ASVs and that the proportion of plant and invertebrate taxa was uneven within these individual samples (Figure 4).

At the population level, analysis on gamma diversities based on *18S* (overall) and *trnL* (plant) data showed that *S. clypeata* and *M. penelope* had similar levels of diversities in their diets, whereas the dietary diversities of *A. crecca* were the most unevenly distributed and the lowest among the three species (Figure 4 and Table S17). Although the diets of *M. penelope* and *A. crecca* shared similar invertebrate (*COI*) taxa richness at the population level, the dietary taxa in *A. crecca* were more unevenly distributed than those of *M. penelope*, and the invertebrate diversity of *A. crecca* became more similar to that of *S. clypeata* as q values increased.

The dietary niche of *M. penelope* and *S. clypeata* largely overlapped, whereas *A. crecca* had a more distinct dietary composition (Figures 1 and 2), as revealed by the three genetic markers based on both Jaccard and Bray–Curtis dissimilarities (Figure 5 and Figure S2). All plant and invertebrate diets were significantly segregated among three duck species (PERMANOVA, p-value = 0.001) (Table S19). However, homogeneous intraspecific dispersion was only detected in the abundance-based invertebrate diets (*COI* beta dispersion = 0.071), indicating that the significant separation of *trnL* and *18S* diets might be due to the intraspecific heterogeneity rather than interspecific diet variations.

According to the SIMPER results, a group of food taxa contributed to the dietary difference between *A. crecca* and the other two duck species (Tables S20–S22). For example, *A. crecca* consumed a higher proportion of streptophytes, including asters (Tables S5–S7), dipterans like typical mosquitoes, such as *Culex* spp., and sponge (Tables S8–S10), while consuming fewer items in Poaceae, particularly *P. australis*, Myrtales, Acanthaceae (Tables S5–S7), algae (Tables S2–S4), and the clitellate oligochaete worm, *P. frici* (Tables S8–S10), compared to *M. penelope* and *S. clypeata*. Although the diets of *M. penelope* and *S. clypeata* were similar, they were slightly differentiated by the consumption of a few taxa (Table S21). For instance, plants in Asteraceae, Acanthaceae, *P. australis*, Apocynaceae (Tables S5–S7), as well as red algae, copepods in *Pseudodiaptomus*, insects in Neoptera, and ostracods in Loxoconchidae (Tables S2–S4) were consumed more by *S. clypeata* than *M. penelope*. In contrast, plants in Poaceae (Tables S5–S7) and algae (Tables S2–S4 and S8–S10) were consumed more by *M. penelope* compared to *S. clypeata*.

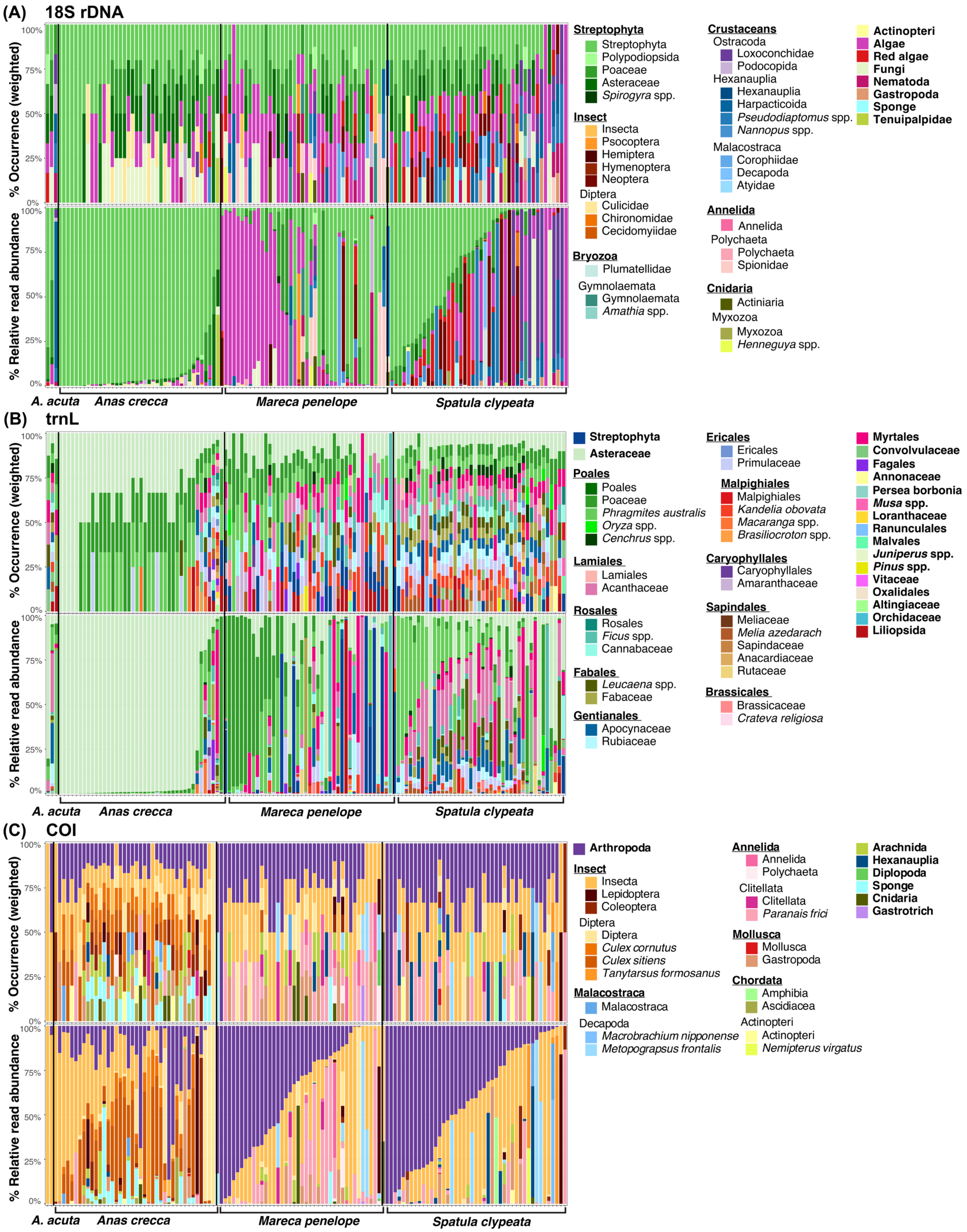

FIGURE 2 | Legend on next page.

FIGURE 2 | Dietary compositions of individual fecal samples of wintering ducks were detected using (A) *18S* rDNA, (B) *trnL*, and (C) *COI* markers. We used samples from *Anas acuta* ($n=3$ for *18S* and *trnL*, $n=2$ for *COI*), *Anas crecca* ($n=41$ for *18S* and *COI*; $n=42$ for *trnL*), *Mareca penelope* ($n=41$ for *18S* and *COI*; $n=42$ for *trnL*), and *Spatula clypeata* ($n=44$ for *18S*, $n=42$ for *trnL*, and $n=45$ for *COI*). Low-abundance taxa (RRA $\leq 0.1\%$) were grouped into broader taxonomic categories (e.g., order or family level) if they shared a higher taxonomic classification with abundant taxa that were at higher taxonomic levels. Taxa without shared classifications were presented individually, regardless of their abundance. The weighted percentage of occurrence and relative read abundance of each taxonomic category is shown, with each colored bar representing one anatid individual (see Tables S11–S16 for details).

4 | Discussion

While ducks are generally considered omnivorous, certain duck species, such as *A. crecca* in Hong Kong, demonstrate distinct dietary niches compared to their sympatric counterparts. The analysis using the three markers indicates that *M. penelope* and *S. clypeata* exhibited omnivorous foraging behavior during their winter stay in Hong Kong. In contrast, although *A. crecca*'s diet included some prey taxa such as insects and gastropods, it was predominantly herbivorous throughout its wintering period in Hong Kong. Additionally, *A. crecca* exhibited less variation in composition across individual samples, while samples of *S. clypeata* exhibited more variations in their overall and animal compositions. Overall, the dietary items in the samples were dominated by several taxa, with an uneven proportion of plant and invertebrate taxa among individual samples. *Anas crecca* showed lower diversity in its plant diet but higher diversity in its animal diet. Conversely, *S. clypeata* exhibited the highest diversity in its plant diet and the lowest diversity in its animal diet.

In this research, DNA metabarcoding was utilized to analyze fecal DNA from duck species wintering in a wetland complex in Hong Kong. By employing multiple markers, including *18S*, *trnL*, and *COI*, DNA metabarcoding offered a more comprehensive analysis of the diets of these species compared to approaches using fewer markers or traditional techniques like microscopy (Da Silva et al. 2019). This multi-marker approach is particularly advantageous for animals with complex dietary habits, such as omnivores that consume plants and animals. Unlike other markers which target specific taxonomic groups, the *18S* marker offers crucial insights into the relative proportions of food items spanning different kingdoms and phyla (Huang et al. 2021, 2022; Wei et al. 2024) in the diets of the ducks. A study in Japan using DNA metabarcoding with *trnL* and *COI* markers also reveals the diets of several duck species during the winter period (Ando et al. 2023), which uncovered a high diversity of consumed plant and invertebrate taxa. For example, analysis with *trnL* showed that *A. crecca* in Japan consumed at least 15 plant species from 12 families. The majority of plant materials were from the majority of plant materials were from the Araceae, Nymphaeaceae, and Poaceae families but not from Asteraceae (83% RRA in this study) (Ando et al. 2023). Additionally, *COI* data indicated that *A. crecca* consumed at least 15 families of arthropods, mollusks, or rotifers. However, reliance on specific markers like *trnL* or *COI* alone limits the ability to accurately assess the relative proportions of plant versus animal taxa in the diet. The inclusion of a universal marker such as *18S* in our study provides more insights into the relative proportion of higher taxonomic groups, e.g., plant and animal taxa, and demonstrates that some duck species, e.g., *A. crecca* in this study, are more specialized.

Comparing our results with those of studies conducted globally, we found that the dietary compositions of these duck species varied to different degrees across geographic locations. This variation underscores the flexibility of these species in their diets and suggests that their adaptable foraging strategies likely contribute to the sustainability of their populations. For example, *A. crecca* in Hong Kong showed the highest consumption of streptophytes, accounting for 94% RRA. Further examination revealed that the streptophytes consumed were mainly asters and Poaceae grasses. In the Great Salt Lake, Utah, USA, a study examining the intestinal tracts above the gizzards of co-occurring *A. crecca* and *S. clypeata* wintering ducks found that both species consumed similar food taxa. During winter, both species consume over 70% of animal matter (including brine shrimp cysts) while increasing their intake of plant materials during fall and spring (Roberts and Conover 2014), which dramatically contrasts with the findings in Hong Kong. However, similar to the *A. crecca* in Hong Kong, *A. crecca* wintering in the Camargue, southern France, consumed a high proportion of plant seeds (> 80%) and significantly fewer invertebrates (< 16%) during fall and winter. The plant species found in their gullets included seven species from Cyperaceae, Poaceae, Potamogetonaceae, Amaranthaceae, and green algae (Brochet et al. 2012), but not from Asteraceae (83% RRA in this study). Another study on the diets of wintering *A. crecca* in Kern, California, USA, which examined the contents of oesophagi, found that plant seeds accounted for about 62% of their diet during fall and winter, with the remainder being animal matter. *Anas crecca* consumed more than nine plant species in this region, mainly from Poaceae and Lythraceae (Euliss Jr. and Harris 1987). The observed diverse dietary compositions of wintering *A. crecca* across various geographic locations might be attributed to differences in local floral and faunal communities. Additionally, this dietary variability highlights the adaptability and flexibl foraging strategies of *A. crecca*.

For *S. clypeata*, streptophytes, including asters and Poaceae grasses, were major components of its diet in Hong Kong, accounting for 36% RRA, along with various other taxa. Additionally, it exhibited a higher consumption rate of arthropods (36% RRA) compared to local *A. crecca* and *M. penelope*. A study analyzing the gizzards of wintering *S. clypeata* from 12 states in the USA revealed different proportions of plant (66%) and animal (35%) contents (McAtee 1922) compared to the results of this study. The USA study identified a diverse range of items, including at least eight species of gastropods, eight species of Dytiscidae (water beetles), eight species of ostracods, and 53 species of plants, such as from families Poaceae, Potamogetonaceae, and Boraginaceae, among others. However, in another study conducted in Texas, USA, *S. clypeata* were predominantly herbivorous (plant > 93.7%), with the contents of the esophagus mainly comprising at least 22 plant species from

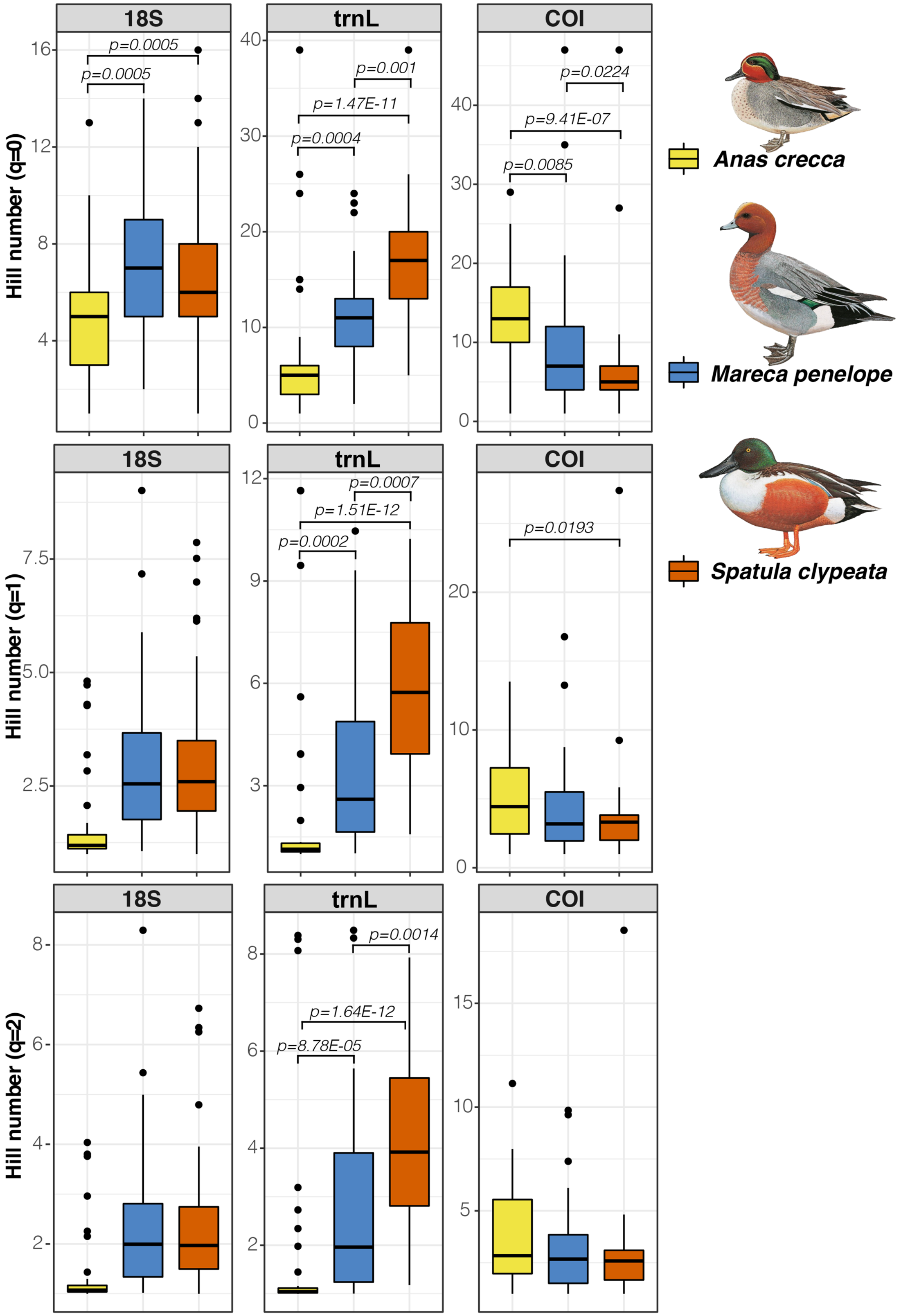

FIGURE 3 | Legend on next page.

e.g., the Polygonaceae and Poaceae families, as well as some gastropod species (Collins et al. 2017), in contrast to the Poaceae, Asteraceae, and Acanthaceae families, and arthropods (> 36% in *18S*) observed in our study. Furthermore, a study examining the contents of gizzards and gullets of *S. clypeata* wintering in Lake Tonga in Algeria reveals that their diets consisted entirely

FIGURE 3 | Alpha diversities of the dietary compositions in three species of ducks, characterized using *18S* rDNA, *trnL*, and *COI* markers. We analyzed fecal samples from *Spatula clypeata* ($n = 44$ for *18S*, $n = 42$ for *trnL*, and $n = 45$ for *COI*), *Mareca penelope* ($n = 41$ for *18S* and *COI*; $n = 42$ for *trnL*), and *Anas crecca* ($n = 41$ for *18S* and *COI*; $n = 42$ for *trnL*). The Hill numbers were calculated for three levels of diversity ($q = 0$, 1, and 2), with increasing weight given to the abundance of dietary taxa. Each colored box represents the interquartile range, with the median indicated by a line. The whiskers extend to the highest and the lowest values within the 1.5× interquartile range, and the black dots represent outliers. The *p*-values of significant differences between groups are shown above the boxes (see Table S18 for details). Illustrations of ducks were reproduced with the permission of Lynx Edicions.

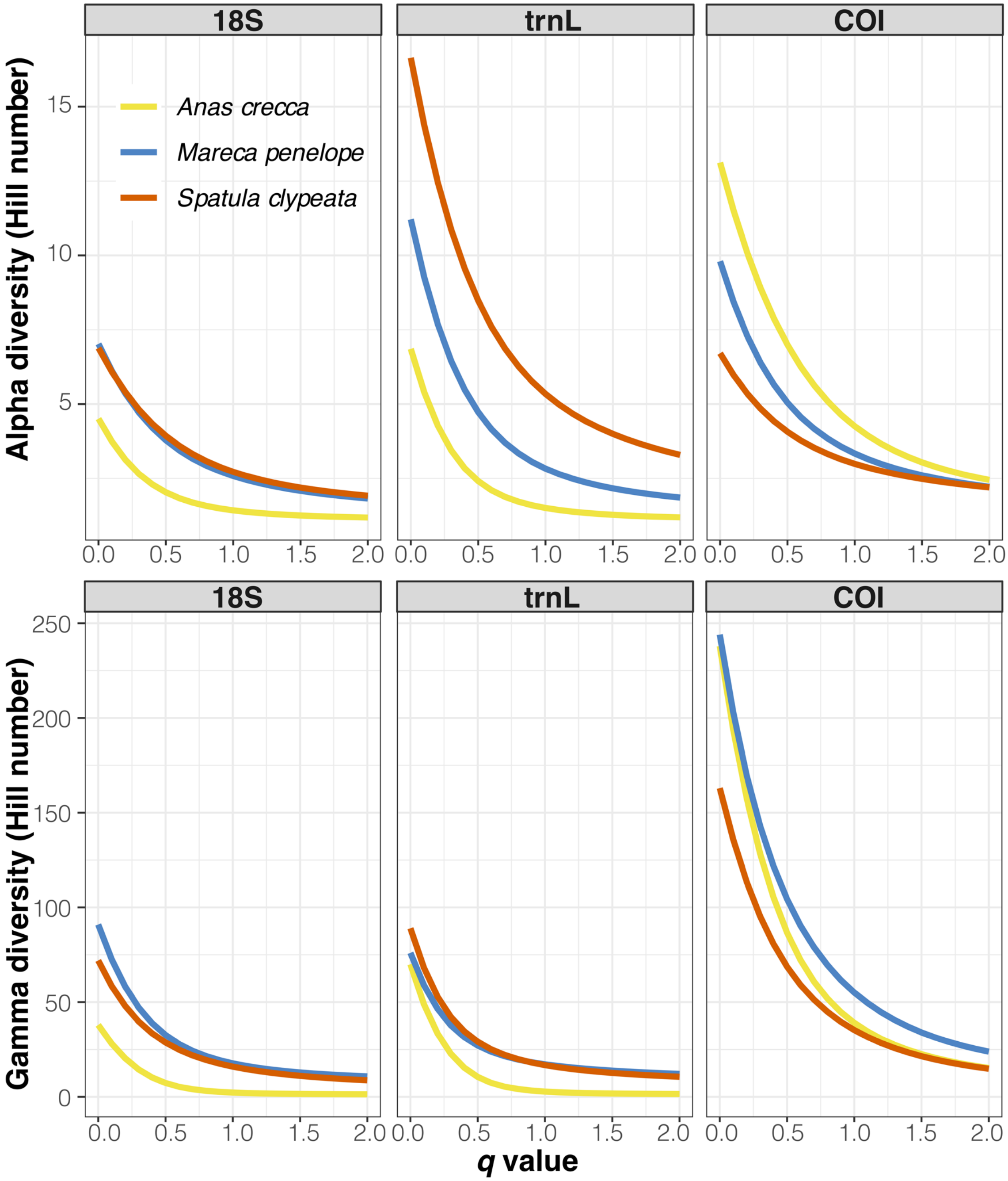

FIGURE 4 | Diversity profiles of the dietary compositions in three duck species, characterized using *18S* rDNA, *trnL*, and *COI* markers. We analyzed fecal samples from *Spatula clypeata* ($n = 44$ for *18S*, $n = 42$ for *trnL*, and $n = 45$ for *COI*), *Mareca penelope* ($n = 41$ for *18S* and *COI*; $n = 42$ for *trnL*), and *Anas crecca* ($n = 41$ for *18S* and *COI*; $n = 42$ for *trnL*). The alpha and gamma diversities are presented as Hill numbers, with increasing orders of diversity *q* (see Table S17 for details).

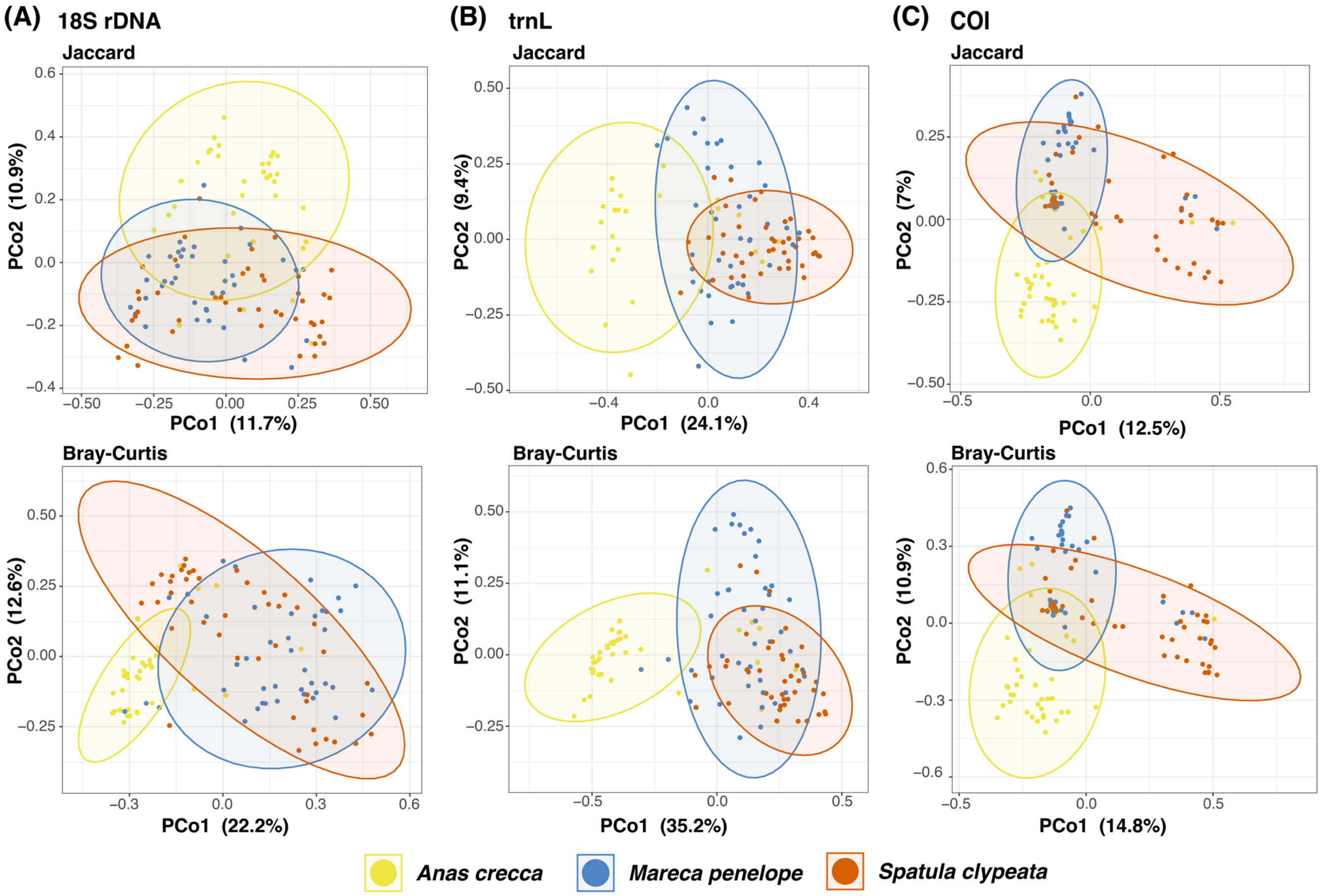

FIGURE 5 | Principal coordinate analysis (PCoA) of dietary compositions in three duck species, detected using (A) *18S* rDNA, (B) *trnL*, and (C) *COI* markers. We analyzed fecal samples from *Spatula clypeata* ($n=44$ for *18S*, $n=42$ for *trnL*, and $n=45$ for *COI*), *Mareca penelope* ($n=41$ for *18S* and *COI*; $n=42$ for *trnL*), and *Anas crecca* ($n=41$ for *18S* and *COI*; $n=42$ for *trnL*). The analysis is based on Bray–Curtis distances estimated from relative read abundance data and binary Jaccard distances calculated from occurrence data. The percentages of variation in diet compositions are shown in brackets along the axes (see Tables S19–S22 for details).

of plant materials without any inclusion of animal matter (Ayaichia et al. 2018). The study reported seven plant species, and the ducks predominantly consumed those from Typhaceae, Cyperaceae, Haloragaceae, and Ceratophyllaceae families (Ayaichia et al. 2018). In a similar study conducted in South Texas, a notable difference was observed in the dietary compositions of *S. clypeata* between freshwater and saltwater habitats. The study reveals that more animal matter was consumed in saltwater habitats (over 80%) than in freshwater habitats (50%). The researchers identified animal components in their esophagus and proventriculus that belonged to seven orders and representatives from one phylum, three classes, two families, and one genus. Notably, the primary animal matter consumed in saltwater environments included ostracods, foraminiferans, gastropods, and copepods (Tietje and Teer 1996). The diets of *S. clypeata* examined in the Japan study, similarly using DNA metabarcoding, showed a lower diversity than our findings. The study indicated that the primary food sources for *S. clypeata* included various species of nonbiting midges, mosquitoes, as well as plants from the Nelumbonaceae and Araceae families, which were distinct from those in Hong Kong (Ando et al. 2023).

Although research on the dietary compositions of *M. penelope* is limited, the dietary variations among *M. penelope* across different geographical locations have also been noted. Plant matter contributed a great proportion (40% RRA) to the diet of *M. penelope* wintering in Hong Kong; the plant matter is primarily composed of species from Poaceae, Asteraceae, and families within Myrtales. Arthropods, including insects and malacostracans, accounted for 77% RRA within the invertebrates consumed. In Vejlerne, Denmark, *M. penelope*'s diet mainly consisted of plant species from Poaceae, Juncaceae, Rosaceae, and Fabaceae, as analyzed using DNA metabarcoding (Svendsen et al. 2023). In Japan, wintering *M. penelope* mainly consumed plant species in the families Nelumbonaceae, Araceae, Ranunculaceae, Apiaceae, and Poaceae (Ando et al. 2023). The diverse dietary compositions of wintering *S. clypeata* and *M. penelope* in various geographic locations also emphasize variations in local floral and faunal communities, as well as the adaptability and flexible foraging tactics of these species. Our findings on *A. acuta* broadly align with the limited dietary studies of wintering populations in other regions. Specifically, conspecifics in the Central Valley of California (Euliss Jr. and Harris 1987) and the Gulf Coast of Texas (Ballard et al. 2004), similar to those in Mai Po, primarily consumed wetland plants, reflecting a common trend of herbivory in winter. Additionally, their diets included a smaller proportion of invertebrates, which are readily available in their wetland habitats.

However, it is worth noting that comparing findings from dietary studies across different regions is challenging due to various factors. These factors include variations in the sampling seasons, digestive parts or materials examined, taxonomic levels at which food items were identified, and differences in how studies analyzed their data and reported their results (Dessborn et al. 2011).

In addition to the interspecific dietary variability, our study further highlights the flexibility and adaptability of duck diets through observed intraspecific dietary variations among samples from the same habitat. While research on individual dietary variations among different duck species remains limited, our findings indicate that the overall and plant-based diets of *A. crecca* displayed much lower variability among individual samples than those of *M. penelope* and *S. clypeata*, in terms of both taxa abundance and occurrence. The compositions of most *A. crecca* samples were dominated by asters. In contrast, individual samples of *M. penelope* and *S. clypeata* were highly variable in their dietary composition. These intraspecific dietary variations among duck samples demonstrate their ability to adapt to diverse habitats and flexible food choices based on the availability of resources in the habitats. Such adaptability likely contributes to the widespread abundance of duck populations globally.

Previous studies on the foraging methods of the three duck species suggest that the foraging behaviors of *A. crecca* and *S. clypeata* are more similar to each other than to that of *M. penelope* (Klimas et al. 2022; Kooloos et al. 1989; Guillemain, Martin, and Fritz 2002). However, we observed a higher similarity between the diets of *S. clypeata* and *M. penelope*. The reason for this similarity therefore remains uncertain. *Anas crecca* generally forages via dabbling, upending, or grazing (Pöysä 1987), primarily feeding at night during winter (Guillemain, Fritz, and Duncan 2002). Previous studies have reported that the plant materials found in the digestive tracts of *A. crecca* mainly consisted of seeds (Olney 1963), with vegetation shoots accounting for a limited proportion (Klimas et al. 2022). Limited research indicates that *A. crecca* exhibits selective feeding behavior, preferring small to medium-sized plant seeds (<4 mm) and prey (Klimas et al. 2022). Studies in France revealed that *S. clypeata* and *M. penelope* forage differently during winter, with *S. clypeata* engaging in dabbling or foraging deep in the water column by dipping and upending (Guillemain et al. 2000b, 2000a), while *M. penelope* predominantly grazed (Guillemain, Martin, and Fritz 2002). Similar to *A. crecca*, *S. clypeata* were mainly granivorous (Ayaichia et al. 2018). Previous research has proposed a sieving mechanism for *S. clypeata*, enabling it to filter and select food particles smaller than 4 mm (Kooloos et al. 1989). According to previous studies, *S. clypeata* was observed foraging during both day and night time (Guillemain et al. 2000b, 2000a; Guillemain, Fritz, and Duncan 2002). A study on the foraging behavior of *M. penelope* found that *M. penelope* primarily grazes on green shoots (Mathers and Montgomery 1997). During winter, *M. penelope* showed the highest peck rates on grass with a height of 30 mm, with peck rates decreasing on both taller and shorter grasslands (Durant and Fritz 2006). Other studies observed that most *M. penelope* individuals engaged in water dabbling for shoots during the observation period, with a small percentage involved in dig feeding, peck feeding, or upending depending on the tidal level (Mathers and Montgomery 1996) and they mainly foraged in the daytime (Von Känel 1981). Since the DNA metabarcoding method cannot determine which specific parts of plants the duck species consumed, the observed dietary similarity between *S. clypeata* and *M. penelope* may be due to their feeding on different parts of the same plants, given their distinct foraging behaviors. However, this hypothesis requires further investigation.

While certain plant species like *P. australis* identified in the diets of the duck species in this study have been documented as food sources for their conspecifics in other regions, our study also reveals the presence of other plant species not previously reported in studies of duck diets. For example, *Ficus* and *K. obovata*, a kind of mangrove found in the Mai Po wetland, were consumed by the four duck species we studied. Furthermore, our research identified certain invertebrates that had not been previously documented in duck diets, such as *M. frontalis*, *P. frici*, and *T. formosanus*.

Although only the upper portions of the feces were collected to minimize environmental contamination, it is still possible that some of the detected taxa originated from the environment. These nontarget taxa might have introduced biases into our dietary analyses. For example, they could have a greater impact on the wPOO than on RRA, especially if the nontargets were present in much smaller quantities compared to the target taxa. Future studies could incorporate environmental samples from foraging areas to better understand the extent of environmental contamination. To enhance the accuracy of dietary composition analyses, including technical replicates for each sample would be advantageous. In this study, despite the absence of technical replicates, we minimized false positive reads and contamination by applying marker-specific abundance thresholds derived from mock community analyses and by removing ASVs associated with negative controls.

We successfully achieved our study objectives, making this one of the few investigations employing DNA metabarcoding to analyze the dietary compositions of wintering duck species. Based on our research findings, we suggest that wet grasslands dominated by herbaceous plants, along with aquatic environments teeming with small aquatic invertebrates or zooplankton, serve as crucial foraging grounds for duck species wintering in Mai Po. To attract migratory duck species to winter in Mai Po, it is essential to focus on managing the Ramsar site and its surrounding areas. This includes maintaining or expanding pond areas and enhancing the abundance and diversity of herbaceous plant species, especially plants in Asteraceae, Poales, and Lamiales, in proximity to these ponds. Given that the studied duck species are primarily filter feeders and grazers, prioritizing grassland management, improvement, and restoration within the Ramsar site is key to promoting the growth of herbaceous plants, ensuring the availability of food resources during migration, and maintaining the health of their wintering habitats. Additionally, we recommend conducting a temporal study by collecting samples from these duck species over multiple years to deepen our understanding of their dietary spectra and variation.

Author Contributions

Conceptualization: S.Y.W.S., Y.-H.S., and I.W.Y.S. Methodology: S.Y.W.S. and E.S.K.P. Resources: S.Y.W.S. and I.W.Y.S. Investigation: E.S.K.P.,

L.Y.C., D.K.L., and S.Y.W.S. Formal analysis: P.-Y.H. Visualization: P.-Y.H. Writing – original draft: E.S.K.P. and P.-Y.H. Writing – review and editing: E.S.K.P., P.-Y.H., I.W.Y.S., Y.-H.S., and S.Y.W.S. Supervision: S.Y.W.S. Project administration: S.Y.W.S. Funding acquisition: S.Y.W.S.

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Disclosure

Permission to reproduce material from other sources: Illustrations of anatids used in Figure 3 were reproduced with the permission of Lynx Edicions.

Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The datasets generated for this study can be found in the NCBI Sequence Read Archive (BioProject ID PRJNA1285626).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Appendix S1:** edn370210-sup-0001-AppendixS1.pdf. **Appendix S2:** edn370210-sup-0002-AppendixS2.xlsx.