

ORIGINAL ARTICLE

Dietary diversity of multiple shorebird species in an Asian subtropical wetland unveiled by DNA metabarcoding

Pei-Yu Huang¹  | Emily Shui Kei Poon^{1,2}  | Lai Ying Chan¹ | David Tsz Chung Chan¹  | Stella Huynh¹ | Ivy Wai Yan So³ | Yik-Hei Sung⁴  | Simon Yung Wa Sin¹ 

¹School of Biological Sciences, The University of Hong Kong, Hong Kong, China

²The School of Life Sciences, The Chinese University of Hong Kong, Shatin, Hong Kong, China

³Wetland and Fauna Conservation Division, Agriculture, Fisheries and Conservation Department, Hong Kong SAR Government, Hong Kong, China

⁴Science Unit, Lingnan University, Tuen Mun, Hong Kong, China

Correspondence

Simon Yung Wa Sin, School of Biological Sciences, The University of Hong Kong, Hong Kong, China.
Email: yungwa.sin@gmail.com

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Abstract

Global declines in shorebird populations resulting from foraging habitat loss have been recently reported, and the situation within the East Asian-Australasian Flyway (EAAF) is particularly concerning. Despite previous studies that analyzed the foraging niches of shorebirds worldwide, the dietary niche dynamics of shorebirds coexisting in Asia are very poorly understood. This study is therefore among the early few that aim to unveil the trophic organization of shorebirds in a subtropical wetland within the EAAF which is vital for species conservation. Our study first determined the dietary spectra of more than 10 shorebird species, such as *Calidris ferruginea* (near threatened), *Charadrius leschenaultii*, and *Pluvialis squatarola*, by applying DNA metabarcoding with 18S and COI markers to fecal DNA. The diet of *Tringa stagnatilis* was also characterized, which was previously undescribed. Shorebirds that occurred in the wetland consumed a variety of food items, primarily a high abundance of malacostracans, mollusks, annelids, insects, and some arachnids. Different proportions of plant materials were also detected in many shorebird species. Using the data, we then revealed clear patterns of inter- and intraspecific variations between these shorebirds. Importantly, we specifically compared the similarities of the spring diets among seven sympatric shorebird species. We found that the dietary compositions of the seven species have segregated from each other to varying levels, but the many similar taxa we identified in the diets among these shorebirds imply that these populations of shorebirds could be competing at different levels. Thus, any reductions in the abundance and diversity of these important food resources would likely intensify their inter- and intraspecific competition, and simultaneously threaten the survival of multiple species. With these findings, conservation measures must be taken to protect and monitor the vital food resources for these energy-deprived shorebirds during migration.

KEYWORDS

conservation, diet partitioning, environmental DNA, foraging ecology, intraspecific dietary variation, migratory birds

Pei-Yu Huang and Emily Shui Kei Poon contributed equally to this work and share the first authorship.

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1 | INTRODUCTION

Shorebirds (order Charadriiformes) are medium-sized wading birds, and most of them are migratory (Piersma & Lindström, 2004). During the non-breeding season, migrants rely on a limited number of wetlands as their staging or wintering grounds after long flights, which are mostly located in coastal estuaries, bays, or inland wetlands, to obtain food for subsequent journeys (Butler et al., 2001). Previous studies on the feeding habits of migratory shorebirds have shown that most shorebird species are carnivorous, which takes advantage of the high abundance and diversity of intertidal macroinvertebrates inhabiting wetlands (Micael & Navedo, 2018). Because a large number of shorebirds often exhibit similar resource requirements and aggregate to form multispecies foraging flocks at the same site and time, these migrants are some of the most important predators in wetland habitats (Joulami et al., 2019). Based on previous studies on shorebird foraging ecology, the occurrence of shorebirds in high density and diversity in a wetland can be attributed to the high abundance and availability of their macroinvertebrate prey or to the niche differentiation between sympatric shorebirds (Bocher et al., 2014).

Most studies on shorebird foraging ecology in recent decades have been extensively conducted on species that have used wetlands along the American Flyways (Faria et al., 2018; Martínez-Curci et al., 2015; Novčić, 2019) and the African-Eurasian Flyway (Bocher et al., 2014; Lourenço et al., 2016, 2017). Among these studies, several have aimed to investigate the role of resource niche partitioning as a mechanism that maintains the diversity and stable coexistence of shorebird species within their communities. Their findings generally supported the ecological theory that sympatric but competing shorebird species in a heterogeneous habitat often exhibit certain levels of niche partitioning, for example, dietary segregation (Lourenço et al., 2017; Martínez-Curci et al., 2015). These studies suggest that dietary niche partitioning between and within shorebird species is possible because of their adaptive variations in phenotypes and behaviors (Durell, 2000). These adaptive variations facilitate their dietary segregation through one or multiple aspects, including their different choices on target prey, foraging habitats, and migration or foraging time (Catry et al., 2014). Within some shorebird species, conspecifics can even differentiate their diets between sexes or age groups to reduce intraspecific competition by targeting either different prey species or different sizes of the same prey species (Hall et al., 2021). However, despite the findings from these studies, the dietary spectra and niche dynamics of shorebirds occurring in Asia are still very poorly understood (Hua et al., 2015). It was shown that the foraging behaviors of some shorebird species and their consequent diets, even of highly stereotyped ones, could vary with geographical locations (Masero et al., 2007; Turpie & Hockey, 1997). Hence, to understand the dietary niche dynamics of shorebirds occurring in Asia, it is necessary to conduct empirical research on the shorebirds that have been using the East Asian-Australasian Flyway (EAAF).

In addition, wetland loss and degradation, environmental pollution, invasive alien species, and the broad effects of climate change

are some major threats that are currently impacting biodiversity worldwide (Melville et al., 2016). In recent decades, global shrinkages of shorebird populations have been documented, and the situation within the EAAF is of particular concern (Chan et al., 2019; Studds et al., 2017). As millions of shorebirds migrate along the EAAF every year (Hansen et al., 2016), any reductions in the abundance and diversity of prey in wetlands can intensify both the inter- and intraspecific competition between sympatric shorebirds, which may further threaten their survival and population sustainability (Zhang et al., 2018). Therefore, understanding the dietary niche dynamics of shorebirds has always been important from a conservation perspective because knowledge is essential to the prediction and assessment of the negative effects caused by anthropogenic threats to shorebirds using the flyway. Among the coastal sites in the EAAF, Hong Kong is in a subtropical region, a central spot in the migratory corridors. An important part of this spot is the Mai Po Inner Deep Bay Ramsar Site (Mai Po), which comprises several habitat types in 1500 hectares, including an extensive intertidal mudflat (Sung et al., 2021). It is frequented by shorebirds as a staging or wintering ground annually. An average of over 30,000 birds of approximately 50 species of shorebirds visited Mai Po every year during 2016–2021 (Anon, 2020). According to the IUCN Red List of Threatened Species, 20 percent of these species are considered globally threatened or near-threatened (IUCN, 2021). *Calidris ferruginea* (curlew sandpiper, Near Threatened), *Charadrius leschenaultii* (greater sand-plover), and *Pluvialis squatarola* (black-bellied plover) are several examples that are known to aggregate in substantial numbers at the Mai Po en route to the northern breeding grounds during their spring passage every year (Anon, 2020). Thus, Mai Po has offered us a valuable opportunity to explore the foraging ecology of these migratory shorebirds.

However, trophic niche partitioning that maintains species coexistence has always been difficult to measure. Traditional methods used in the dietary studies of shorebirds were mostly lethal and involved microscopic examination of their digested contents in formalin-fixed digestive systems of sacrificed birds, often in small numbers (Rundle, 1982). Identifying prey taken by foraging shorebirds merely through observation has also been highly challenging because of, for example, their steady probing in sediment, the small sizes of their invertebrate prey, and rapid movements in expansive foraging areas (Novčić et al., 2021). Considering the lack of knowledge about the foraging ecology of shorebirds migrating in Asia, this study applied DNA metabarcoding, a noninvasive approach, to investigate the diets of more than 10 shorebird species that used a subtropical wetland within the EAAF. Moreover, the dietary compositions of some of our studied species, such as *Tringa stagnatilis* (marsh sandpiper), have never been determined previously. By collecting more than 190 fecal samples from these shorebirds, we aim to (1) determine the dietary compositions of sampled shorebirds; (2) examine the inter- and intraspecific dietary variations of these shorebird species; and (3) estimate the levels of dietary segregation of seven sympatric shorebird species while at Mai Po during their spring passage by comparing the

similarities of their dietary compositions. Our findings not only shed light on the shorebird community organization in Asia but also essentially contribute insights into the species conservation and habitat management for shorebirds using the EAAF, especially globally threatened species.

2 | MATERIALS AND METHODS

2.1 | Sample collection and DNA metabarcoding

The north-western part (22°29'48.5"N 114°02'02.5"E) of Hong Kong is a wetland in a shallow bay which is an extensive intertidal mudflat backed by a mangrove forest, reedbeds, and traditionally managed aquaculture ponds (Huang et al., 2021). We collected fecal samples in the wetland in Mai Po Inner Deep Bay Ramsar Site (Mai Po) (22°29'20.7"N 114°02'09.9"E), and permissions to enter Mai Po where access is restricted by law were obtained. Approvals for experiments on animals were granted by the Department of Health (license no. 21-603 in DH/HT&A/8/2/3 Pt. 27) and the HKU Committee on the Use of Live Animals (license no. 5769-21). Special Permit under Wild Animals Protection Ordinance for the trapping and handling of wild birds was also obtained. Shorebirds migrated to Mai Po were captured by mist net during 8–11 p.m. between January 2019 and September 2020. Each bird was identified to species by morphology. To avoid cross-contamination from multiple shorebird species, only one or several individuals of the same species were temporarily kept in one breathable container for defecations. All captured birds were released back into the wild afterward. Each fresh fecal sample was collected inside the containers using a sterilized disposable spatula. Recapturing and resampling the same individual was possible, but the chance was very low.

In this study, we have collected 191 fecal samples from 22 shorebird species in 13 genera [including *Calidris ferruginea* (curlew sandpiper), *Charadrius leschenaultii* (greater sand-plover), *Charadrius mongolus* (lesser sand-plover), *Pluvialis squatarola* (black-bellied plover), *Tringa stagnatilis* (marsh sandpiper), *Tringa totanus* (common red-shank), *Xenus cinereus* (Terek sandpiper), *Actitis hypoleucos* (common sandpiper), *Arenaria interpres* (ruddy turnstone), *Calidris alpina* (dunlin), *Calidris canutus* (red knot), *Calidris ruficollis* (red-necked stint), *Calidris tenuirostris* (great knot), *Charadrius alexandrinus* (Kentish plover), *Gallinago megala* (Swinhoe's snipe), *Glareola maldivarum* (oriental pratincole), *Limicola falcinellus* (broad-billed sandpiper), *Limosa* (black-tailed godwit), *Numenius phaeopus* (Eurasian whimbrel), *Pluvialis fulva* (Pacific golden plover), *Recurvirostra avosetta* (pied avocet), and *Tringa nebularia* (common greenshank); Table S1].

Each sample was preserved in absolute ethanol in the field and stored at -80°C on the same day until DNA extraction. The QIAamp Fast DNA Stool Mini Kit (Qiagen) was used to extract the fecal DNA. We included mock communities and negative controls during DNA extraction in our analyses (Appendix S1 and Table S2a). We quantified the fecal DNA using the Qubit dsDNA high-sensitivity (HS) assays on a Qubit 4 Fluorometer (Invitrogen).

Previous shorebird studies have reported that the major prey of shorebirds included arthropods, such as crustaceans and insects. Therefore, we used a universal genetic marker (18s_SSU3_F: 5'GGTCTGTGATGCCCTTAGATG3' and 18s_SSU3_R: 5'GGTGTGTACAAAGGGCAGGG3') that amplifies the V7 region of 18S small subunit of nuclear ribosomal DNA (rDNA; ca.170bp) (McInnes et al., 2017). We also used another COI marker specific to macroinvertebrates (COI_III_B_F: 5'CCIGAYATRGICITYCCICG3' and COI_III_C_R: 5'GGIGGRTAICIGTTCAICCC3'), such as class Insecta and Gastropoda, that targets the Folmer region of mitochondrial cytochrome c oxidase I (COI; ca. 86bp) (Shokralla et al., 2015). All fecal DNA samples, mock communities, and negative controls were used for library preparation using 18S and COI markers through a two-step polymerase chain reaction (PCR; Appendix S1). A library multiplex was created for each marker by pooling libraries from different samples in an equimolar ratio. Each library was sequenced to a depth of approximately 400k reads on a NovaSeq instrument (PE 150bp reads; Illumina) by Novogene Corporation.

2.2 | Bioinformatics

The demultiplexed raw paired-end fastq reads of 18S and COI markers were preprocessed by paired read merging, adapter trimming, and quality filtering. Paired-end reads were merged using USEARCH v11.0.667 (Edgar, 2010) with the -fastq_mergepairs function. PCR primer sequences were trimmed using CUTADAPT v2.4 (Martin, 2011) with the *linked adapter* mode (max_error_rate = 0.15). We kept only those merged reads that matched the 18S or COI primer sequences. The quality of reads was assessed using the FastQC v0.11.8 (Andrews et al., 2010) and the -fastq_eestats2 command of VSEARCH (Rognes et al., 2016). Then, we retained the high-quality reads that are within the target lengths (18S: 130–175bp; COI: 60–85bp) and with the expected number of errors per read <1 (-fastq_maxee 1), using the -fastq_filter function in VSEARCH. All preprocessed reads were dereplicated (VSEARCH -derep_full-length), from which the amplicon sequence variants (ASVs) were then generated by removing the chimeras and singletons (with abundance <0.0001% of all reads, that is, 125 reads for 18S and 207 reads for COI) using USEARCH -unnoise3 (Edgar, 2016b; Edgar & Flyvbjerg, 2015). We clustered all preprocessed reads into ASVs by using a 99% similarity threshold (VSEARCH -usearch_global -id 0.99).

Taxonomic classification was conducted in two steps to achieve higher taxonomic resolution. We firstly assigned each ASV to the lowest identifiable taxonomic level using the SINTAX algorithm in USEARCH (Edgar, 2016a) with a bootstrap cutoff of 0.7. The ribosomal RNA database SILVA (release 132, July 2017; Quast et al., 2013) and mitochondrial reference database MIDORI (release GB240; Machida et al., 2017) were used for 18S and COI datasets, respectively. We again searched ASVs from both datasets against the NCBI database (non-redundant nucleotide sequences) and obtained the best 1000 blast hits with similarity above 99% and

e-value $<1e-50$ for 18S or $<1e-10$ for COI. Afterward, we assigned the lowest common taxonomic level shared by 95% of 18S blast hits (≥ 100 bps) or 80% of COI blast hits (≥ 70 bps) using BASTA (Kahlke & Ralph, 2019) with the algorithm of the lowest common ancestor (LCA). The results were then combined by assigning ASVs with lower ranks of taxonomies.

Potential false-positive reads in the samples were filtered out by applying thresholds defined by mock communities. Contaminant ASVs defined by negative controls were also eliminated. Non-dietary items (e.g., Aves, Bacteria, and Protists) and those with low taxonomic resolution (e.g., Eukaryota and Chordata) were discarded. After the above treatments, samples with read numbers <500 were removed, guided by the read number-based rarefaction curves. The mean numbers of reads per sample for 18S and COI are 121,139 and 126,147, respectively (Appendix S1 and Table S3).

2.3 | Data analysis

We used all 18S data of 191 samples from 22 shorebird species and COI data of 173 samples from 20 shorebird species (*R. avosetta* and *A. interpres* were excluded for COI after data filtration because of the low numbers of reads) for further data analyses. In the 18S dataset, 32 taxonomic categories were summarized from 57 taxa in 102 ASVs, while in the COI dataset, 27 taxonomic categories were summarized from 61 taxa in 133 ASVs (Appendix S1 and Table S3). The lowest identifiable taxonomic levels in the 18S dataset included 10 classes, 18 orders, six families, 10 genera, and 13 species (Table S3a), while the COI dataset included three phyla, four classes, seven orders, 10 families, seven genera, and 30 species (Table S3b). We summarized the data as (1) percentage of read count for each taxon in a sample (relative read abundance, RRA), (2) percentage of occurrence for each taxon in a sample (weighted percentage of occurrence, wPOO), and (3) proportion of samples in which a taxon is detected (frequency of occurrence, FOO) (Deagle et al., 2019; Lee et al., 2021). The RRA/wPOO at the population level is presented as the mean of RRA/wPOO of all individual samples of a shorebird species, whereas FOO estimates the frequency of incidence of a taxon within all samples of a shorebird species. The results were visualized with the R packages ggplot2 v3.3.5 (Wickham, 2016), alluvial v0.12.3 (Brunson, 2020), and pheatmap v1.0.12 (Kolde, 2019).

2.3.1 | Data analysis on seven sympatric shorebird species during spring

For our objective 3, we only analyzed seven sympatric shorebird species for which sample sizes are ≥ 5 and we only used feces that were collected between February and May in 2020 during the spring migration period (18S, $n = 131$; COI, $n = 120$). The seven shorebird species were *Charadrius leschenaultii*, *Calidris ferruginea*, *Pluvialis squatarola*, *Tringa stagnatilis*, *Xenus cinereus*, *Charadrius mongolus*, and *Tringa totanus* (Table S1). We evaluated the relationship between

sample size and the dietary Chao2 species richness by rarefaction curves (Figure S1) based on the hill numbers of order $q = 0$ calculated from ASVs or taxa using the R package iNext (Hsieh et al., 2016).

Diversity analyses of the dietary compositions of the seven shorebird species were based on Hill numbers (represented as "D") (Hill, 1973) and were carried out by using the R package hilldiv v1.5.1 (Alberdi & Gilbert, 2019b). The sensitivity of Hill number toward abundant ASVs is modulated by the q value (the order of diversity) and more weight is given to the abundant ASV when the q value increases. The richness is indicated by the Hill number of order $q = 0$ (0D) in which only the occurrence of each ASV is considered. Hill number of order $q = 1$ (1D) considers both richness and evenness, which is equivalent to the exponential of Shannon's diversity index. For the order of diversity $q = 2$ (2D), abundant ASVs are overweighed, thus yielding Hill number equivalent to the multiplicative inverse of Simpson's dominance index (Jost, 2006). Pairwise diversity comparisons at sample level between shorebird species were performed using the Kruskal-Wallis test, followed by a posthoc Dunn test, using Benjamini-Hochberg correction ($p < 0.05$) with the function `div_test` of hilldiv. Diversities based on Hill numbers can be used further to calculate alpha, beta, and gamma diversities, which engage a multiplicative definition ${}^qD_\gamma = {}^qD_\alpha \times {}^qD_\beta$. Alpha diversity (${}^qD_\alpha$) measures diversity at the sample level and gamma diversity (${}^qD_\gamma$) at the population or species level, which were computed by the function `div_profile` in hilldiv. Beta diversity (${}^qD_\beta$) denotes the differences between individual samples, which is derived from dividing γ -diversity by α -diversity (Chao et al., 2012; Jost, 2007).

We inferred the differences in taxa compositions of samples between shorebird species by calculating the pairwise binary Jaccard dissimilarity distances based on the occurrence of each ASV, and pairwise Bray-Curtis dissimilarity distances based on the fourth root transformed RRA of each ASV. We visualized the results with principal coordinates analysis (PCoA) using the functions `ordinate` and `plot_ordination` in R package phyloseq v1.30.0 (McMurdie & Holmes, 2013). We performed hierarchical clustering analysis using Ward's method in `hclust` function. Permutational multivariate analysis of variance (PERMANOVA) test was performed to evaluate the separation of dietary compositions between the shorebird species, which was estimated by the centroid and dispersion of diet compositions of individual samples in each group in a measure space. Homogeneity of intra-group beta-dispersion ($p > 0.05$), inferred with the function `adonis` in `vegan` v2.5.7 (Oksanen et al., 2019), is the prerequisite for significant interspecific variation for the PERMANOVA test. We tested pairwise PERMANOVAs of the shorebird species using the pairwise `adonis` (Martinez-Arbizu, 2019) and evaluated their beta-dispersions with the `betadisper` function in `vegan`. Similarity percentage (SIMPER) was calculated to assess the contribution of an individual taxon to the variations between shorebird species and was tested using the non-parametric Kruskal-Wallis rank-sum test by the functions `simper.pretty` and `kruskal.pretty` in R scripts `simper.pretty.R` and `R_kruskal.R` (Steinberger, 2018). Only taxa contributing $>1\%$ of the variance ($p < 0.05$) are presented. PERMANOVAs and SIMPER analyses of RRA were performed based on Bray-Curtis

distances, and those of occurrence data were conducted based on binary Jaccard dissimilarity distances.

To describe the interspecific dissimilarity and overlap of dietary compositions between the seven shorebird species in spring, we presented both pairwise Bray–Curtis and Jaccard dissimilarity index (0: similar; 1: dissimilar) and Pianka's niche overlap index (Pianka, 1974) based on the RRA and occurrence data of ASVs. Pianka's index, calculated using the R package *spaa* v0.2.2 (Zhang et al., 2016), ranges from 0 to 1 indicating no overlap to complete overlap. Statistical significances of the pairwise Pianka's index were evaluated with reference to 1000 permutations of a null model that randomized the dietary items values but maintained the observed dietary breadth of each shorebird species, using *ra3* randomization algorithm from the R package *EcoSimR* (Gotelli et al., 2020).

3 | RESULTS

3.1 | Overall dietary composition

The overall diet of shorebirds was mainly composed of food items (>1% RRA in 18S or COI datasets) belonging to the phyla Arthropoda (including the orders Decapoda and Isopoda in the class Malacostraca; the orders Diptera, Coleoptera, Blattodea, and Psocoptera in the class Insecta; the order Podocopida in the class Ostracoda; and the order Sarcopiformes in the class Arachnida), Mollusca (including the classes Bivalvia and Gastropoda), clade Streptophyta (class Magnoliopsida), and Annelida (including classes Polychaeta). Other minor food items, such as taxa in the phylum Cnidaria, the class Actinopterygii (phylum Chordata), and the class Maxillopoda (phylum Arthropoda), were also detected (Figure 1; Table S4).

Malacostracans constituted almost half of the overall diet of shorebirds (47.7% RRA and 67% FOO by 18S) in which decapods were the major component. *Gelasimus* spp. (fiddle crabs), *Macrophthalmus* spp. (crabs in Indo-Pacific), *Metapenaeus* spp. (prawns), and *Emerita analoga* (Pacific sand crabs) were several decapod taxa detected by COI (Figure 1; Table S4). Another major component was mollusks (16.5% RRA and 29.3% FOO by 18S). Gastropods, including taxa in the order Stenothyridae (aquatic snails) and *Tarebia granifera* (quilted melania) detected by COI, were the major constituents of mollusks. Interestingly, we detected more than 10% of Magnoliopsida (flowering plants), including species in the order Poales (grasses, bromeliads, and sedges) and *Ruppia maritima* (beaked tasselweed), in the samples. For annelids (9.7% RRA and 28.8% FOO by 18S), apart from the class Clitellata, species in the class Polychaeta (bristle worms) were also detected and those in the family Nereididae (ragworms) accounted for most of them, followed by *Laonome* spp. (marine tube worms) (Figure 1; Table S4).

In addition, we detected a diverse group of insects (7.3% RRA and 23% FOO by 18S) in the shorebird diets, particularly dipterans, such as *Rhipidia sejuga* (crane flies), *Drosophila melanogaster* (small fruit flies), *Hermetia illucens* (black soldier flies), *Musca*

domestica (houseflies), and those in the family Tephritidae (fruit flies). Coleopterans, such as those in the family Chrysomelidae (leaf beetles) and *Bembidion niloticum* (ground beetles), were also revealed by COI. Other minor taxa, such as *Blattella germanica* (German cockroaches) in the order Blattodea, *Saldula* spp. (shore bugs) in the order Hemiptera, and *Nanopsocus oceanicus* (barklice) in the Psocoptera, were also identified. Bivalves (2.6% RRA and 9.4% FOO by 18S), such as taxa in the order Cardiida (marine clams) and *Solen strictus* (Gould's razor shells), were also detected but in small proportions (Figure 1; Table S4). Other less abundant taxa, including the classes Arachnida (joint-legged invertebrates, e.g., the order Sarcopiformes and species in the order Araneae, such as *Tetragnatha* spp.), Ostracoda (seed shrimps, e.g., the order Podocopida), Maxillopoda (barnacles and copepods), cnidarians (orders Actiniaria and Bivalvulida), and actinopterygians (ray-finned fishes), contributed 0.2%–3.1% RRA by 18S (Figure 1a,b; Table S4). Large proportions of insects and other arthropods detected by COI were unable to be resolved to lower taxonomic levels; thus, the diversity in the overall shorebird diet here could have been underestimated (Figures 1c,d, 2b and 3b; Table S4b).

3.2 | Variations in the diet compositions between shorebird species and individuals

Our results revealed distinct patterns of inter- and intraspecific variations between the diets of shorebirds (Figures 2a,b and 3a,b; Tables S5–S7). *Charadrius leschenaultii* (18S, $n = 45$; COI, $n = 39$; Table S1) mainly consumed malacostracans, particularly decapods (52.5% RRA and 80% FOO by 18S). They also fed on several taxa groups of insects (22.8% RRA and 60% FOO by 18S; 76.8% RRA and 100% FOO by COI), including dipterans (e.g., *R. sejuga*, *D. melanogaster*, and Tephritidae flies), coleopterans (e.g., *B. niloticum* and Chrysomelidae beetles), and others (e.g., *B. germanica* and *Saldula* spp.). They only consumed a very small proportion of mollusks [e.g., bivalves, gastropods, and polyplacophore *Cyanoplax keepiana* (chitons)] (Figures 2 and 3; Tables S5–S7). We found that *C. mongolus* ($n = 7$ for both 18S and COI) preyed upon Podocopida ostracods (56% RRA and 57.1% FOO by 18S), malacostracans (26% RRA and 42.9% FOO by 18S, e.g., decapods), polychaetes (e.g., Nereid worms), insects (e.g., dipterans *D. melanogaster* and Tephritidae flies; psocids; coleopterans Chrysomelidae beetles), and only a very small proportion of gastropods (Figures 2 and 3; Tables S5–S7). For *C. alexandrinus* (18S, $n = 3$; COI, $n = 2$), malacostracans and Magnoliopsida plants accounted for almost all taxa detected by 18S, and insects contributed to all COI reads (Figures 2 and 3; Tables S5–S7).

Calidris ferruginea (18S, $n = 43$; COI, $n = 41$) consumed not only malacostracans [31.5% RRA and 55.8% FOO by 18S; e.g., decapods (such as *Metapenaeus* spp.), isopods, and amphipod *Caleidoscopsis* spp.] but also annelids (20.9% RRA and 44.2% FOO by 18S; e.g., polychaetes, such as the Nereid worms and *Laonome* spp., and clitellates), mollusks (e.g., gastropods, bivalves, such as the Cardiida clams, and polyplacophore *C. keepiana*), Magnoliopsida

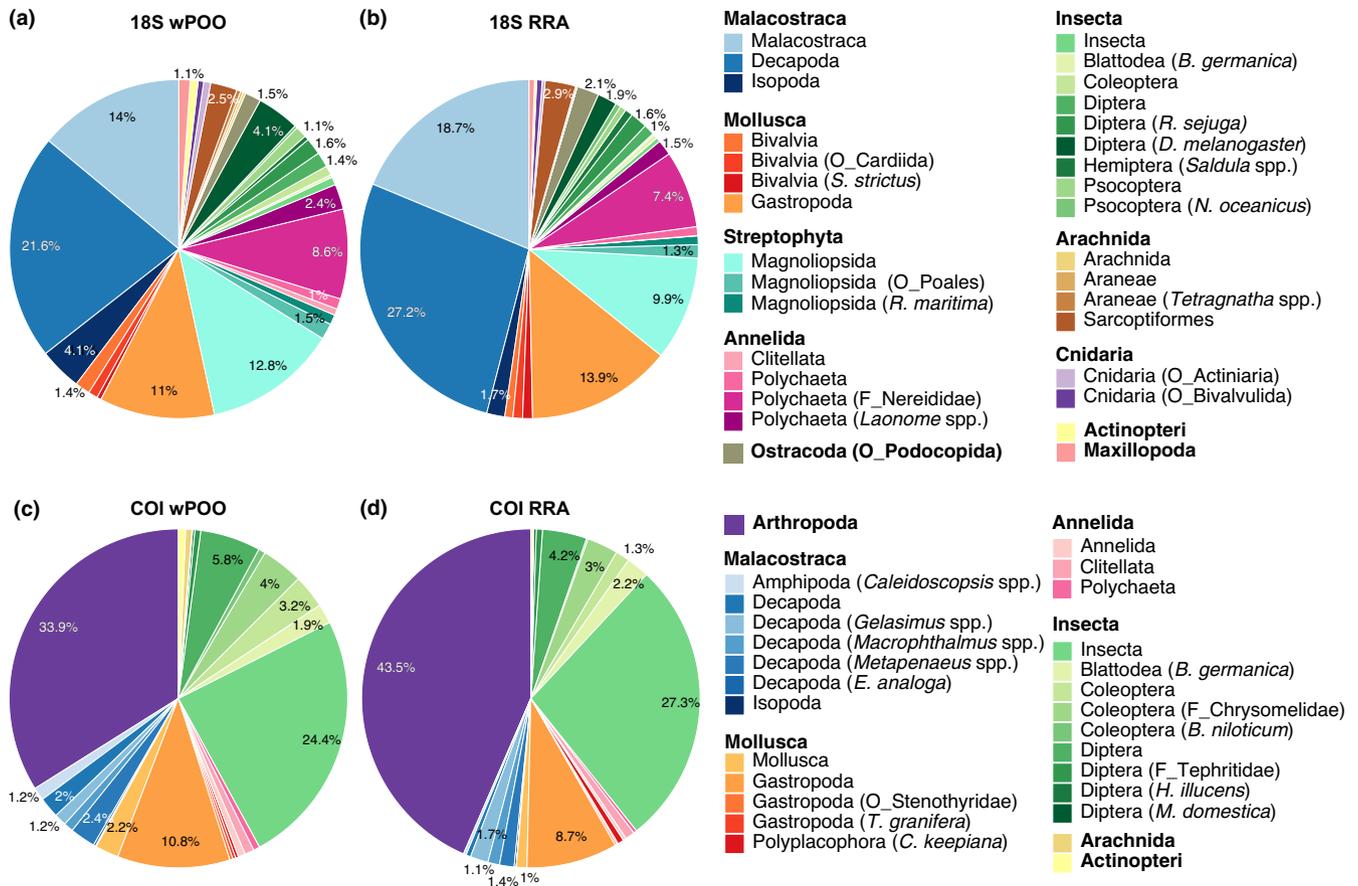


FIGURE 1 Dietary compositions of shorebirds determined using the 18S rDNA and COI markers on fecal DNA. (a, b) In the 18S dataset ($n = 191$), 32 dietary categories were summarized from 57 taxonomic groups identified from 102 ASVs. (c, d) In the COI dataset ($n = 173$), 27 dietary categories were summarized from 61 taxonomic groups identified from 133 ASVs. Each taxon was categorized to the lowest taxonomic level when the number of reads was $>0.1\%$ of all taxa detected. Otherwise, the taxa were grouped into higher level of taxonomic category. Weighted percentages of occurrence (wPOO) (a, c) and relative read abundance (RRA) (b, d) of dietary categories are shown as color blocks. Only categories with RRA or wPOO $>1\%$ are indicated. F, family; O, order (see Table S4 for details).

plants (e.g., Poales and *R. maritima*), and others in smaller proportions [including species in the orders Sarcoptiformes (mites), Actiniaria (sea anemones), Bivalvulida (myxosporean parasites), Maxillopoda, and Actinopteri]. Insects were also detected by COI (36.5% RRA and 58.5% FOO; e.g., *B. germanica*, the Chrysomelidae beetles, and dipterans; Figures 2 and 3; Tables S5–S7). *C. alpina* (18S, $n = 8$; COI, $n = 7$) consumed many mollusks (54.5% RRA and 87.5% FOO by 18S; 34.5% RRA and 71.4% FOO by COI), mostly gastropods, and Cardiida clams were detected in a small proportion. Malacostracans were detected in lower amounts (e.g., isopods), followed by Magnoliopsida plants (e.g., Poales) and polychaetes (e.g., *Laonome* spp. and the Nereid worms; Figures 2 and 3; Tables S5–S7). *Calidris ruficollis* (18S, $n = 2$; COI, $n = 3$) mainly consumed insects (*N. oceanicus* and *D. melanogaster*), Sarcoptiformes mites, some malacostracans (decapods, including *Metapenaeus* spp., and amphipod *Caleidoscopsis* spp.), and Magnoliopsida plants. For *C. tenuirostris* and *C. canutus* ($n = 1$ – 2), gastropods were predominantly detected by 18S in their diets. Additionally, some insects were detected by COI in *C. tenuirostris* (Figures 2 and 3; Tables S5–S7).

Pluvialis squatarola (18S, $n = 23$; COI, $n = 20$) mainly fed on malacostracans (43.2% RRA and 56.5% FOO by 18S), such as decapods (e.g., *Metapenaeus* spp.), isopods, and amphipod *Caleidoscopsis* spp. They also preyed on mollusks (39.9% RRA and 60.9% FOO by 18S), particularly gastropods and bivalves (e.g., *S. strictus* and the Cardiida clams), followed by annelids [e.g., polychaetes (the Nereid worms and *Laonome* spp.) and clitellates] and Magnoliopsida plants. Species in the Actiniaria and the Sarcoptiformes were detected in very small proportions. Insects were also detected by COI (41% RRA and 60% FOO; e.g., dipterans; Figures 2 and 3; Tables S5–S7). For *P. fulva* (18S, $n = 3$; COI, $n = 4$), gastropods, Magnoliopsida plants, *Laonome* polychaetes, and beetles were detected in their diet (Figures 2 and 3; Tables S5–S7).

Tringa totanus (18S, $n = 16$; COI, $n = 14$) mostly consumed malacostracans [61.4% RRA and 81.3% FOO by 18S; e.g., decapods (such as *Metapenaeus* spp., *E. analoga*, and *Macrophthalmus* spp.), isopods, and amphipod *Caleidoscopsis* spp.]. Magnoliopsida plants, gastropods, and polychaetes (e.g., *Laonome* spp. and the Nereid worms) were detected in lower amounts. Some other taxa were detected in smaller proportions, including insects (e.g., psocids, *D. melanogaster*,

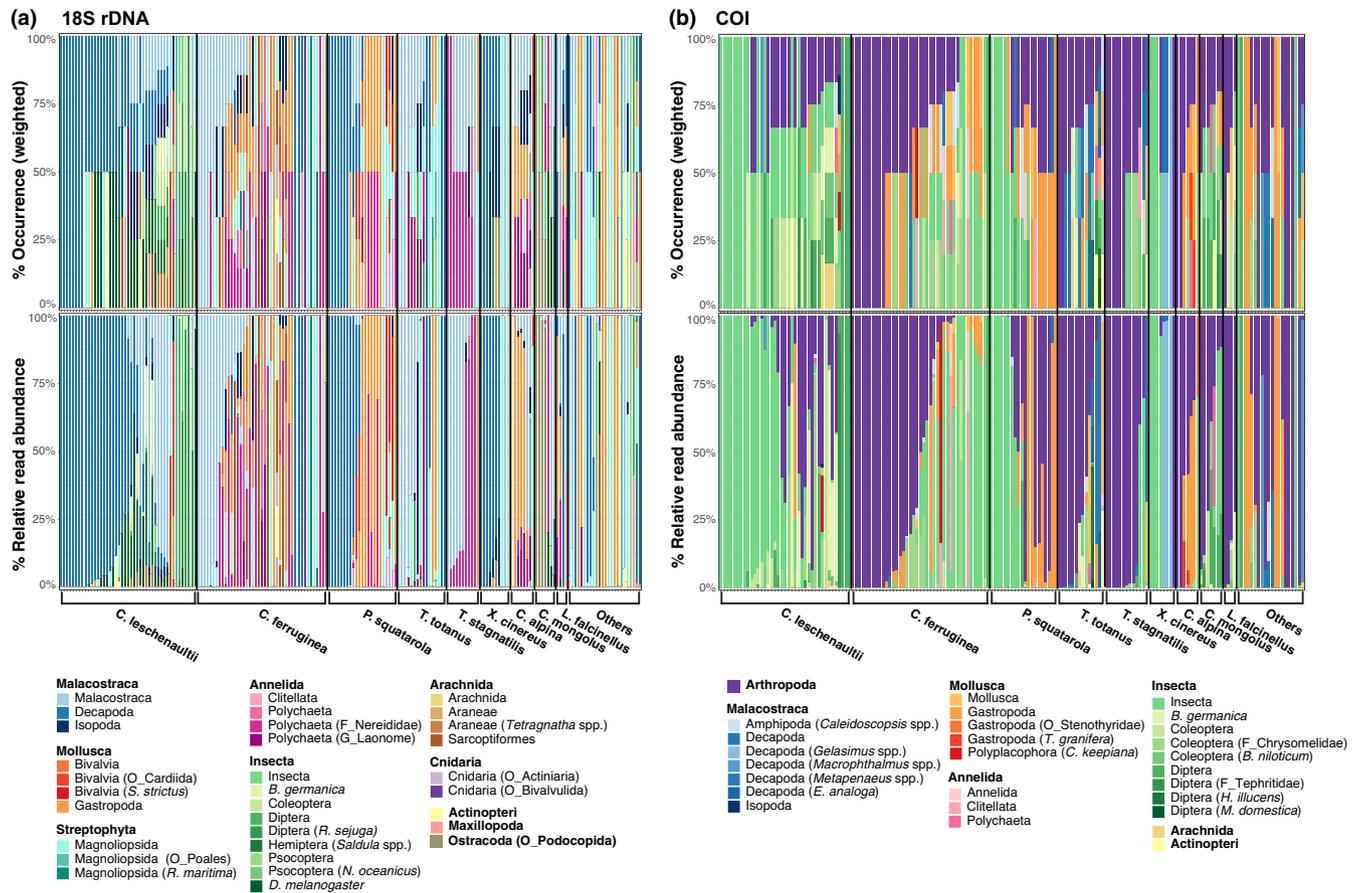


FIGURE 2 Taxa proportions in individual fecal samples of shorebirds detected using (a) 18S rDNA ($n = 191$) and (b) COI ($n = 173$) markers. Weighted percentage of occurrence (upper panel) and relative read abundance (lower panel) of each taxon are shown. Colored bars represent 32 and 27 dietary categories based on 18S and COI markers, respectively. Shorebird species in the “others” (from left to right) include (a) *Charadrius alexandrinus* ($n = 3$), *Pluvialis fulva* ($n = 3$), *Numenius phaeopus* ($n = 3$), *Calidris ruficollis* ($n = 2$), *Calidris canutus* ($n = 2$), *Recurvirostra avosetta* ($n = 2$), *Calidris tenuirostris* ($n = 2$), *Limosa limosa* ($n = 2$), *Glareola maldivarum* ($n = 1$), *Arenaria interpres* ($n = 1$), *Gallinago megala* ($n = 1$), *Actitis hypoleucos* ($n = 1$), and *Tringa nebularia* ($n = 1$); (b) *Charadrius alexandrinus* ($n = 2$), *Pluvialis fulva* ($n = 4$), *Numenius phaeopus* ($n = 2$), *Calidris ruficollis* ($n = 3$), *Calidris canutus* ($n = 2$), *Calidris tenuirostris* ($n = 1$), *Limosa limosa* ($n = 2$), *Gallinago megala* ($n = 1$), *Glareola maldivarum* ($n = 1$), *Actitis hypoleucos* ($n = 1$), and *Tringa nebularia* ($n = 1$). F, family; O, order (see Tables S1 and S7 for details).

H. illucens, *M. domestica*, Tephritidae flies, Chrysomelidae beetles, and *B. germanica*), fishes, and arachnids (Figures 2 and 3; Tables S5–S7). *T. stagnatilis* (18S, $n = 11$; COI, $n = 13$) consumed malacostracans (52.1% RRA and 72.7% FOO by 18S; e.g., isopods and decapods, including *Metapenaeus* spp.), Nereid worms (38% RRA and 81.8% FOO by 18S), gastropods, and a very limited amount of Magnoliopsida plants. Insects were detected by COI, including the Chrysomelidae beetles and dipterans (Figures 2 and 3; Tables S5–S7). Decapods, such as *Macrophthalmus* spp., accounted for almost all detected taxa in the *Tringa nebularia* sample ($n = 1$; Figures 2 and 3; Tables S5–S7).

Xenus cinereus (18S, $n = 10$; COI, $n = 8$) preyed upon many malacostracans (78.6% RRA and 80% FOO by 18S), mostly decapods (e.g., *Gelasimus* spp.) and some isopods. They also consumed Magnoliopsida plants (20% RRA and 20% FOO by 18S), insects (49.1% RRA and 50% FOO by COI; e.g., *R. sejuga*), Podocopida ostracods, and spiders (Figures 2 and 3; Tables S5–S7). *Limicola falcinellus* ($n = 4$) mainly consumed malacostracans (50.4% RRA and 100% FOO by 18S; e.g., decapods and isopods), gastropods (26% RRA

and 50% FOO by 18S), polychaetes (mostly the Nereid worms), and the Maxillopoda. Insects [e.g., *B. germanica*, coleopterans (including Chrysomelidae beetles and *B. niloticum*) and *D. melanogaster*], mollusks, and Poales were also detected (Figures 2 and 3; Tables S5–S7). The diet compositions of the remaining shorebird species ($n = 1$ –3 per species), including *Actitis hypoleucos*, *Arenaria interpres*, *Gallinago megala*, *Glareola maldivarum*, *Limosa limosa*, *Numenius phaeopus*, and *Recurvirostra avosetta*, are presented in Figures 2 and 3; Tables S5–S7.

3.3 | Diversities of the spring diets of seven shorebird species at the individual and population levels

3.3.1 | Alpha diversity

At the individual (or sample) level, despite the alpha diversities of the diet compositions detected by 18S (referred to as “18S diet

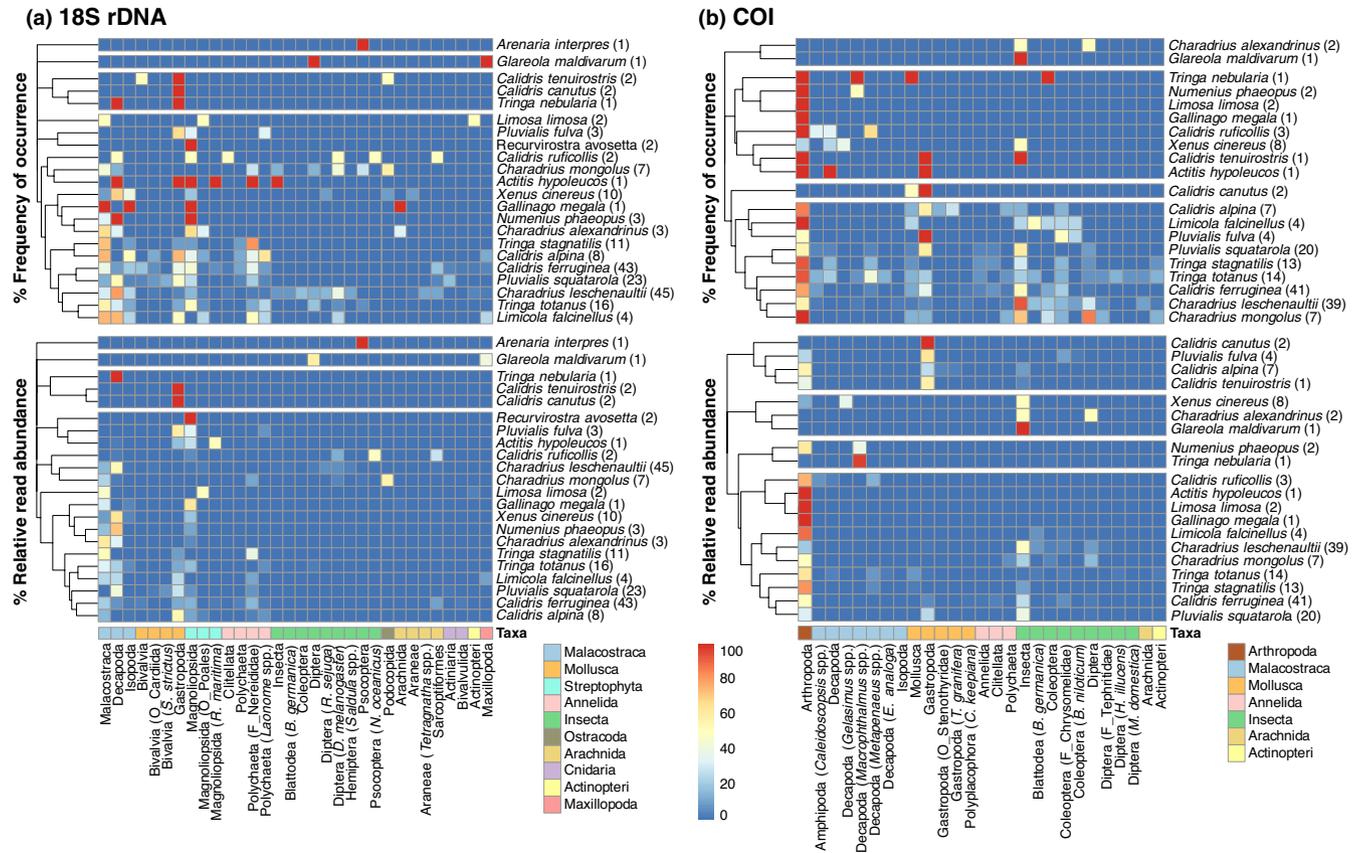


FIGURE 3 Heatmap of mean frequencies of occurrence (FOO, upper panel) and relative read abundance (RRA, lower panel) of dietary taxa in shorebird species identified using (a) 18S rDNA and (b) COI markers. Hierarchical clustering dendrograms for FOO were generated from the incidence-based Jaccard dissimilarities and for RRA from abundance-based Bray–Curtis dissimilarities of the dietary taxa compositions between shorebird species. The number in each bracket indicates the sample size of each species. Dietary categories in higher taxonomic categories are indicated as color blocks (see Tables S5 and S6 for details).

compositions” hereafter) being similar between the seven shorebird species, the alpha diversities of the diet compositions detected by COI (referred to as “COI diet compositions” hereafter) of *C. ferruginea*, *C. mongolus*, *T. stagnatilis*, and *T. totanus* were significantly higher than those of *X. cinereus* ($q = 1$ and 2 , $p < 0.05$) (Figures 4, 5, S2 and S3; Table S8). The detailed diet compositions of shorebirds at the species and individual sample levels during spring are shown in Figures 4, S2 and S3, Tables S4 and S9–S11.

While the variation between *C. ferruginea* and *X. cinereus* might be contributed by the difference in their sample sizes, *C. mongolus*, *T. stagnatilis*, and *T. totanus*, which had similar sample sizes as *X. cinereus* (Table S1), had 1.7–2.4 times more effective numbers of dietary ASVs detected by COI in an individual sample than *X. cinereus* ($q = 1$ and 2) (Alberdi & Gilbert, 2019a; Figure 5; Table S12).

3.3.2 | Gamma diversity

At the population (or species) level, although the species richness in the diets of *C. ferruginea* and *C. leschenaultii* was the highest (e.g., ${}^0D = 33$ – 38 by 18S; might be attributed to their large sample sizes, Table S12a; Figure 6), the dietary taxa were unevenly

consumed among individuals within the populations of these shorebird species. For example, in the *C. leschenaultii* population, the effective numbers of dietary ASVs drastically dropped by 80%–90% and 83%–92% with increased q values based on 18S and COI, respectively (${}^0D = 33$, ${}^1D = 6.5$, and ${}^2D = 3.4$ by 18S; ${}^0D = 49$, ${}^1D = 8.4$, and ${}^2D = 4.1$ by COI, Table S12; Figure 6; Alberdi & Gilbert, 2019a). This might be mainly due to the far more frequently and abundantly consumed malacostracans and insects within the *C. leschenaultii* population in spring (Figure S2a–c; Table S11). Another example of shorebird species having dietary taxa unevenly consumed by their population is *T. stagnatilis*. There was a dramatic drop in the gamma diversities when $q > 0$ (${}^0D = 17$, ${}^1D = 4.6$, and ${}^2D = 3.1$, Table S12) based on COI, together with the low gamma diversities revealed by 18S (${}^0D = 4$, ${}^1D = 2.1$, and ${}^2D = 2$) (Figure 6). The results indicated that the overall diet of *T. stagnatilis* in spring was only dominated by a limited number of dietary taxa (Figure S2; Table S11). When we compared *X. cinereus* to *C. mongolus* and *T. totanus*, which all had similarly smaller sample sizes, *X. cinereus* showed lower diet diversities at both the individual and population levels by the two markers (Figures 5 and 6), implying that *X. cinereus* diets are considerably specialized (Figure S2; Tables S11 and S12).

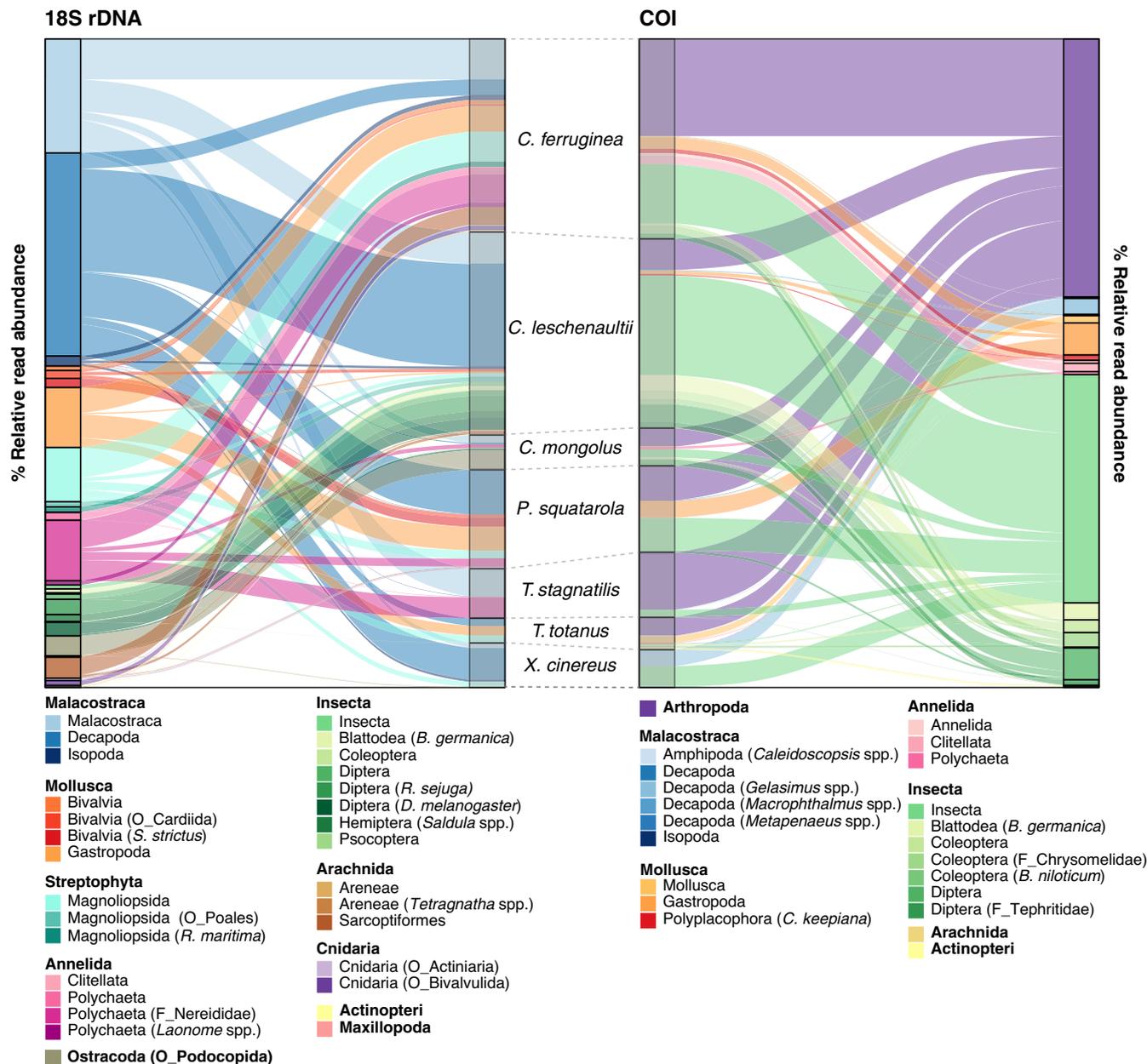


FIGURE 4 Relative read abundance of the dietary taxa of seven shorebird species in spring (February–May 2020) detected by 18S rDNA (left panel) and COI (right panel). Colors of the dietary strata and alluvia represent 30 and 22 dietary categories detected by 18S and COI markers, respectively. The width of each shorebird species stratum is proportional to its sample size (Table S1). The detailed numbers of relative read abundance of dietary taxa detected using 18S rDNA and COI are shown in Tables S9a and S10a, respectively.

3.3.3 | Dietary segregation

Based on the results of Bray–Curtis and Jaccard PCoA (Figure 7), Ward's hierarchical clustering (Figure S4), pairwise Bray–Curtis and Jaccard dissimilarity index (Table S13), pairwise Pianka's overlap index (Figures S5 and S6; Tables S13 and S14), and pairwise PERMANOVAs (Table S15), we found that most of the diet compositions of the seven shorebird species partially overlapped with each other, yet some exhibited a significant degree of segregation despite their different sampling sizes (PERMANOVA: 18S, $p = 0.001$; COI, $p = 0.001$). However, this might also be contributed by the non-homogeneous dispersion of individual samples within each shorebird

species (beta-dispersion: 18S, $p = 0.00003$; COI, $p = 0.017$). The dissimilarity and overlap indices range from no overlap (Pianka's index = 0, Bray–Curtis/Jaccard dissimilarity index = 1) to high overlap for RRA (Table S13a,c) or intermediate overlap for the occurrence of ASVs (Table S13b,d). Despite some overlap, the 18S diet compositions of *C. ferruginea* and *C. leschenaultii* were different, as shown by both abundance-based Bray–Curtis and incidence-based Jaccard PCoA (Figure 7; dissimilarities = 0.71–0.82, Table S13a,b). The variation was mainly contributed by the decapods (50.5% RRA and 15.8%–46.1% simpler variations, Tables S9a and S15a,b) and insects (*R. sejuga* and *D. melanogaster*, 5.9%–7.5% RRA and 4.4%–5.6% simpler variations) found in the *C. leschenaultii* diet as well as

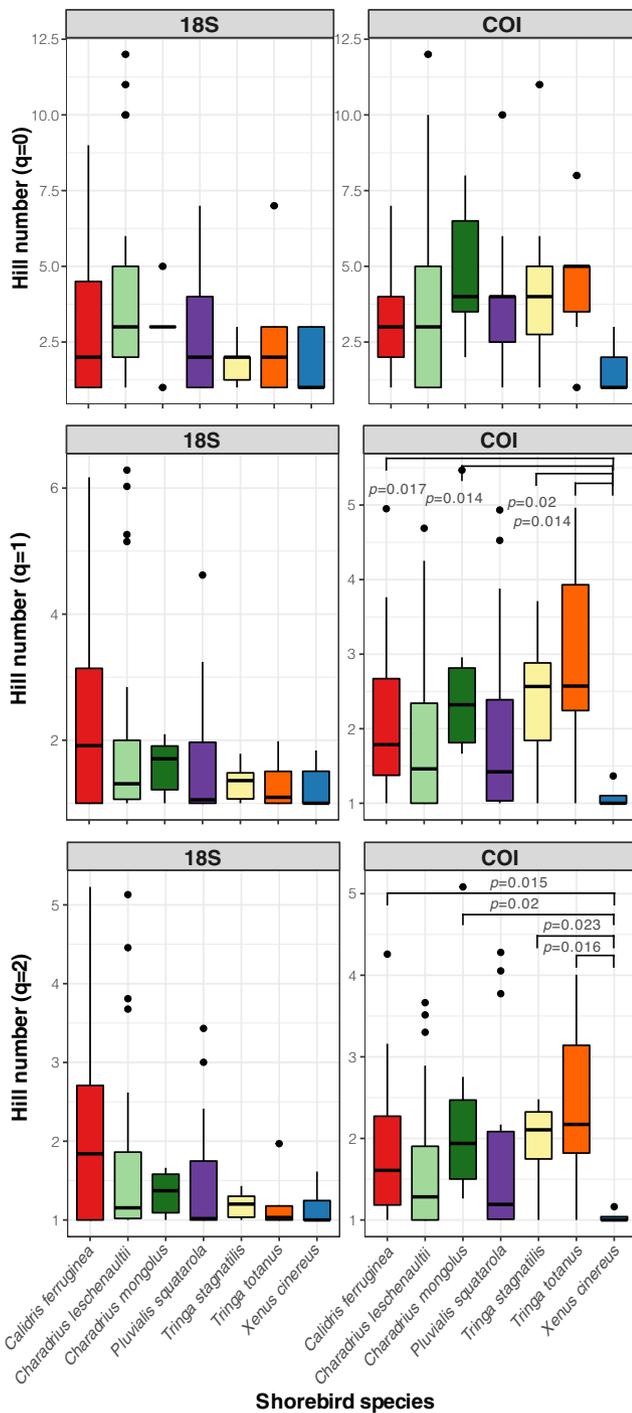


FIGURE 5 Alpha diversities of the dietary compositions in spring (February–May 2020) of seven shorebird species, characterized by 18S rDNA ($n = 131$, left panel) and COI ($n = 120$, right panel) markers. Hill numbers were obtained for $q = 0$, $q = 1$, and $q = 2$, with the abundance of dietary taxa being weighted increasingly. Each colored box represents the interquartile range with the median indicated by a line within the box. The upper and lower whiskers extend to the highest and the lowest values within the $1.5 \times$ interquartile range. Black dots are outliers. P -values of significantly different groups are shown above boxes. See Table S1 for sample sizes.

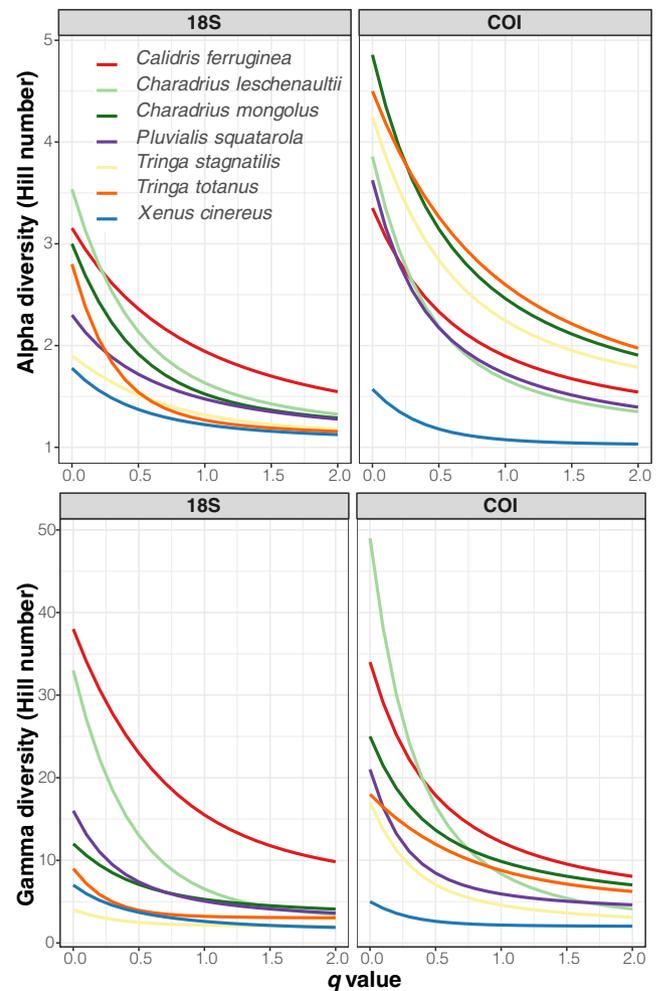


FIGURE 6 Diversity profiles of the dietary compositions in spring (February–May 2020) of seven shorebird species, characterized by 18S rDNA (left panel) and COI (right panel). Alpha diversities and gamma diversities are indicated as hill numbers with the increasing orders of diversity q . See Table S1 for sample sizes.

the abundant Magnoliopsida plants and Nereid polychaetes (14.5%–18.4% RRA and 2.5%–6.5%) detected in the *C. ferruginea* diet. Their COI diet compositions showed similar segregation patterns except that slightly more individuals of *C. leschenaultii* clustered with those of *C. ferruginea* (Figures 7 and S4).

Moreover, we found that the overall diet (both 18S and COI) of half of the *P. squatarola* population was similar to that of *C. leschenaultii*, while the diets of the rest resembled the *C. ferruginea* diet, and the overall diet largely overlapped with that of *T. totanus* (Figure 7; Table S13). The 18S diet of *P. squatarola* also overlapped with that of *X. cinereus*. For *X. cinereus*, their overall diet shared more similarity with that of *C. leschenaultii* than *C. ferruginea* (Figure 7; Table S13). *Xenus cinereus* diets were very different from those of *T. stagnatilis* (Figure 7; Table S13), which was mostly contributed by the decapods (71.1% RRA and 23.9%–37.6% variations by 18S, Tables S9a–c and S15a,b) and insects (56.1% RRA and 32.8%

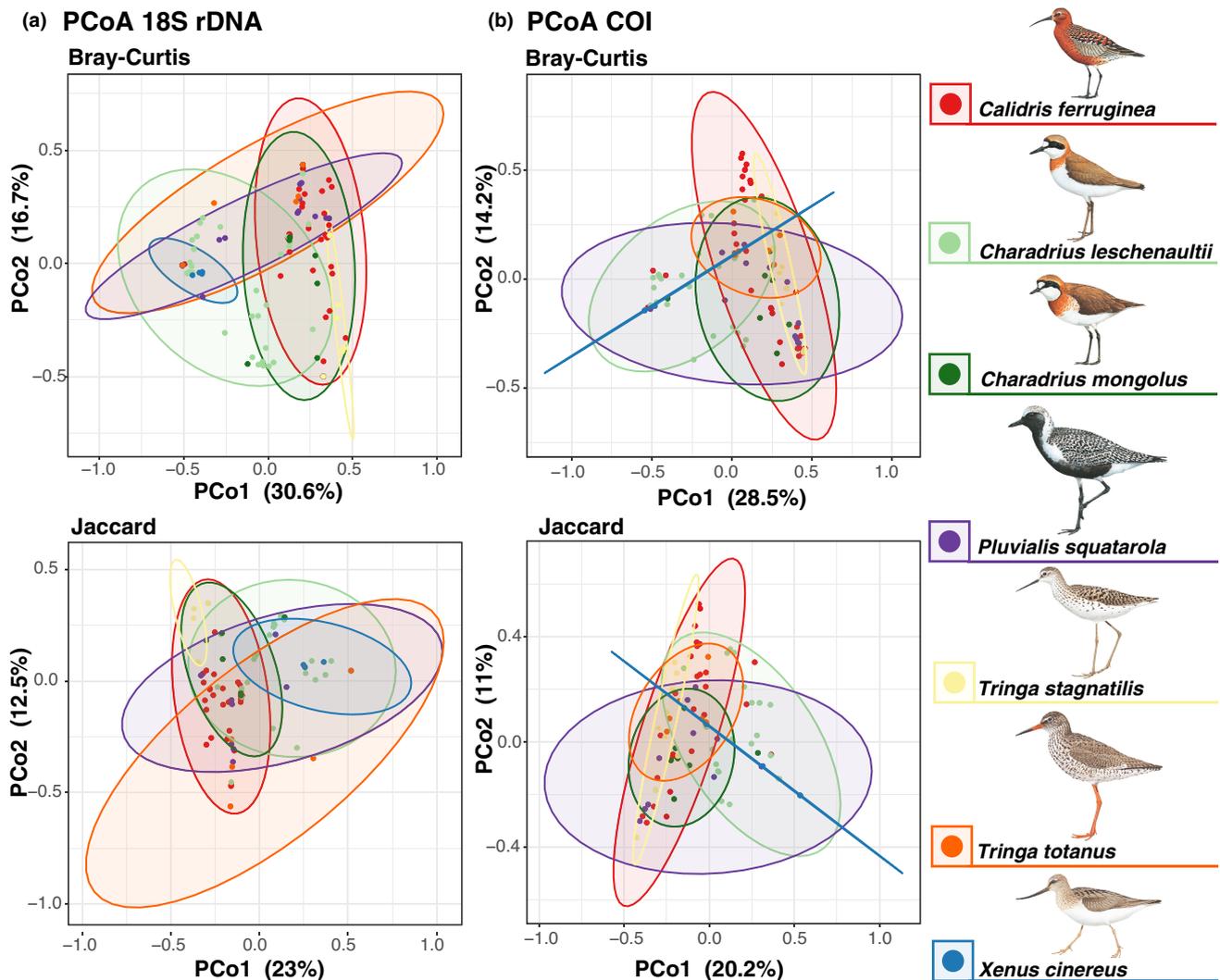


FIGURE 7 Principal coordinate analysis (PCoA) of the dietary compositions in spring (February–May 2020) of seven major shorebirds, detected by (a) 18S rDNA and (b) COI markers, based on Bray–Curtis distances estimated from relative read abundance data (upper panel) and binary Jaccard distances calculated from occurrence data (lower panel). The percentages of variations in diet compositions are shown in brackets along axes. Data ellipses were plotted based on the multivariate t -distribution at a level of 0.95 using function `stat_ellipse` in R package `ggplot2` v3.3.5 (Wickham, 2016). See Table S1 for sample sizes. Illustrations of shorebirds were reproduced with the permission of Lynx Edicions.

abundance-based variation by COI, Tables S10a–c and S15c,d) abundantly consumed by *X. cinereus* as well as other malacostracans and Nereid polychaetes dominantly eaten by *T. stagnatilis* (41.8%–56.6% RRA and 18.4%–32.7% variations by 18S). Notably, *Gelasimus* spp. were only detected in the COI diet of *X. cinereus* (42.3% RRA), which contributed 25.4%–30.1% of the abundance-based variation between *X. cinereus* and three other species, that is, *C. ferruginea*, *C. leschenaultii*, and *P. squatarola* (Table S15c,d). *Gelasimus* spp. also contributed to the significantly non-overlapping COI diets between *X. cinereus* and other shorebird species based on the occurrence data (Table S13d).

For closely related species, although *C. mongolus* and *C. leschenaultii* belong to the same genus *Charadrius*, the overall diet of *Charadrius mongolus* based on abundance overlapped with that of *Calidris ferruginea* more than that of *Charadrius leschenaultii*

(Figure 7; Table S13). The COI diet composition of *C. mongolus* was also found to overlap with those of *P. squatarola* and *Tringa* spp. (Figure 7; Table S13). In another pair, *T. stagnatilis* and *T. totanus*, despite belonging to the genus *Tringa*, the COI dietary items consumed by the population of *T. totanus* ($q > 0$, gamma diversities = 6.2–8.8, Table S12b) were twice as diverse as those consumed by the *T. stagnatilis* population ($q > 0$, gamma diversities = 3.1–4.6) (Figure 6). The 18S diets of *T. stagnatilis* and *T. totanus* were completely segregated from each other (Pianka's index = 0, Table S13a,b) while the COI diets partially overlapped (Table S13c,d) as shown by the PCoA and hierarchical clustering (Figures 7 and S4).

For intraspecific variations, when we compared the individual diets of *C. ferruginea* with those of *C. leschenaultii* (both $n \geq 35$), higher intraspecific variations in diets were observed in *C. ferruginea* (beta diversity of $q > 0$: 18S = 6.4–8 vs. 2.6–4; COI = 5.2–6.4 vs. 3.1–5,

Table S12). Among the species with smaller sampling sizes ($n = 5-9$), less intraspecific variation in diets was observed in *X. cinereus* (beta diversity of $q > 0$: $18S = 1.7-2.1$; $COI = 2$) than in *C. mongolus* and *T. totanus* (beta diversity of $q > 0$: $18S = 2.5-3.5$; $COI = 3.2-4$) (Figure S2).

4 | DISCUSSION

4.1 | Interspecific dietary segregation during spring passage

Our results have demonstrated the patterns of dietary segregation between sympatric shorebird species in an important staging ground in the EAAF during their spring passage. Compared to *P. squatarola* and *C. ferruginea*, the greater sand-plover *C. leschenaultii* in Mai Po targeted malacostracans as their principal prey, especially decapods and some insects, but apparently very little or even no annelids and mollusks. Available information on the feeding habits of *C. leschenaultii* elsewhere is very limited. Hockey et al. (1999) showed that *C. leschenaultii* in Mida Creek, Kenya also mainly fed on crabs and some undetermined invertebrates (Hockey et al., 1999). *Charadrius leschenaultii* in Kenya was observed to hunt visually by day for large and active epifauna by searching large areas of mudflats or saltmarshes in a run-and-pause manner, a typical foraging mode for most plovers. This foraging mode could support the observation that sessile or slow-moving prey with weak visual cues to predators, such as sediment burrowing annelids and mollusks, were infrequently preyed upon by *C. leschenaultii* only in Mai Po.

Contrary to *C. leschenaultii* and *P. squatarola*, the curlew sandpiper *C. ferruginea* (Near Threatened) in Mai Po had a diverse diet and is more of a generalist. The dietary compositions of *C. ferruginea* here were similar to those of their conspecifics that occurred in South Africa and Australia (Dann, 2000; Puttick, 1978). In Mai Po, they ate many annelids, mollusks, malacostracans, and some insects, with Nereid worms, including *Neanthes glandicineta*, being one of their major prey. A previous local survey showed that *N. glandicineta* is the most abundant Polychaeta species in the mudflat of Mai Po (Shen et al., 2010). A few studies have demonstrated that *C. ferruginea* is a diurnal tactile forager, which often steadily probes with its long downcurved bill on the substrate to catch unseen prey while standing in or out of shallow water (Dann, 2000; Khomenko, 2003; Puttick, 1978). This foraging behavior probably relates to the high prevalence of mudflat infauna, such as polychaetes and gastropods, in our *C. ferruginea* diets.

For the black-bellied plover *P. squatarola*, our results showed that the dietary spectrum of *P. squatarola* partially overlapped with those of both *C. ferruginea* and *C. leschenaultii* in Mai Po. *Pluvialis squatarola* primarily predated decapods in malacostracans, gastropods, and bivalves (including *Solen strictus*) in mollusks, but consumed annelids and insects in smaller proportions. Comparisons to the diets of its conspecifics occurring elsewhere, for example, the United Kingdom and southern Africa, showed differences in compositions at a high

taxonomic level (Dit Durell & Kelly, 1990; Pienkowski, 1982; Turpie & Hockey, 1997), reflecting that *P. squatarola* could be opportunistic foragers. A few studies proposed that their diets could vary in accordance with the prey species abundance in a particular habitat (Baker, 1974; Pienkowski, 1982; Turpie & Hockey, 1997). For instance, in the United Kingdom, *P. squatarola* at Lindisfarne mostly ate polychaetes from microhabitats where polychaetes were the densest, while bivalves, gastropods, and crustaceans were consumed less often (Pienkowski, 1982). Moreover, a couple of studies reported that behavioral plasticity was observed in some Charadriinae species, including *P. squatarola*. They found that *P. squatarola* occasionally used the sandpiper-like foraging method to frequently peck prey from the shallow water column when the tide covered the mudflat. They proposed that this might give competitive advantages to this species to expand their foraging niches (Dit Durell & Kelly, 1990; Masero et al., 2007).

Despite the smaller sample size of the Terek sandpiper *X. cinereus*, this species might have the most specialized diet among the sympatric shorebird species in Mai Po. It almost exclusively preyed on decapods, including *Gelasimus* spp., with only small proportions of insects, isopods, ostracodes, and spiders. Foraging behavior studies on *X. cinereus* have been conducted in other regions of Asia, for example, South Korea and Thailand, which showed that they were very well adapted for catching crabs, such as *Scopimera* and *Uca* spp., in sediment by often sprinting to chase active prey and making deep probes with their distinctive long upcurved bills to reach crab burrows (Biljlsma & de Roder, 1991; Piersma, 1986). Another sandpiper considered to have relatively low diversity in diets was *T. stagnatilis*, whose diets in other places are very poorly known. In this study, *T. stagnatilis* seemingly specialized on malacostracans and nereid polychaetes. Unlike *C. ferruginea* and *P. squatarola*, which very often caught annelids as well as mollusks from the feeding grounds at Mai Po, *T. stagnatilis* barely consumed mollusks.

4.2 | Intraspecific variations in dietary compositions

Our study has provided clear evidence of intraspecific variations in shorebird dietary compositions at the individual sample level. The intraspecific variations within a few of our shorebird species unveiled by DNA metabarcoding were pronounced, and this remains true for the individuals occurring in spring. For example, some individuals of *C. ferruginea* and *P. squatarola* mainly consumed decapods, while their conspecifics primarily ate mollusks and polychaetes. These intraspecific dietary variations could be attributed to several factors, such as phenotypic differences (e.g., bill lengths), body conditions (e.g., health), and life experiences (e.g., acquired skills) of conspecifics, which can be associated with age and sex (Durell, 2000). These factors can further affect the social status (e.g., agonistic interactions among conspecifics; Duijns & Piersma, 2014), foraging proficiencies, food/microhabitat preference, and the consequent dietary compositions among conspecifics. Differences in the diets of

conspecifics with various life-history traits have been reported elsewhere (Alves et al., 2013; Catry et al., 2012; Duijns & Piersma, 2014). In the case of *Calidris mauri* (western sandpiper) in North America during spring, epifaunal invertebrates made up a higher proportion of the diets of males, which were lighter in weight and had shorter bills than females, while polychaetes dominated the diets of adult females, which had longer bill lengths (Hall et al., 2021). In *T. totanus*, subordinate individuals were also excluded from preferred feeding areas and were forced to feed on alternative prey (Cresswell, 1994).

Although some shorebird species can forage both diurnally and nocturnally, foraging time during the day was unlikely the factor underlying the intraspecific variations found in this study. All fecal sampling for this study was consistently performed between dusk and midnight, so the dietary compositions observed were primarily made up of the food they consumed shortly before sampling (e.g., 20–50 min for *Calidris canutus*; Zhang et al., 2019). However, there was the possibility that some individual shorebirds might have just arrived at Mai Po on the day they were sampled and the food they consumed elsewhere might contribute to the observed variations.

4.3 | Highlights on the overall and specific dietary compositions

By using the DNA metabarcoding technique, our study has uncovered a group of consumed taxa that might not be able to be identified as food items by traditional approaches. In one of our findings, a high abundance of ostracods was detected in 4/7 samples of *C. mongolus*, and ostracods have not yet been detected as their dietary component (Aarif, 2009). Ostracods are very small crustaceans (max. 30 mm in size), and those in the order Podocopida are typically 1 mm in size. Similar to *C. leschenaultii* and *P. squatarola*, *C. mongolus* are plovers (subfamily Charadriinae), which are obligate visual foragers. They share several characteristic phenotypic traits, including large eyes and short bills, which are adaptations for targeting large prey by sight. Here, it is uncertain what species or sizes of ostracods *C. mongolus* fed on, but *C. mongolus* elsewhere were reported to forage at night occasionally (Wiersma et al., 2020), and thus, the plausible explanations for this anomaly might be that *C. mongolus* targeted bioluminescent ostracods that they could see at night as mentioned. They might also exhibit behavioral plasticity (Masero et al., 2007; Paulson, 1990) and be able to forage like sandpipers during the daytime. Alternatively, *C. mongolus* might ingest the ostracods through secondary or accidental consumption.

Moreover, our results have shown that some individuals of many of our studied species, *C. ferruginea* for instance, consumed a certain amount of flowering plant materials, including *Ruppia maritima* (widgeon grass) in the order Alismatales and plant species in the order Poales. *Ruppia maritima* was also reported to occur in several ponds within Mai Po in 2020 (SMEC, 2021). Ntiemoa-Baidu et al. (1998) also found *R. maritima* seeds in the diet of *C. ferruginea* in coastal lagoons in Ghana (Ntiemoa-Baidu et al., 1998). Although plant materials, such as diaspores, were found in the guts or fecal contents

of shorebirds in several studies (Andrei et al., 2009; Brooks, 1967; Rundle, 1982), the literature reporting observations on shorebirds eating plants was very limited, and many dietary studies suggested that shorebirds likely ingested plants secondarily or accidentally. Thus, the functional role of migratory shorebirds as major passive dispersers of plants by endozoochory as well as the associations between plant dispersal patterns and movements of shorebird species have only been gaining more attention and discussion in recent years (Green et al., 2002, 2016; Lovas-Kiss et al., 2019). Shorebirds are highly mobile both daily at a site and seasonally across continents, for example, *C. ferruginea* uses stopover sites on a few continents, such as the Yellow Sea region, the Island of Java, and southern Borneo in the EAAF (Lisovski et al., 2021). Given the abundance of shorebirds, this finding has profound implications for the regional survival and range expansion of plant species and ultimately the preservation of wetland plant diversity and ecosystem functions in Asian and Australasian areas.

4.4 | Methodological limitations

In this study, our results have shown that the more fecal samples obtained from a shorebird species, the clearer the patterns of inter- and intraspecific variations in diets could be revealed. Thus, the levels of inter- and intraspecific dietary variations in the shorebird species with smaller sample sizes, such as *T. totanus* and *C. mongolus*, could only be more effectively estimated if more samples were available from these species (Figure S1). We suggest that for rare shorebird species, temporal sampling can be conducted across years to increase the sample size, which would allow us to obtain more complete dietary spectra. In addition, although we collected our fecal samples from clean containers used for keeping birds to minimize environmental contamination of our samples, it was possible that certain taxa detected were due to secondary or accidental consumption. For example, small non-target living organisms or environmental DNA in or on the sediments or prey could be detected in our dataset. These non-target taxa could have introduced biases in our dietary analyses in different ways, for example, it may have a larger influence on the wPOO than RRA if the small organism or eDNA ingested by secondary or accidental consumption are in much smaller amounts compared to the target prey. We suggest future studies include environmental samples from the foraging grounds, such as sediment and specimens of the target prey, to gain more insights into the identities of the consumed non-target taxa.

5 | CONCLUSIONS

The ecological roles of shorebirds are gaining more recognition worldwide and they are the key dispersers across continents in the field (Green et al., 2002, 2016; Lovas-Kiss et al., 2019). Our study revealed the foraging dynamics of a group of diverse shorebird species that used Mai Po, a central site in the EAAF, as foraging grounds.

Shorebird species or individuals have evolved various adaptations that facilitate their diet specialization to minimize both inter- and intraspecific competition (Durell, 2000). However, despite these adaptations, our results showed that the diets of most sympatric species partially overlapped during spring, suggesting these sympatric species as well as their conspecifics could compete for the same kinds of food sources to various degrees. Therefore, any reductions in the abundance and diversities of important prey taxa groups, for example, species in Decapoda, Polychaeta, and Mollusca, would likely intensify the inter- and intraspecific competition among them. This would simultaneously threaten the sustainability of multiple shorebird populations depending on these food taxa. In addition, this study illustrated the dietary compositions of more than one hundred shorebird individuals visiting Mai Po, providing us with many insights into their food demands that are crucial to their survival and possible conservation. It is imperative to protect and regularly monitor foraging grounds that harbor diverse communities of macroinvertebrates, which are essential to refuel energy-deprived birds during migration periods.

AUTHOR CONTRIBUTIONS

S.Y.W.S., Y.-H.S., and I.W.Y.S. designed research; S.Y.W.S., E.S.K.P., L.Y.C., and D.T.C.C. performed research; P.-Y.H. and S.H. analyzed data; P.-Y.H. and E.S.K.P. wrote the paper, and all authors contributed to the revised versions.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

Raw 18S rDNA and COI sequencing reads and sample information in this study are available in the NCBI Sequence Read Archive (SRA) under BioProject accession PRJNA807646 (www.ncbi.nlm.nih.gov/bioproject/PRJNA807646).

ORCID

Pei-Yu Huang  <https://orcid.org/0000-0001-5297-7719>

Emily Shui Kei Poon  <https://orcid.org/0000-0002-5705-9981>

David Tsz Chung Chan  <https://orcid.org/0000-0002-6886-3713>

Yik-Hei Sung  <https://orcid.org/0000-0003-0026-8624>

Simon Yung Wa Sin  <https://orcid.org/0000-0003-2484-2897>

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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