Foraging niche partitioning of three *Myotis* bat species and marine fish consumption by *Myotis pilosus* in a subtropical East Asian region

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Abstract

Most bats are insectivorous, but some species have evolved the ability to prey on fish. Although piscivory has been confirmed in the Rickett's big-footed myotis (*Myotis pilosus*), the extent of piscivory of other cohabiting *Myotis* species is uncertain. This study aims to explore the dietary niches and fish consumption of three *Myotis* species in a subtropical East Asian region, and specifically the fish diet of *M. pilosus*. Our findings reveal, for the first time, that *M. pilosus* consumes marine fishes, in contrast to previous research conducted in inland regions that suggested year-round consumption of cyprinids in freshwater habitats. We also observed seasonal variation in the diets of *M. pilosus*. It predominately hunted wide-banded hardyhead silverside, sailfin flying fish, and shorthead anchovy during the wet season, while mainly preying upon mullets during the dry months. In more inland areas, *M. pilosus* was found to primarily feed on invasive freshwater poeciliids. Furthermore, *M. pilosus* consumed more fish during the dry season, while there was a greater consumption of insects during the wet months. Most notably among our findings is the consumption of fish by one individual of Chinese myotis (*M. chinensis*) and two individuals of Horsfield's myotis (*M. horsfieldii*), indicating that

they are potentially piscivorous. We revealed that both *M. horsfieldii* and *M. pilosus* consumed water striders, suggesting that foraging of aquatic insects could be driving the evolution of fishing behavior. Our findings have also shed light on the flexibility of foraging behavior in piscivorous bats.

1. Introduction

Bats are the only mammals capable of sustained flight. They use echolocation during flight to navigate and forage for food nocturnally. There are over 1400 known bat species worldwide, forming a diverse group that have adapted to a wide range of diets (Taylor, 2019). The majority of bats are insectivorous, feeding primarily on insects, while others are frugivorous and nectarivorous bats, which consume fruit and nectar, respectively (Ramírez-Fráncel et al., 2022). In addition to insects, certain bat species have adapted to feed on other animals; for instance, sanguivorous vampire bats consume blood (Riskin and Carter, 2023), and piscivorous bats prey on fish (Aizpurua and Alberdi, 2018). Piscivorous bats can be found among two distinct genera: *Noctilio* (bulldog bats) and *Myotis* (mouse-eared bats). Among these, only the *N. leporinus* (greater bulldog bat) in Latin America has been confirmed as mainly piscivorous (Brooke, 1994). Three other species display ‘limited’ fishing behaviors, including *M. vivesi* (fish-eating myotis) in the Gulf of California (Otálora-Ardila et al., 2013), *M. pilosus* (Rickett's big-footed myotis) in southern and eastern China, Vietnam, and Laos (Ma et al., 2003), and *M. capaccinii* (long-fingered myotis)

in the Mediterranean Sea (Aihartza et al., 2003). Additionally, several species have ‘unconfirmed’ fishing behaviors, such as *N. albiventris* (lesser bulldog bat), *M. daubentonii* (Daubenton's myotis), and *M. macropus* (large-footed myotis).

The evolution of fishing behavior in bats has long fascinated scientists, and comprehending the dietary compositions of various piscivorous bats is crucial for understanding their foraging ecology and unravelling the intricacies of these evolutionary processes. While fishing behavior is an important hunting strategy, it is worth noting that none of these species are obligate piscivorous (Aizpurua et al., 2014). Fish-eating bats also prey upon arthropods to varying extents, and the prevalence and intensity of fishing or hunting arthropods can be influenced by different factors such as their morphological traits, prey availability, and interspecific competition within their habitats (Chang et al., 2019).

In Asia, *M. pilosus* is relatively large for its genus, exhibiting a body length ranging from 51 to 65 mm (Wilson and Mittermeier 2019). They possess remarkably enormous feet with enlarged, laterally compressed claws, resembling those of *N. leporinus* (Fish et al., 1991, Ospina-Garcés et al., 2016). However, it was not until 2003 that the piscivorous nature of *M. pilosus* was confirmed (Ma et al., 2003). The bats capture insects in mid-air and use their hind limbs to employ a trawling method for catching fish (Jiang et al., 2003). While fish remains have been discovered in their diets in some regions, regular fish consumption has only been verified in Beijing in northern China (Ma et al., 2006). Studies conducted in Beijing, Shandong, and Guizhou have shed light on the dietary habits of *M. pilosus* (Ma et al., 2006, Chang et

al., 2019, Ma et al., 2003, Wang et al., 2024). These studies reported on the piscivorous and insectivorous feeding behaviors of *M. pilosus*, with species from the order Cypriniformes (cyprinids) being their primary fish prey. In Beijing, they found that *M. pilosus* consumed grass carp (*Ctenopharyngodon idella*), Eurasian carp (*Cyprinus carpio*), pale chub (*Zacco* spp.), and Amur minnow (*Rhynchocypris* spp.) (Ma et al., 2006, Chang et al., 2019). In Shandong, in addition to grass carp and Eurasian carp, they also found bitterlings (*Rhodeus* spp.) to be their primary prey (Chang et al., 2019). These fish species are similarly found in freshwater habitats within inland areas (Froese and Pauly, 2024). Additionally, *M. pilosus* was found to consume at least seven orders of insects, such as Coleoptera (beetles), Lepidoptera (moths and butterflies), Hemiptera, Diptera, and more (Ma et al., 2006, Chang et al., 2019).

However, discrepancies in the extent of fishing incidence have been observed among these studies. One possible factor contributing to these discrepancies could be the variations in spatiotemporal prey availability across different sampling locations and seasons. Further research on their fish and insect consumption is crucial to gain a better understanding of how resource variation or resource partitioning is associated with its adaptations to the environment through foraging strategy. Thus, it is necessary to investigate the dietary compositions of *M. pilosus* in various locations or habitats across seasons to provide further insights into their fishing behavior. However, beyond the few populations studied, the dietary composition of *M. pilosus* remains largely unknown.

There have been speculations that several other Asiatic *Myotis* species, such as *M. macrotarsus* (pallid large-footed myotis), *M. horsfieldii* (Horsfield's myotis), *M. hasseltii* (lesser large-footed myotis), *M. stalkeri* (Kei myotis), *M.* adversus (large-footed bat), *M. macrodactylus* (big-footed myotis), and *M. macropus*, might be piscivorous due to their large hind feet and claws (Aizpurua and Alberdi, 2018). Notably, *M. horsfieldii* is a native bat species in South Asia. Although it is smaller compared to *M. pilosus*, with a body length ranging from 44 to 51 mm, its capability to directly capture insects from water surfaces (Wilson and Mittermeier 2019) suggests a potential for piscivory. It is widely believed that the fishing behavior in bats evolved from their insectivorous habits, with the hypothesis that capturing insects from water represents an intermediate stage in this behavior (Aizpurua et al., 2013). Despite the relatively large hind feet of *M. horsfieldii*, exceeding half the length of the tibia, and their observed circular flight patterns above open water surfaces at a close distance of a few centimeters to search for insects, there is currently a lack of evidence for fishing behavior in *M. horsfieldii* (Aizpurua and Alberdi, 2018). Furthermore, their dietary composition has never been reported to date, leaving unanswered questions regarding whether fish forms part of its diet and what insect species they prey upon.

Hong Kong, situated at the southern coast of China, is the distribution range of *Myotis* species (Shek, 2006). It is a semi-island known for its diverse habitats, including various types of forests, coastal areas, wetlands, marine, and freshwater habitats, each hosting a wide array of arthropod and fish species (Dudgeon and

Corlett, 2004). In this region, four *Myotis* species coexist, including *M. pilosus*, *M. horsfieldii*, *M. chinensis* (Chinese myotis), and *M. muricola* (whiskered myotis, locally rare). *Myotis chinensis* is bigger in size than *M. pilosus*, with a body length ranging from 91 to 97 mm (Wilson and Mittermeier 2019), and possesses relatively large hind feet compared to some other bat species. However, there are only a few studies conducted in China reporting its diets. Despite its large size, it is believed that *M. chinensis* exclusively feeds on insects. They are capable of preying on larger insects and capturing ground-dwelling insects through picking behavior, primarily consuming species in Coleoptera (e.g., carabid beetles), Orthoptera (grasshoppers), and Diptera (flies) according to an earlier study (Ma et al., 2008). Given the uncertainties surrounding the extent of piscivory by different *Myotis* species in the region, this study seeks to investigate the diets of *M. pilosus*, *M. horsfieldii*, and *M. chinensis* in Hong Kong by analyzing their fecal compositions using DNA metabarcoding.

Traditional microscopy for species identification of fecal or stomach contents presents several challenges. The identification of partially digested prey body parts under a microscope relies heavily on the expertise and experience of the analyst. Moreover, the process can be a time-consuming, subjective, and may have limitations in taxonomic resolution, potentially introducing biases or inconsistencies in prey identification (Svendsen et al., 2023, Massey et al., 2021). In contrast, DNA metabarcoding overcomes some of these challenges. It is a high-throughput technique that enables the analysis of a large number of samples and provides a higher

taxonomic resolution even with small amounts of prey DNA (de Sousa et al., 2019, Monterroso et al., 2019).

By analyzing the prey identities and relative quantities of the three *Myotis* species, we can gain insights into the spectra of habitats bat individuals forage and prey they target in a habitat-diverse environment. Notably, both *M. chinensis* and *M. pilosus* are listed as Near Threatened species in the Red List of China's Vertebrates (Jiang et al., 2016), with *M. pilosus* also being classified as a Vulnerable species in the IUCN Red List of Threatened Species (IUCN, 2023, Jiang et al., 2019). Hence, it would be important to further explore the dietary niche partitioning of these sympatric species. The findings would inform us their trophic relationships and what types of food resources and habitats are critical to their population sustainability, and significantly contribute to the conservation efforts for each of these species in the region.

In this study, we aim 1**)** to investigate the dietary compositions of the three *Myotis* species at both individual and species levels, 2) to estimate the dietary diversity within individuals and populations, 3) to assess the effect of environmental and host factors on the dietary compositions of these species, and 4) to determine the patterns of dietary niche partitioning among the *Myotis* species.

2. Materials and Methods

2.1 Sample collection

174 Between 2018 and 2021, we visited water tunnels or caves in Hong Kong to capture
175 *Myotis* bats during wet (April to September) and dry (October to March) season. We
176 refrained from visiting roosting sites during the months when bats were overwintering
177 or breeding/nursing, thus minimizing any potential disturbance. The locations include
178 Lin Ma Hang (LMH01), Tai Lam Chung (TLC01 and TLC02), Pak Tam Chung
179 (PTC), and Sai Kung (SK0, SK02, and SK03) (Fig. S1 and Table S1a-c) (Shek, 2004).
180 We captured the bats using hand-held hoop nets and placed them in breathable
181 sterilized bags. To prevent cross-contamination, each individual bat was kept in a
182 separate sterilized bag. We identified the species and sex of each bat based on its
183 morphology (Poon et al., 2023, Shek, 2006). The fresh feces from each bat were
184 collected and placed in individual 2mL tubes. They were preserved in 100% ethanol
185 and then kept at -80°C until DNA extraction. In total, we have collected fecal samples
186 from 62 *M. pilosus*, 51 *M. horsfieldii*, and 43 *M. chinensis*. Physical parameters of
187 each bat, such as body weight and forearm length, were measured. After collecting
188 samples and recording their physical parameters, all bats were released immediately
189 back to the wild. Approvals for animal experiments were granted by the Department
190 of Health (ref. 19-177 in DH/SHS/8/2/3 Pt. 30), the Committee on the Use of Live
191 Animals (ref. 4963-19), and the Agriculture, Fisheries, and Conservation Department
192 (AFCD; ref. 35 in AF GR CON 09/51 Pt.8).
193

194 ***2.2 DNA extraction and metabarcoding***

195 The QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used to
196 extract the fecal DNA. We included negative controls and mock communities (Table
197 S2a) during DNA extraction to later check for contamination during PCR. We
198 quantified the fecal DNA using the Qubit dsDNA high-sensitivity (HS) assays on an
199 Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific, Massachusetts, US)
200 (Huang et al., 2021).

201 All fecal DNA samples, mock communities (Table S2a), and negative controls
202 were used for library preparation using three genetic markers through two-step PCR
203 (Huang et al., 2022, Huang et al., 2021) (Supplementary Materials and Methods). The
204 first pair of markers used was a universal pair (18s_SSU3_F:
205 5'GGTCTGTGATGCCCTTAGATG3' and 18s_SSU3_R:
206 5'GGTGTGTACAAAGGGCAGGG3') which targets the V7 region of 18S small
207 subunit ribosomal DNA (rDNA; ca. 174bp) (McInnes et al., 2017). This pair of
208 markers provides an overview of the dietary compositions of *Myotis* bats. To
209 investigate whether fish was consumed by the three *Myotis* species, we used the
210 mitochondrial 12S rDNA specific primers (12S_AcMDB07_HK_F:
211 5'GCCTATATACCRCCGTCG3' and 12S_AcMDB07_ R:
212 5'GTACACTTACCATGTTACGACTT3'; ca. 283bp) to amplify fish DNA (Bylemans
213 et al., 2018). Considering the important prey of *Myotis* bats, which consists of
214 arthropods, we used a third pair of markers which amplifies the mitochondrial
215 cytochrome c oxidase subunit I gene (COI; COI_Fwh2_F: 5'
216 GGDACWGGWTGAACWGTWTAYCCHCC3' and COI_Fwh2_R: 5'

217 GTRATWGCHCCDGCTARWACWGG3'; ca. 219bp) (Vamos et al., 2017). This pair
218 of markers offers a higher resolution in identifying the macroinvertebrate species
219 consumed by the bats. For each marker, we generated a library multiplex by pooling
220 libraries from different samples in an equimolar ratio. Subsequently, all three libraries
221 were sequenced on an Illumina NovaSeq (PE 150bp) by Novogene (Hong Kong).
222

223 ***2.3 Sequencing data preprocessing***

224 Raw paired-end DNA reads were merged by using the -fastq_mergepairs function in
225 USEARCH v11.0.667 (Edgar, 2010). Primer sequences were removed with
226 CUTADAPT v2.5 (Martin, 2011). The assessment of trimmed reads quality was
227 completed with FastQC v0.11.9 (Wingett and Andrews, 2018) and VSEARCH
228 v2.18.0 (Rognes et al., 2016). Only high-quality trimmed reads within the target
229 lengths (18S: 130-180bp; COI: 130-210bp; 12S: 140-290bp) were retained for later
230 analysis. These pre-processed reads were then dereplicated by using the -
231 derep_fulllength command in VSEARCH. Chimeras and singletons were removed
232 from the dereplicated reads by using USEARCH. Using the -usearch_global function
233 in VSEARCH, all pre-processed reads were clustered into amplicon sequence variants
234 (ASVs) based on 99% similarity.
235 Using the SINTAX algorithm in USEARCH, each ASV was assigned to the
236 lowest identifiable taxonomic level with a confidential cutoff of 0.7 (Edgar, 2016).
237 The ribosomal RNA database SILVA (Glöckner et al., 2017), the mitochondrial

database MIDORI for eukaryotes (Leray et al., 2022), and MitoFish for fish (Zhu et al., 2023) were used as taxonomic classification reference databases for 18S, COI, and 12S sequences, respectively. We also assigned ASVs against the NCBI non-redundant nucleotide sequences database to obtain the best 1000 blast hits with a similarity higher than 99% and e-value lesser than 1e-50. The BASTA with the lowest common ancestor (LCA) algorithm (Kahlke and Ralph, 2019) helped us assign the lowest common taxonomic level shared by 80% of blast hits. To obtain a high taxonomic classification resolution, the results from SINTAX and LCA were combined by assigning ASVs with lower ranks of taxonomies.

Potential false-positive and contaminant ASVs were eliminated by comparing them with mock communities and negative controls (Table S2b-d). Non-diet ASVs (e.g., Fungi, Bacteria, and Algae) and unclassifiable ASVs (e.g., ASVs that could not be identified beyond the domain level) were discarded from the analysis. After ASVs cleaning, samples with reads number lower than 100 were subsequently excluded. To better present our taxonomic data in figures, we classified each identified taxon as high and low abundance taxon according to whether it occupies over 0.1% number of reads. High abundance taxa were grouped to the lowest taxonomic level, while low abundance taxa were grouped into higher taxonomic level. In this way, the taxa were classified into taxonomic categories (Table S3a-c) (Huang et al., 2022).

2.4 Data analysis

259 After data preprocessing, we used 18S data of 146 samples, 12S data of 49 samples,
260 and COI data of 102 samples for downstream data analysis. In the 18S dataset, a total
261 of 11 taxonomic categories were classified from 91 ASVs. The identifiable taxonomic
262 levels categorized included three classes, eight orders, two families, and one genus
263 (Table S3a). In the 12S dataset, 22 taxonomic categories were classified from 24
264 ASVs. The taxonomic levels included nine families and 12 species (Table S3b). In the
265 COI dataset, 31 taxonomic categories were classified from 676 ASVs. The taxonomic
266 levels included two classes, 12 orders, eight families, 14 genera, and 13 species (Table
267 S3c). In the statistical analysis, we calculated (1) the percentage of read count for each
268 taxon in a sample (relative read abundance, RRA), (2) the percentage of occurrence
269 for each taxon in a sample (weighted percentage of occurrence, wPOO), and (3) the
270 proportion of samples in which a taxon is detected (frequency of occurrence, FOO).
271 The RRA or wPOO at the population level is presented as the mean of RRA or wPOO
272 of all individual samples of a bat species (Lee et al., 2021). We conducted these
273 analysis in R v4.2.1 and visualized with the R package ggplot2 v3.4.2 (Hadley, 2016).
274

275 ***2.4.1 Diet diversity analysis***

276 To determine the relationships between sample numbers and dietary species Chao2
277 diversity, we generated rarefaction curves using hill numbers from ASVs or taxa
278 (q=0) via the R package iNext (Hsieh et al., 2016) (Fig. S2). Using hill numbers based
279 on variant q values (the order of diversity), we estimated the diet diversity of three
280 *Myotis* species at both individual (alpha diversity) and population/species (gamma

281 diversity) levels. Hill number only considers the occurrence of each ASV with q=0.
282 The weight of species abundance in hill number increases when q value increases.
283 The hill number is equivalent to the exponential of Shannon's diversity index and
284 inversion of Simpson's dominance at q=1 and q=2, respectively (Alberdi and Gilbert,
285 2019). Pairwise diversity comparisons at the individual level (alpha) between *Myotis*
286 species were carried out using the Kruskal–Wallis test, followed by Benjamini-
287 Hochberg correction ($p<0.05$).
288 Beta diversity refers to the differences in dietary taxa compositions between
289 individual samples. To analyze beta diversity, we calculated pairwise binary Jaccard
290 dissimilarity distances and pairwise Bray–Curtis dissimilarity distances using the
291 occurrence of each ASV and the fourth root transformed RRA of each ASV,
292 respectively. We visualized the dissimilarities with principal coordinates analysis
293 (PCoA) with the plot_ordination function in R package phyloseq v1.42.0 (McMurdie
294 and Holmes, 2019), and fitted the effect of each taxonomic category into the PCoA
295 result using the envfit and ordiArrowMul function in R package vegan v2.6.4
296 (Oksanen et al., 2022). Pairwise permutational multivariate analysis of variance
297 (PERMANOVA) tests were performed to investigate the separation of dietary
298 compositions between the *Myotis* species. Significant interspecific composition
299 variants observed in PERMANOVA tests are premised on the intraspecific
300 homogeneity, which were tested by betadisper function. We also carried out similarity
301 percentage (SIMPER) analyses to assess the contribution of each taxon to the
302 difference between *Myotis* species by using R scripts simper_pretty.R (Steinberger et

303 al., 2016), which were then checked by the Kruskal-Wallis test by using R scripts
304 R_krusk.R (Steinberger, 2020).
305 Within each species, we performed multiple analyses to determine the
306 contribution of host variables (i.e., sex) and environmental variables (i.e., sampling
307 seasons and sampling locations) to the diversity variation. Data from SK01-03 and
308 PTC were combined for analyses due to their close geographic proximity. Generalized
309 linear models were performed to evaluate the effect of variables on alpha dietary
310 diversity by using the logarithmic hill number of order q=1 as the dependent variable.
311 We visualized the beta diversity dissimilarities of variable groups using PCoA based
312 on Bray-Curtis and Jaccard dissimilarity distances with fitted taxonomic categories.
313 PERMANOVA and corresponding beta-dispersion tests were used to assess the beta
314 diversity variations among variable groups. We identified taxa that significantly
315 contributed to the composition variation among variable groups, using the SIMPER
316 test, which was checked by the Kruskal–Wallis test.
317

318 **3. Results**

319 ***3.1 Dietary compositions of the three* Myotis *bat species***

320 Based on 18S data, the three *Myotis* species primarily preyed upon insects, arachnids,
321 and/or ray-finned fish (Actinopteri) (Fig. 1 and S3). Specifically, moths and/or
322 butterflies (lepidopterans), flies (dipterans), true bugs (hemipterans), and beetles
323 (coleopterans) were the most commonly consumed insect groups. Arachnids such as

324 spiders (Araneae) and mites (Mesostigmata), as well as fish also formed part of their
325 diet.

326 The three *Myotis* species exhibited noticeable interspecific variations in their
327 primary food sources (Fig. 1b-d). *Myotis chinensis* (18S, n=40) primarily consumed
328 insects (70% RRA), particularly bush crickets (*Sasima* spp. in order Orthoptera at
329 39%) and moths and/or butterflies (22%). Additionally, spiders accounted for 27% of
330 their diet (Fig. 1b and Table S4). *Myotis horsfieldii* (18S, n=49) mainly hunted smaller
331 size insects (91%), with flies (55%) and true bugs (16%) being the primary prey. They
332 consumed arachnids (7%) but in smaller proportions (Fig. 1c; Table S4).

333 Fish was mainly found in the diets of *M. pilosus* (n=57; Fig. 1d; Table S4). One
334 sample of *M. chinensis* found to contain 2% fish (Fig. 1a-b, 2, and S4; Table S5a-b).
335 18S data also revealed the presence of fish contents in two samples of *M. horsfieldii*,
336 which were not detected by the 12S (Fig. 1a and 1c; Table S5a-b). To validate the
337 presence of fish in the samples of *M. chinensis* and *M. horsfieldii*, we first confirmed
338 the bat species identity by DNA barcoding using primers SFF_145f and SFF_351r
339 (Walker et al., 2016), and then we confirmed the fish contents of these samples by
340 performing PCR using the 18S and 12S primers (section 2.2) and Sanger sequencing.
341 We concluded that the results were consistent with the DNA metabarcoding finding
342 that fish was identified in these three samples.

343 *Myotis pilosus* (18S, n=57) displayed significant intraspecific differences in diets
344 (Fig. 1 and 2a; Table 6a-b). Approximately one-fifth (11/57) of the individuals were
345 found to exclusively feed on macroinvertebrates, with a majority of these individuals

found in the wet season. These individuals primarily prey on moths and/or butterflies. In contrast, the remaining individuals preyed on fish in varying degrees, constituting 31-100% of their individual diets (Fig. 1a and 1d; Table S5a-b).

Based on the 12S metabarcoding data, 48 out of the 49 individuals that consumed fish were *M. pilosus* (Fig. 2a; Table S6a-c), and the remaining one was *M. chinensis*. Both freshwater, marine, or brackish fish were found in the diet of *M. pilosus*, at least twelve fish species were detected. *Myotis pilosus* primarily hunted marine fish, with Old World silversides (Atherinidae) making up 33% of their diet, including wide-banded hardyhead silverside (*Atherinomorus lacunosus*, max. size <25cm) at 31%. Mullets (Mugilidae) comprised 31% of their diet, with flathead grey mullet (*Mugil cephalus*, common max. size <50cm) making up 17%, and eastern keelback mullet (*Planiliza affinis*, max. size <40cm) and kanda mullet (*Osteomugil engeli*, max. size <48cm) each contributing about 6%. Herrings and sprats (Clupeidae) accounted for 12% of their diet, with silver-stripe round herring (*Spratelloides gracilis*, max. size <11cm) being commonly consumed at 10%. Mosquitofish (*Gambusia affinis*, max. size <5.1cm, from the family Poeciliidae) was the dominant freshwater fish at 13% (Froese and Pauly, 2024). Notably, mosquitofish was mainly consumed by individuals from Tai Lam Chung (Fig. 2; Table S6a-b). The *M. chinensis* individual that consumed fish was from Sai Kung and was found to consume *A. lacunosus*.

The COI data offered a more detailed revelation of the macroinvertebrate prey consumed by the *Myotis* species (Fig. 3 and S5; Table S7a-c). Overall, the majority of prey consisted of dipterans (about 32%), such as lake flies in the family Chironomidae

368 (e.g., *Procladius culiciformis*, *Glyptotendipes tokunagai*, *Tanytarsus formosanus*,
369 etc.); lepidopterans (14%), including grass moths like *Syntonarcha iriastis* and
370 *Cirrhochrista brizoalis*, and macromoth like *Calliteara grotei*; orthopterans (13%),
371 including crickets like *Mecopoda* spp. (bush crickets) and *Gryllotalpa* spp. (mole
372 cricket); and spiders (12%), such as northern golden orb weaver (*Nephila pilipes*)
373 (Fig. 3; Table S7a).

374 The macroinvertebrate orders in diets revealed by COI were consistent with those
375 unveiled by 18S. Notable variations were observed in the dietary compositions among
376 *Myotis* species, as revealed by COI. *Myotis chinensis* (COI, n=34) primarily preyed
377 on arthropods in several orders, such as bush cricket *Mecopoda elongata* (23%) from
378 Orthoptera (29%), *S. iriastis* (8%) from Lepidoptera (16%), and *N. pilipes* (7.5%)
379 from Araneae (26%). Spiders were found in more than 50% of the *M. chinensis*
380 samples (Fig. S5b; Table S7a). In *M. horsfieldii* (COI, n=44), smaller size insects like
381 dipterans accounted for more than 66% of their total diet, with *P. culiciformis* (24%)
382 and *G. tokunagai* (15%) being the main contributors. More than 50% of the *M.*
383 *horsfieldii* samples contained various lake fly species. Lepidopterans and
384 hemipterans, on the other hand, made up 9% and 10% respectively (Fig. 3c; Table
385 S7a). Although a larger proportion of 28% of insects remained unidentified compared
386 to the other two bat species, the primary prey of *M. pilosus* (COI, n=24) consisted of
387 arthropods from various orders, including Lepidoptera (22%), Araneae (14%),
388 Orthoptera (7%), Hemiptera (7%), and Trichoptera (caddisflies, 6.5%) (Fig. 3d; Table
389 S7a).

390

391 ***3.2 Alpha and gamma diversity of consumed taxa***

392 The alpha diversity of individual diets indicated that the diets of *M. horsfieldii*
393 exhibits the highest diversity compared to the other two *Myotis* species, as shown by
394 18S and COI data (Fig. 4 and 5; Table S8a-b). Notably, there is a distinct decrease in
395 hill numbers as the *q* value increases, particularly observed in the macroinvertebrate
396 (COI) composition of *M. horsfieldii* and *M. chinensis* diets (Fig. 5; Table S8b). This
397 suggests that although these bats consume a greater variety of macroinvertebrates
398 compared to *M. pilosus*, their prey compositions are highly uneven, with certain taxa
399 dominating their diets. Furthermore, the macroinvertebrate (COI) compositions of *M.*
400 *chinensis* were more unevenly distributed compared to *M. horsfieldii* (Fig. 5; Table
401 S8b). In the case of individuals of *M. pilosus* that consumed fish, mostly two to three
402 fish species were detected in their diets (12S data; Fig. 2a; Fig. 5). A similar pattern of
403 prey consumption based on 18S data was also observed at the population level as
404 reflected by gamma diversity, with the dietary composition of *M. horsfieldii* being the
405 most diverse (Fig. 5; Table S8b). Despite having the lowest macroinvertebrate (COI)
406 diversity in their diets, *M. pilosus* displayed comparable diversity of overall (18S)
407 diets to *M. chinensis* at the population level (Fig. 5; Table S8b).

408

409 ***3.3 Effects of environmental and host factors on the dietary compositions***

410 According to the PERMANOVA tests conducted using Bray–Curtis and Jaccard
411 dissimilarity distances, the diets of *M. pilosus* were found to differ significantly

between dry and wet season based on 12S (Table S9a-b) and 18S (Table S10a-b) data. Specifically, during the wet season, there was a higher consumption of macroinvertebrates by *M. pilosus*, mainly lepidopterans as evidenced by the 18S data, while in the dry season more fish was consumed. Moreover, the fish compositions of *M. pilosus* from Sai Kung, where a majority of samples that were collected from, exhibited notable variations between the wet and dry seasons (Fig. 2a). The SIMPER analysis results further identified the main contributors to this seasonal difference. Old World silverside (Atherinidae) and shorthead anchovy (*Encrasicholina heteroloba* in Engraullidae, max. size<12cm) were consumed more during the wet season, while mullets (*M. cephalus* and *P. affinis* in Mugilidae) were consumed more during the dry season (Fig. 6a-b and Table S11a-b) (Froese and Pauly, 2024).

Similarly, the diets of *M. chinensis* also differed significantly between the two seasons based on both 18S (Fig. 1a and Table S10a-b) and COI (Fig. 3a and Table S12a-b) data. The 18S data showed that during the wet season, *M. chinensis* mainly consumed moths and/or butterflies (Lepidoptera), while in the dry season, they consumed a large proportion of *Sasima* bush crickets (Orthoptera) (Fig. 1a). Although both GLM and PERMANOVA analyses consistently demonstrated a significant divergence in the seasonal diets of *M. horsfieldii* (Table S12a-b and S13), it's important to note that the small sample size (n=4) from the wet season in the COI dataset may limit the accuracy of these findings. Further investigations are required in the future to confirm this result. Furthermore, the PERMANOVA analysis revealed an additional noteworthy disparity in the diets of the two sexes of

434 *M. horsfieldii* (Table S12a-b), which was mainly attributed to the distinct
435 consumption of dipterans, lepidopterans, and hemipterans by each sex (Table S14a-
436 d).
437
438 ***3.4 Dietary niche partitioning between the three* Myotis *species***
439 Based on the PCoA analysis, 18S and COI data revealed highly similar patterns of
440 dietary niche partitioning among the three *Myotis* species (Fig. 6c-f). The PCoA
441 analysis using Bray–Curtis and Jaccard distances within each of the 18S and COI
442 dataset also revealed consistent patterns. The overall (18S) and macroinvertebrate
443 (COI) diets of *M. horsfieldii* were distinctly differentiated from those of *M. pilosus*
444 and *M. chinensis*, while the dietary compositions of *M. pilosus* and *M. chinensis*
445 were more overlapping with each other (Fig. 6c-f). The SIMPER analysis results
446 indicated the main contributors to this differentiation in *M. horsfieldii* were
447 predominantly dipterans and hemipterans, which constituted a significant portion of
448 their diet (Fig. 6c-d; Table S15a-b). Specifically, the true bugs included water
449 striders (e.g., *Asclepios apicalis*) while dipterans included a group of lake flies (e.g.,
450 *P. culiciformis*, *G. tokunagai*, *Kiefferulus tainanus*, *Polypedilum nubifer*,
451 *Chironomus* spp. etc.) (Fig. 6e-f; Table S16a-b).
452 *Myotis chinensis* also differentiated from *M. horsfieldii* and *M. pilosus* by
453 consuming a considerable proportion of bush crickets (Orthoptera), such as *Sasima*
454 spp. and *M. elongata*, as well as spiders (Araneae, including *N. pilipes*) (Fig. 6c-f;
455 Table S15a-b and S16a-b). On the other hand, the dietary niche of *M. pilosus*

differed from the other two species by including a large portion of fish (Actinoptera) in their diets (Fig. 6c-d and Table S15a-b). *M. pilosus* also differentiated from *M. chinensis* by consuming caddisflies (Trichoptera) (Fig. 6e-f; Table S16a-b).

4. Discussion

Fish eating behavior of *Myotis*

This study provides new insights into the dietary compositions of *M. pilosus* and *M. chinensis* in coastal habitats, expanding on previous research that primarily focused on inland regions of China. Additionally, our research contributes to the understanding of the dietary compositions of *M. horsfieldii*, a species found in southeast and south Asia that has not been previously studied in terms of its foraging ecology. Contrary to earlier studies conducted in inland regions of China, which suggested that *M. pilosus* primarily fed on cyprinids in freshwater habitats year-round (Ma et al., 2006, Chang et al., 2019), our research in Hong Kong unveiled a different dietary pattern. Our findings show that *M. pilosus* in Hong Kong did not prey on cyprinids but instead exhibited a diverse diet, targeting a wide variety of fish prey species from nine different families. These prey species encompassed marine, brackish, and freshwater fish, highlighting the versatility of *M. pilosus* in its feeding habits. Moreover, we observed variations in the species compositions consumed by *M. pilosus* across different seasons. For instance, marine species such as *A. lacunosus*, *Doboatherina duodecimalis* (tropical silverside, max. size 11cm), *Parexocoetus brachypterus* (sailfin flying fish, max. size 13cm), and *E.*

478 *heteroloba* were only fished by *M. pilosus* during the wet season at Sai Kung. This
479 was observed specifically at sampling site SK01 but not the other sites. Worth noting
480 is that SK01 is located 1.7 km or less away from the nearest shore at the southwest,
481 making it a likely site for *M. pilosus* to fish rather than flying north- or eastward to
482 reach the shore, which is located over 3 km away.

483 *Atherinomorus lacunosus*, which forms large schools along sandy shorelines,
484 serves as an important food source for *M. pilosus*. This fish species forages
485 nocturnally, using their large eyes to receive moonlight and their large upturned
486 mouth to capture zooplankton that migrate vertically to the upper water column
487 during nocturnal hours (Skibinski, 2005). This feeding behavior creates large amount
488 of ripples that trawling bats like *M. pilosus* can detect. Another noteworthy prey
489 species is *P. brachypterus*. Sailfin flying fish is commonly found in coastal waters
490 in large shoals and possessing elongated, wing-like pectoral fins, which enable it to
491 leap out of the water and glide rapidly for considerable distances above the surface,
492 an adaptation to evade underwater predators. During the breeding season, spawning
493 flying fish aggregate in abundance near the surface at night, with many vigorously
494 jumping and flying out of the water to release ripe eggs and sperm (Stevens et al.,
495 2003, Digo et al., 2015, Lewis, 1961), exposing them to attack by aerial predators,
496 such as *M. pilosus*.

497 During the dry season in Sai Kung, *M. pilosus* captured the marine fish species
498 *Spratelloides gracilis* and *Sardinella melanura* (blacktip sardinella, common size
499 10cm). Clupeids, such as *S. gracilis* and *S. melanura*, are primarily forage fish known

500 for their high egg production. For instance, *S. gracilis* spawns near the water surface,
501 releasing approximately 1600 eggs per unit body mass (Dalzell and Wankowski,
502 1980). Although previous studies in China have reported crustaceans as prey for *M.*
503 *pilosus* and other bat species (Wang et al., 2024), our study did not identify any
504 crustaceans in the diet of *M. pilosus*. Based on our results, one notable target species
505 for *M. pilosus* is *M. cephalus*, along with other mullet species, during dry months.
506 *Mugil cephalus* is known for congregating in schools over sand or mud in coastal and
507 brackish waters. In Hong Kong, this species exhibits two short spawning peaks in
508 winter (dry) months, resulting in a large numbers of juveniles appearing inshore,
509 especially in estuaries, during February and March (Sadovy and Cornish, 2000).
510 In the Tai Lam Chung woodland area, which is an inland, *M. pilosus* primarily
511 preys on the exotic freshwater poeciliid, *G. affinis*. The southern shore is obstructed
512 by highland with an elevation of at least 400m, and it is at least 9 km away from the
513 nearest shore in any direction (Fig. S1). *Gambusia affinis* is a freshwater species and a
514 widespread non-native fish in Hong Kong, originating from North America and
515 released by the Hong Kong Government for mosquito control purposes around 1940
516 (Dudgeon and Corlett, 2004). Surveys on the distribution of *G. affinis* in Hong Kong
517 have reported its presence in the lower reaches of the closest streams within less than
518 1km of TLC forest (Tsang and Dudgeon, 2021a). Experiments conducted on the
519 ecological effects of *G. affinis* have shown that it reduces the abundance and richness
520 of invertebrates and alters assemblage compositions in Hong Kong wetland (Tsang
521 and Dudgeon, 2021b). Our findings suggest that *M. pilosus* may play a potential role

522 in controlling invasive poeciliids in the local ecosystem. Similar findings have been
523 reported in *M. capaccinii* in northwest Israel and eastern Iberian Peninsula, where *M.*
524 *capaccinii* fed on exotic *Gambusia* spp. (Aizpurua et al., 2014, Levin et al., 2006).
525 Mosquito fish frequently swim close to water surface, using their upturned mouth to
526 break the surface and capture floating insects. Furthermore, the decrease in oxygen
527 during the night may compel these fish to come to the water surface for breathing
528 (Aizpurua et al., 2014, Levin et al., 2006).

529 Our study has made an intriguing discovery regarding the feeding habits of *M.*
530 *chinensis* and *M. horsfieldii*. We reveal a few individuals of *M. chinensis* and *M.*
531 *horsfieldii* from Sai Kung have included fish in their diet. Interestingly, we have
532 found the first case of *M. horsfieldii* preyed on *Asclepios apicalis*, a water strider
533 species that is also targeted by *M. pilosus*. While most water striders are known for
534 their capacity to glide effortlessly on the calm freshwater surface, *Asclepios* spp. are
535 considered as sea skaters which are found primarily in brackish water along coasts
536 (Poolprasert et al., 2022, Andersen and Foster, 1992). *Myotis horsfieldii* might be
537 drawn to this food source due to the consistent ripples produced by *Asclepios* as
538 they manoeuvre across water's surface. Reports on *M. horsfieldii*'s foraging behavior
539 are limited, but one literature mentions that *M. horsfieldii* typically flies in circles
540 around 10 cm above water to skim insects, and they roost near water sources
541 (Wilson and Mittermeier 2019). Our finding suggests that *M. horsfieldii* may
542 capture fish while foraging insects over waters. In addition, our study found
543 evidence that *M. chinensis* consumes the marine *A. lacunosus*, which is a significant

544 discovery as there is limited information available on the foraging behavior of *M.*
545 *chinensis*. Despite these findings, it should be noted that fish was only detected in a
546 few individuals of *M. horsfieldii* and *M. chinensis*. Therefore, further investigation is
547 needed to determine whether these bats actively engage in fishing or if they simply
548 mistakenly catch live or dead fish on the water surface while foraging for insects.
549 Additional research is necessary to understand the piscivorous behavior of these two
550 *Myotis* species. Our discovery that these two *Myotis* species, which were not
551 previously known to consume fish, do in fact include fish in their diets has significant
552 implications for the evolution of fishing behavior. The active foraging of aquatic
553 insects by bats may lead to occasional consumption of fish, which in turn creates the
554 selective pressure that drives the evolution of fishing behavior.
555
556 **Foraging niches of the three *Myotis* species**
557 We observed a higher proportion of fish in the diet of *M. pilosus* during the dry
558 season compared to the wet season, with a significant increase in insect components
559 during the wet season, which is consistent with Ma et. al. (2006) but different from
560 Chang et al. (2018). Our results showed that this increase in insect consumption was
561 due to a 23% rise in the number of individuals preyed on macroinvertebrates during
562 the wet season (23/36) compared to the dry season (5/21). Furthermore, while there
563 were some variations in the insect dietary compositions compared to previous
564 research, our study revealed a similar finding to Chang et al. (2019) in terms of *M.*
565 *pilosus* primarily preying on lepidopterans in the wet season. Based on the nocturnal

566 behavior of most moths and the fact that the lepidopterans identified in our study are
567 all moth species, such as *C. brizoalis* and *C. grotei* consumed by *M. pilosus*, we
568 suggest that most of the lepidopterans preyed upon by *M. pilosus* are moths. Surveys
569 on the abundance of forest invertebrates in Hong Kong have shown that local
570 lepidopterans vary seasonally, with both number and biomass experiencing winter
571 (dry season) lows and early summer (in May during wet season) maxima (Kai and
572 Corlett, 2002). During the wet season, local *M. pilosus* also targeted trichopterans.
573 Trichopterans are moth-like and closely related to lepidopterans, which are nocturnal
574 and commonly associated with freshwater bodies (De Moor and Ivanov, 2008).
575 Here, we provide additional evidence for the specialization of *M. pilosus* in
576 fishing, as we have consistently found fish in its diet throughout the year. It is worth
577 noting that the abundance of lepidopterans in local terrestrial habitat is higher
578 during summer (Kai and Corlett, 2002), this high abundance likely explains why
579 some individuals of *M. pilosus* have shifted their target prey from fish to insects
580 during this time. This shift may be driven by higher energetic profit associated with
581 capturing a larger number of moths compared to capturing fish. Despite the higher
582 nutritional value of a single fish compared to a single moth, catching fish likely
583 incurs a greater energy cost (Aizpurua et al., 2013). This is due to the heavier body
584 mass of fish and the increased effort required to accurately detect fish underwater
585 and subsequently pull it out of the water (Aihartza et al., 2008). This results in lower
586 capture efficiency when targeting fish (Altenbach, 1989). Therefore, our study

587 highlights the adaptive feeding behavior of *M. pilosus*, which adjusts its diet based
588 on prey availability, underscoring the significance of this feeding strategy.
589 Our results show that there is a greater overlap in the macroinvertebrate diet
590 between *M. pilosus* and *M. chinensis*, while the diet of *M. horsfieldii* is distinct from
591 the other two species. We found that local *M. chinensis*, similar to *M. pilosus*, also
592 hunted a higher proportion of lepidopterans, such as grass moth, *S. iriastis*, during the
593 wet season. Males of this grass moth generate ultrasonic sound by rubbing their
594 genitalia against one of their sternites to attract mates from considerable distances.
595 The sounds emitted consist of initial pulse occurs at about 42 kHz, followed by two
596 subsequent pulses at about 57 kHz (Gwynne and Edwards, 1986). These sound pulses
597 fall within the detectable frequency range of *M. chinensis*. Another noteworthy
598 foraging habit shared between *M. chinensis* and *M. pilosus* is their considerable
599 consumption of non-flying nocturnal arthropods, specifically spiders like the
600 northern golden orb weaver (*N. pilipes*). *Nephila pilipes* typically build intricate
601 webs to capture prey on bushes and trees near water sources (Harvey et al., 2007).
602 Spiders have been documented as part of the diet of several other *Myotis* species,
603 such as *M. myotis*, *M. emarginatus*, *M. lucifugus*, *M. nattereri*, *M. evotis*, and *M.*
604 *septentrionalis* (Goiti et al., 2011, Maucieri and Barclay, 2021, Swift and Racey,
605 2002, Kaupas and Barclay, 2018). However, previous studies did not report spiders
606 as a food source for *Myotis* bats in China (Ma et al., 2003, Ma et al., 2008, Ma et al.,
607 2006). Moreover, *M. chinensis* mainly preyed on orthopterans, especially bush
608 crickets, such as *Mecopoda* and *Sasima* spp., during the dry season. The consumption

609 of spiders and orthopterans suggests that *M. chinensis* is capable of foraging in
610 cluttered environments of woodlands and gleans arthropods from various substrate
611 surfaces, such as spider webs and/or foliage in bushes. While there are some
612 geographical variations in dietary composition, our findings agree with Ma et al.
613 (2008), which remarked that conspecifics in Beijing prey on 'non-wing beating'
614 insects such as coleopterans (e.g., ground beetles in Carabidae) and orthopterans
615 while lacking hymenopterans (sawflies, wasps, bees, and ants) and lepidopterans
616 (Ma et al., 2008).

617 Based on our results, we found that the diet of *M. horsfieldii* is distinctive,
618 which prominently includes a high consumption of smaller flying insects,
619 specifically dipterans. Notably, *M. horsfieldii* preys on a wide variety of lake fly
620 species from the Chironomidae family, leading to a higher dietary diversity
621 compared to *M. chinensis* and *M. pilosus*. Conversely, our observations indicate that
622 spiders, orthopterans, and coleopterans were seldom found in their excrement. In the
623 forests of Hong Kong, dipterans constitute the majority of the insect biomass, and
624 their abundance did not exhibit seasonal variation (Kai and Corlett, 2002).
625 Numerous chironomid species bear a striking resemblance to mosquitoes and adults
626 swarms in terrestrial habitats serve as vital sources of food for bats (Puig-Montserrat
627 et al., 2020, Beck, 1995). Local *M. horsfieldii* also targets hemipterans and
628 lepidopterans, though to a lesser extent compared to dipterans.

629 Our findings indicate that *M. pilous* is primarily a fish-eating bat, while *M.*
630 *chinensis* and *M. horsfieldii* primarily feed on insects. One plausible reason for this

631 dietary differentiation pattern could be differences in their morphological
632 characteristics. Previous study suggested that the body size of aerial hunting bats
633 limits their weight-carrying capacity, and fishing bat species tend to have relatively
634 larger body sizes and longer hind feet that give them morphological advantages to
635 fish hunting (Chang et al., 2019). Despite *M. chinensis* (91-97mm body length, 25-
636 30g) (Wilson and Mittermeier 2019) and *M. pilosus* (51-65mm, 11.7-32.5g)
637 exhibiting larger body sizes and heavier body masses, *M. pilosus* share a similar
638 hind foot to forearm length ratio (0.31±0.014) to other fishing bats (0.31±0.051)
639 (Chang et al., 2019), while the ratio for *M. chinensis* (0.26) is more similar to those
640 of other non-fishing bats (0.25±0.038). *Myotis horsfieldii* (44-51mm, 5.0-7.5g)
641 (Wilson and Mittermeier 2019) is smaller in size compared to the other two species.
642 It has a low value ratio (0.23), which is also more akin to other non-fishing trawling
643 bats (Chang et al., 2019). Apart from the influence of morphological adaptations on
644 prey capturing ability, other factors contributing to the dietary differentiation pattern
645 could include differences in prey detection ability through variations in echolocation
646 call structures during foraging (Aizpurua and Alberdi, 2018). Moreover,
647 physiological adaptations such as the ability to metabolize salt from marine fish
648 (Carpenter, 1968) or effectively digest exoskeleton of insects (Liu et al., 2014),
649 could also contribute to the observed dietary differences among the three *Myotis*
650 species. However, limited information is available on these aspects of the three
651 *Myotis* species.

652 This dietary study uncovered the overlapping macroinvertebrate diets of *M.*
653 *pilosus* and *M. chinensis*, as well as the distinct diet of *M. horsfieldii*, with the latter
654 being documented for the first time. Both *M. pilosus* and *M. chinensis* are
655 considered regionally threatened. The potential interspecific competition observed
656 in our findings, particularly between *M. pilosus* and *M. chinensis* during wet season,
657 underscores the importance of protecting macroinvertebrate food sources in local
658 terrestrial ecosystem to prevent intensifying competition among the congenerics. To
659 safeguard the sustainability of vulnerable *M. pilosus*, it is also crucial to preserve the
660 integrity of marine, brackish, and freshwater ecosystems in Hong Kong, as these
661 diverse environments serve as essential foraging grounds for *M. pilosus* to capture
662 the diverse range of fish species.
663

664 **5. Conflict of Interest**

665 The authors declare that the research was conducted in the absence of any commercial
666 or financial relationships that could be construed as a potential conflict of interest.
667

668 **6. Data Availability Statement**

669 Raw sequencing data will be uploaded to the NCBI Sequence Read Archive (SRA).
670 Accession number will be provided after the manuscript is accepted.
671

672 **Author contributions**

S.Y.W.S. Conceptualization; E.S.K.P. and S.Y.W.S. Methodology; E.S.K.P., J.C.T.C., D.T.C.C., C.T.S., W.C.T., and S.Y.W.S. Investigation; X.W. Formal analysis; C.T.S., W.C.T., and S.Y.W.S. Resources; X.W. Visualization; X.W. and E.S.K.P. Writing - Original Draft; E.S.K.P., S.Y.W.S. and all authors Writing - Review & Editing; S.Y.W.S. Supervision; S.Y.W.S. Project administration; S.Y.W.S. Funding acquisition.

Statement on ethics

Approvals for animal experiments were granted by the Department of Health (ref. 19-177 in DH/SHS/8/2/3 Pt. 30), the University of Hong Kong (ref. 4963-19), and the Agriculture, Fisheries, and Conservation Department (AFCD; ref. 35 in AF GR CON 09/51 Pt.8).

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7. Figures Legends and Tables

695 **Fig. 1**. Dietary composition of *Myotis* bat species based on taxa detected in fecal
696 samples using the 18S rDNA marker. The relative read abundance of each taxon were
697 visualized at both (a) individual level (n=146) and (b-d) species level for (b) *M.*
698 *chinensis* (n=40), (c) *M. horsfieldii* (n=49), and (d) *M. pilosus* (n=57). Prey in classes
699 with read abundance lower than 0.1% of all taxa detected were grouped into "Others".
700 Only taxa with abundance higher than 1% in relative read abundance are shown. F,
701 Family. O, Order. Bat photos © Agriculture, Fisheries and Conservation Department.

702 **Fig. 2**. Dietary composition of *Myotis* bat species based on taxa detected in fecal
703 samples using the 12S rDNA marker. The relative read abundance of each taxon were
704 visualized at both (a) individual level [*M. chinensis* (n=1) and *M. pilosus* (n=48)] and
705 (b) species level (*M. pilosus*, n=48). Individual bars were grouped by sampling
706 locations and seasons. Prey in classes with read abundance lower than 0.1% of all taxa
707 detected were grouped into "Others". Only taxa with abundance higher than 1% in
708 relative read abundance are shown. Refer to Fig. S1 for locations. SK, Sai Kung
709 (including Pak Tam Chung); TLC, Tai Lam Chung; LMH, Lin Ma Hang.

710 **Fig. 3**. Dietary composition of *Myotis* bat species based on taxa detected in fecal
711 samples using the COI marker. The relative read abundance of each taxon was
712 visualized at both (a) individual level (n=102) and (b-d) species level for (b) *M.*
713 *chinensis* (n=34), (c) *M. horsfieldii* (n=44), and (d) *M. pilosus* (n=24). Prey in classes
714 with read abundance lower than 0.1% of all taxa detected were grouped into "Others".
715 Only taxa with abundance higher than 1% in relative read abundance are shown. G,

716 Genus. F, Family. O, Order.

717 **Fig. 4**. Pairwise alpha diversity comparison between *Myotis* species. Alpha diversity
718 is represented by using the hill numbers of order q=0 (upper panel), q=1 (middle
719 panel), and q=2 (lower panel), which were calculated based on the ASVs abundance
720 detected by18S rDNA (left panel) and COI (right panel) marker at individual level.
721 Only the p-values of species pairs which have significant different alpha diversity
722 were shown (p<0.05).

723 **Fig. 5** Dietary diversity for each *Myotis* represented in the hill numbers of variant
724 order q. The hill numbers were calculated at individual level and species level to
725 estimate the (a) alpha diversity and (b) gamma diversity, respectively. The ASVs
726 abundance of each sample were detected by using 18S rDNA (left panel), COI
727 (middle panel), and 12S rDNA (right panel) marker gene.

728 **Fig. 6** Principal coordinate analysis (PCoA) of dietary compositions in (a, b) *Myotis*
729 *pilosus* and (c-f) three *Myotis* species based on (a, c, e) the pairwise Bray–Curtis
730 dissimilarity distances calculated from the fourth root transformed RRA of each ASV,
731 and (b, d, f) the pairwise Jaccard dissimilarity distances calculated from the
732 occurrence of each ASV. Each point corresponds to one sample, with the color
733 indicates (a, b) sampling locations, seasons, and (c-f) *Myotis* species. The ASVs were
734 identified by the (a, b) 12S rDNA, (c, d) 18S rDNA, and (e, f) COI marker gene.
735 Taxonomic categories significantly contributing to the difference between groups
736 were shown (see Table S11, S15, and S16). Refer to Fig. S1 for locations. SK, Sai
737 Kung (including Pak Tam Chung); TLC, Tai Lam Chung; LMH, Lin Ma Hang.

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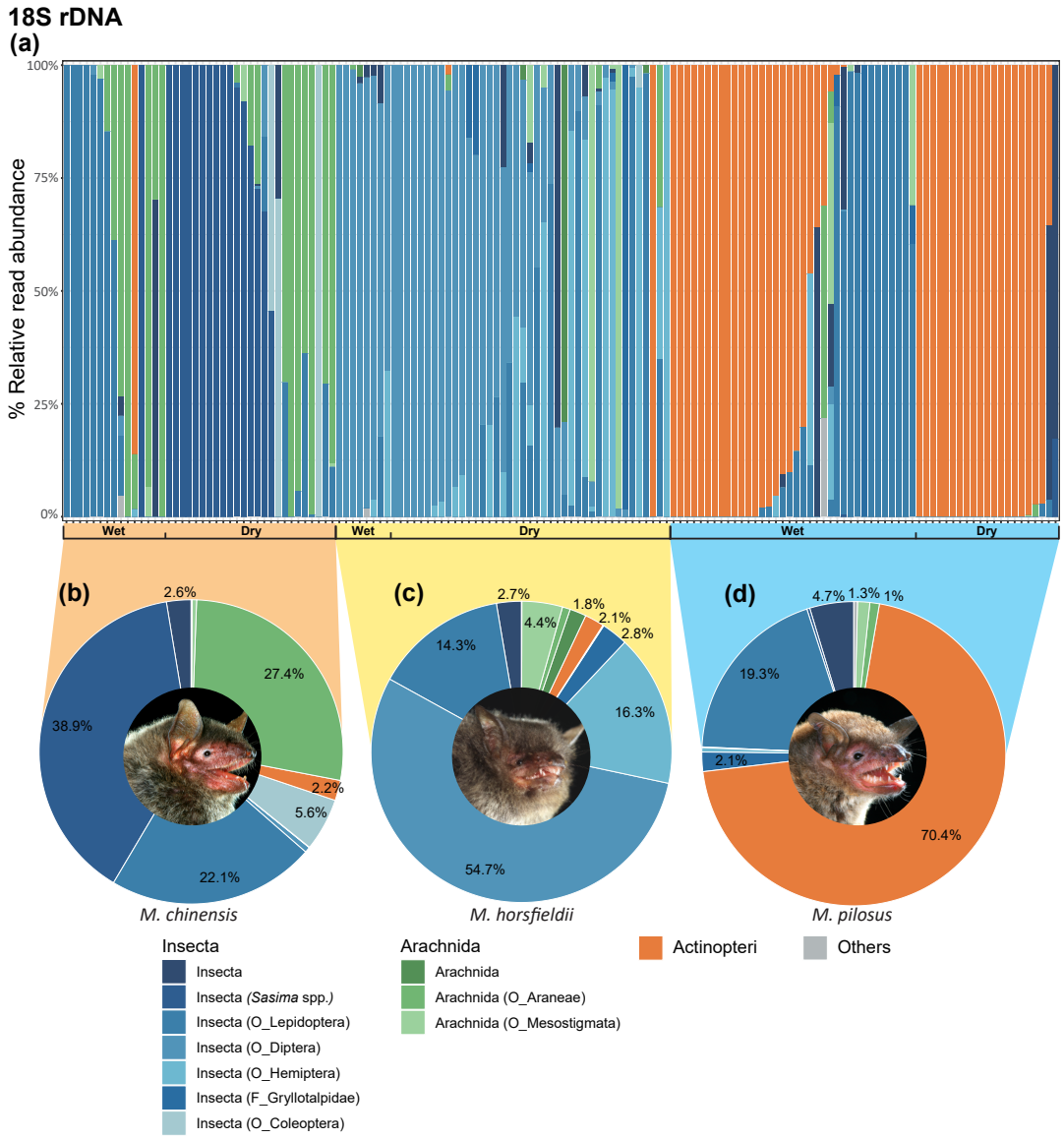
18S rDNA
(a)
% Relative read abundance
100%
75%
50%
25%
0%
Wet
Dry
Wet
Dry
Wet
Dry
(b)
2.6%
27.4%
38.9%
2.2%
5.6%
22.1%
M. chinensis
(c)
2.7%
1.8%
4.4%
2.1%
2.8%
14.3%
16.3%
54.7%
M. horsfieldii
(d)
4.7%
1.3%
1%
19.3%
2.1%
70.4%
M. pilosus
Insecta
Insecta
Insecta (Sasima spp.)
Insecta (O_Lepidoptera)
Insecta (O_Diptera)
Insecta (O_Hemiptera)
Insecta (F_Gryllotalpidae)
Insecta (O_Coleoptera)
Arachnida
Arachnida
Arachnida (O_Araneae)
Arachnida (O_Mesostigmata)
Actinopteri
Others

Figure 1

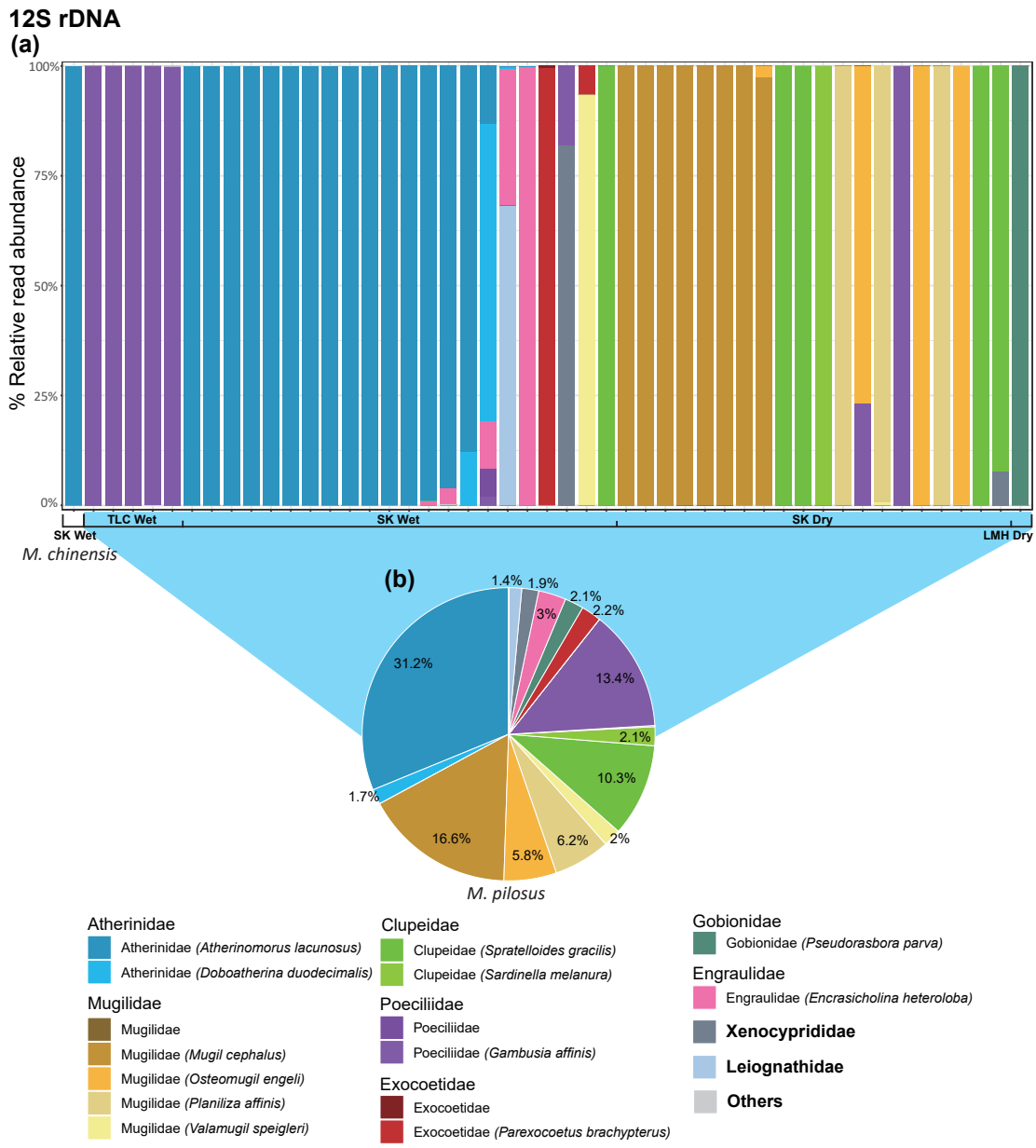
12S rDNA
(a)
% Relative read abundance
100%
75%
50%
25%
0%
TLC Wet
SK Wet
SK Dry
SK Wet
LMH Dry
M. chinensis
(b)
1.4%
1.9%
3%
2.1%
2.2%
13.4%
2.1%
10.3%
2%
6.2%
5.8%
16.6%
1.7%
31.2%
M. pilosus
Atherinidae
Atherinidae (Atherinomorus lacunosus)
Atherinidae (Doboatherina duodecimalis)
Mugilidae
Mugilidae
Mugilidae (Mugil cephalus)
Mugilidae (Osteomugil engeli)
Mugilidae (Planiliza affinis)
Mugilidae (Valamugil speigleri)
Clupeidae
Clupeidae (Spratelloides gracilis)
Clupeidae (Sardinella melanura)
Poeciliidae
Poeciliidae
Poeciliidae (Gambusia affinis)
Exocoetidae
Exocoetidae
Exocoetidae (Parexocoetus brachypterus)
Gobionidae
Gobionidae (Pseudorasbora parva)
Engraulidae
Engraulidae (Encrasicholina heteroloba)
Xenocyprididae
Leiognathidae
Others

Figure 2

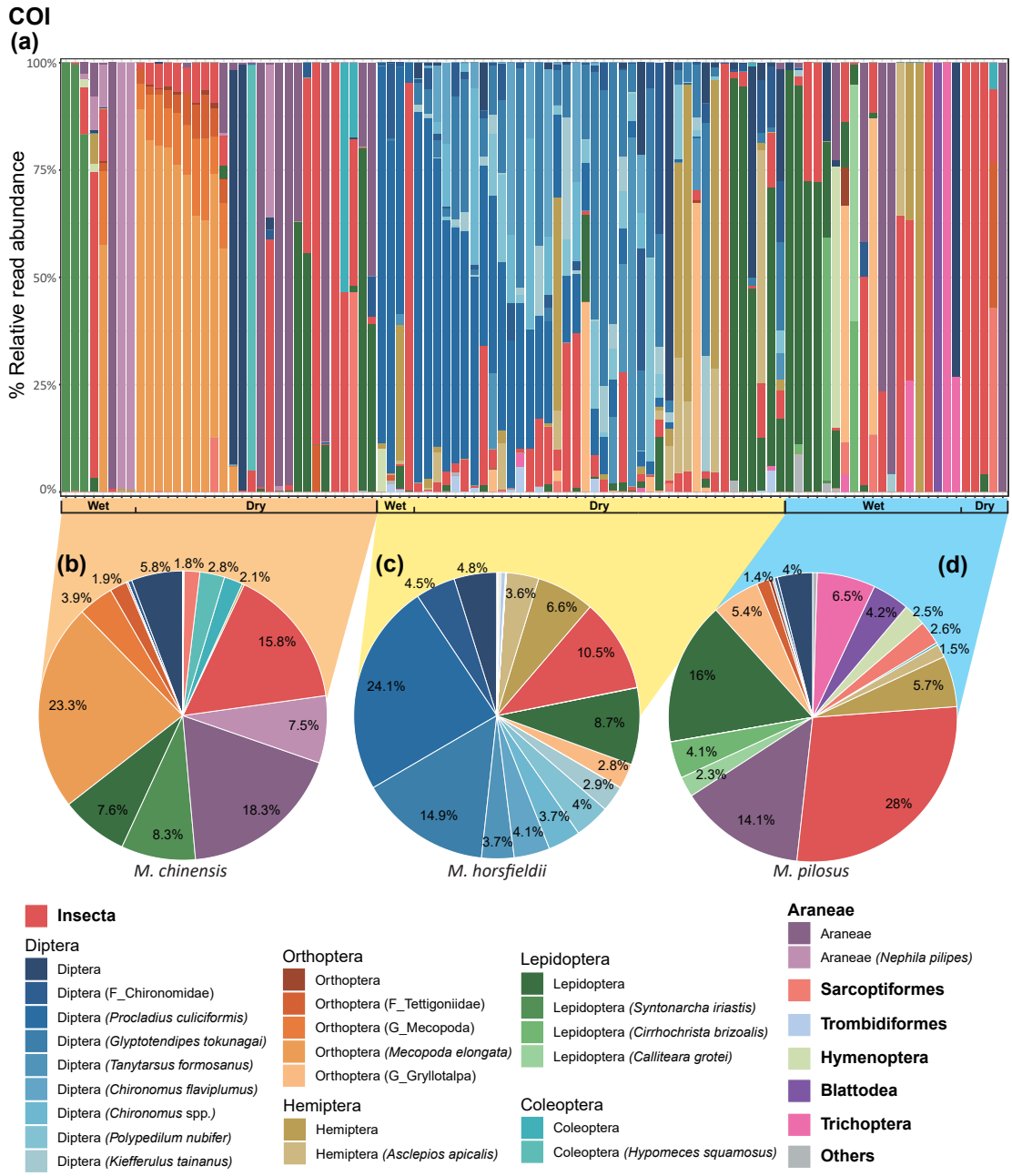
COI
(a)
% Relative read abundance
100%
75%
50%
25%
0%
Wet
Dry
Wet
Dry
Wet
Dry
(b)
5.8%
1.8%
2.8%
2.1%
1.9%
3.9%
15.8%
7.5%
23.3%
18.3%
7.6%
8.3%
M. chinensis
(c)
4.8%
4.5%
3.6%
6.6%
10.5%
24.1%
8.7%
2.8%
2.9%
4%
3.7%
4.1%
3.7%
14.9%
M. horsfieldii
(d)
1.4%
4%
6.5%
5.4%
4.2%
2.5%
2.6%
1.5%
16%
5.7%
4.1%
2.3%
28%
14.1%
M. pilosus
Insecta
Diptera
Diptera
Diptera (F_Chironomidae)
Diptera (Procladius culiciformis)
Diptera (Glyptotendipes tokunagai)
Diptera (Tanytarsus formosanus)
Diptera (Chironomus flaviplumus)
Diptera (Chironomus spp.)
Diptera (Polypedilum nubifer)
Diptera (Kiefferulus tainanus)
Orthoptera
Orthoptera
Orthoptera (F_Tettigoniidae)
Orthoptera (G_Mecopoda)
Orthoptera (Mecopoda elongata)
Orthoptera (G_Gryllotalpa)
Hemiptera
Hemiptera
Hemiptera (Asclepios apicalis)
Lepidoptera
Lepidoptera
Lepidoptera (Syntonarcha iriastis)
Lepidoptera (Cirrhochrista brizoalis)
Lepidoptera (Calliteara grotei)
Coleoptera
Coleoptera
Coleoptera (Hypomeces squamosus)
Araneae
Araneae
Araneae (Nephila pilipes)
Sarcoptiformes
Trombidiformes
Hymenoptera
Blattodea
Trichoptera
Others

Figure 3

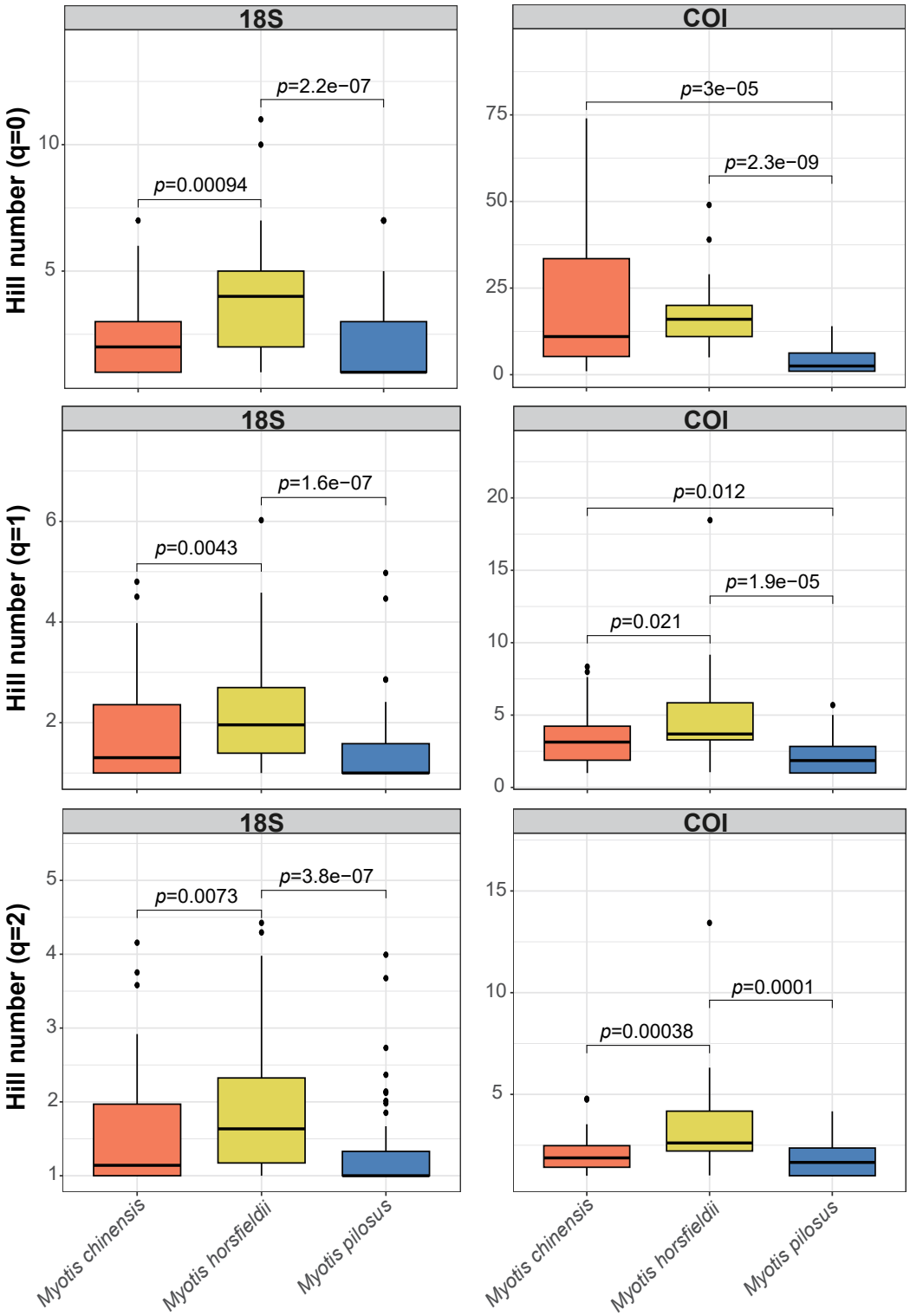
18S
COI
Hill number (q=0)
Hill number (q=1)
Hill number (q=2)
p=2.2e−07
p=0.00094
p=3e−05
p=2.3e−09
p=1.6e−07
p=0.0043
p=0.012
p=1.9e−05
p=0.021
p=3.8e−07
p=0.0073
p=0.0001
p=0.00038
Myotis chinensis
Myotis horsfieldii
Myotis pilosus

Figure 4

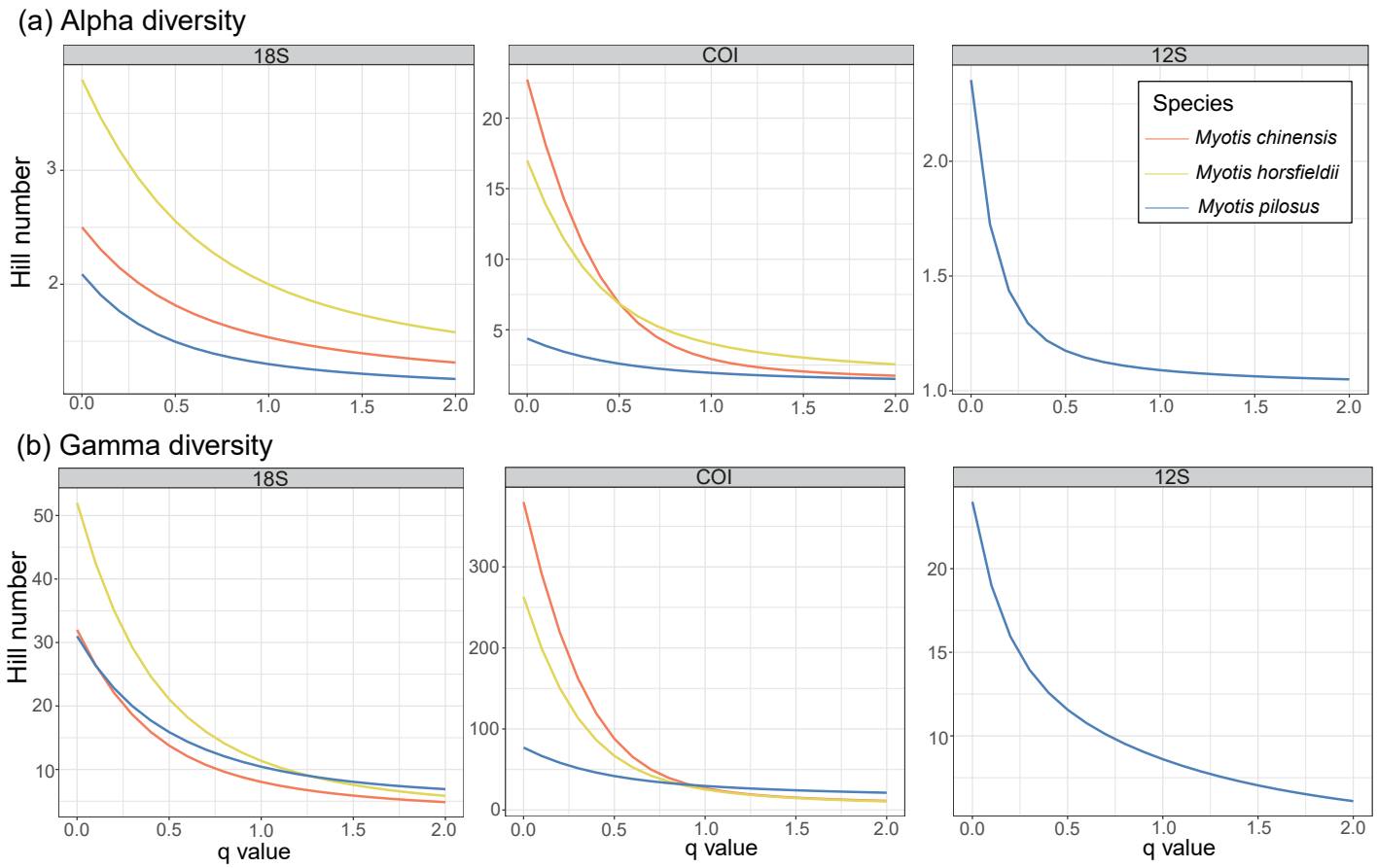
(a) Alpha diversity
18S
COI
12S
Species
Myotis chinensis
Myotis horsfieldii
Myotis pilosus
Hill number
(b) Gamma diversity
18S
COI
12S
Hill number
q value
q value
q value

Figure 5

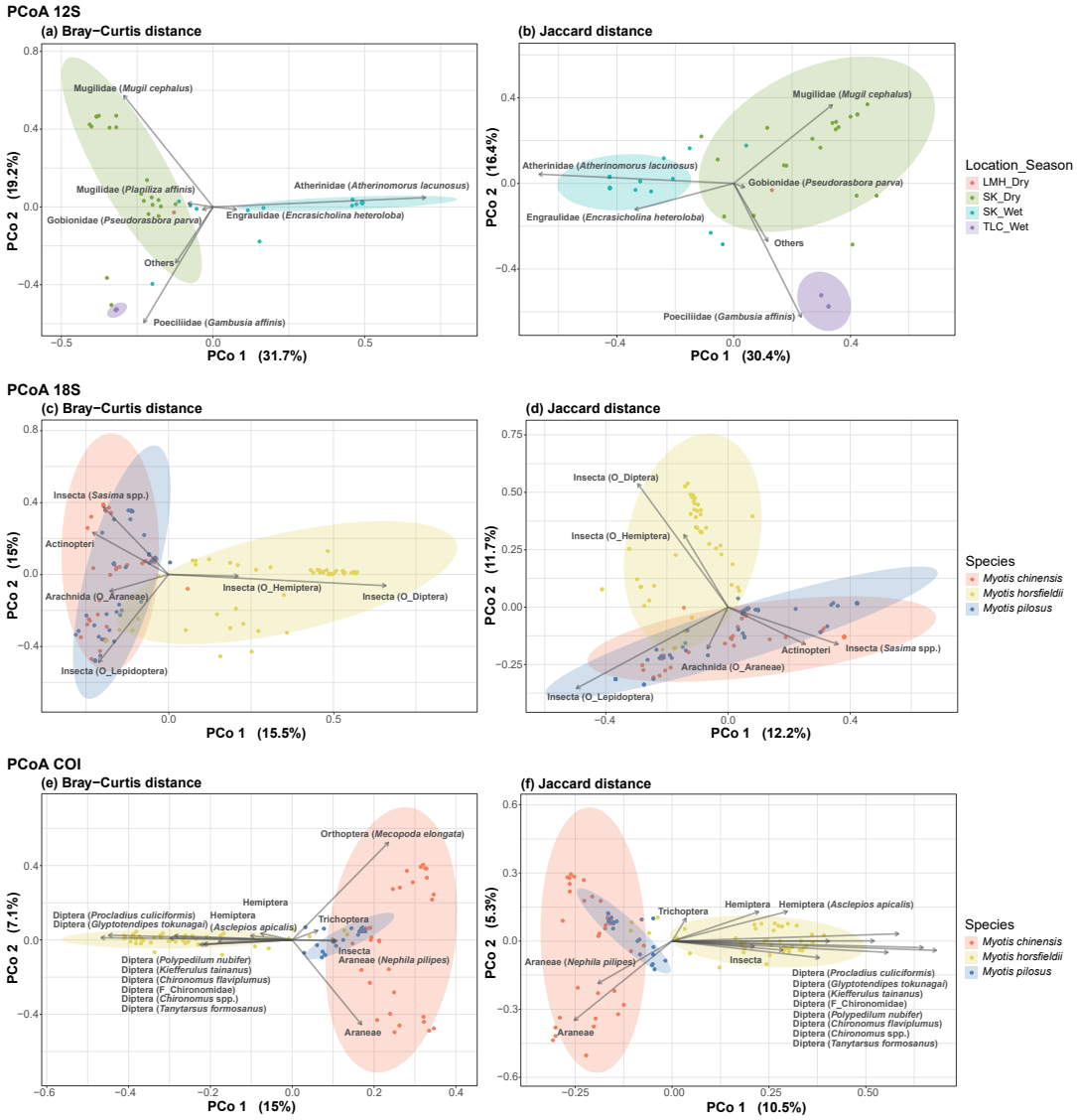
PCoA 12S
(a) Bray−Curtis distance
Mugilidae (Mugil cephalus)
Mugilidae (Planiliza affinis)
Gobionidae (Pseudorasbora parva)
Atherinidae (Atherinomorus lacunosus)
Engraulidae (Encrasicholina heteroloba)
Others
Poeciliidae (Gambusia affinis)
PCo 1 (31.7%)
PCo 2 (19.2%)
(b) Jaccard distance
Mugilidae (Mugil cephalus)
Atherinidae (Atherinomorus lacunosus)
Gobionidae (Pseudorasbora parva)
Engraulidae (Encrasicholina heteroloba)
Others
Poeciliidae (Gambusia affinis)
PCo 1 (30.4%)
PCo 2 (16.4%)
Location_Season
LMH_Dry
SK_Dry
SK_Wet
TLC_Wet
PCoA 18S
(c) Bray−Curtis distance
Insecta (Sasima spp.)
Actinopteri
Arachnida (O_Araneae)
Insecta (O_Lepidoptera)
Insecta (O_Hemiptera)
Insecta (O_Diptera)
PCo 1 (15.5%)
PCo 2 (15%)
(d) Jaccard distance
Insecta (O_Diptera)
Insecta (O_Hemiptera)
Actinopteri
Insecta (Sasima spp.)
Arachnida (O_Araneae)
Insecta (O_Lepidoptera)
PCo 1 (12.2%)
PCo 2 (11.7%)
Species
Myotis chinensis
Myotis horsfieldii
Myotis pilosus
PCoA COI
(e) Bray−Curtis distance
Orthoptera (Mecopoda elongata)
Hemiptera
Diptera (Procladius culiciformis)
Diptera (Glyptotendipes tokunagai)
Hemiptera (Asclepios apicalis)
Trichoptera
Insecta
Araneae (Nephila pilipes)
Diptera (Polypedilum nubifer)
Diptera (Kiefferulus tainanus)
Diptera (Chironomus flaviplumus)
Diptera (F_Chironomidae)
Diptera (Chironomus spp.)
Diptera (Tanytarsus formosanus)
Araneae
PCo 1 (15%)
PCo 2 (7.1%)
(f) Jaccard distance
Trichoptera
Hemiptera
Hemiptera (Asclepios apicalis)
Araneae (Nephila pilipes)
Insecta
Araneae
Diptera (Procladius culiciformis)
Diptera (Glyptotendipes tokunagai)
Diptera (Kiefferulus tainanus)
Diptera (F_Chironomidae)
Diptera (Polypedilum nubifer)
Diptera (Chironomus flaviplumus)
Diptera (Chironomus spp.)
Diptera (Tanytarsus formosanus)
PCo 1 (10.5%)
PCo 2 (5.3%)
Species
Myotis chinensis
Myotis horsfieldii
Myotis pilosus

Figure 6

Supplementary Materials

Foraging niche partitioning of three *Myotis* bat species and marine fish consumption by *Myotis pilosus* in a subtropical East Asian region

Supplementary materials and methods

Supplementary figures S1-S5

Supplementary Materials and Methods

Preparation of DNA metabarcoding libraries through 2-step PCRs

For 18S rDNA libraries, 1st step PCRs were carried out in 30 µl reactions containing 6 µl of 5X GoTaq Flexi Buffer, 0.6 µl of 10 mM dNTP Mix, 3.6 µl of 25 mM $MgCl_2$, 0.15 µl of 5 U/µl GoTaq G2 Flexi DNA Polymerase, 1 µl of extracted DNA, 0.6 µl of each assigned 10 µM forward and reverse primer uniquely tagged with heterogeneity spacer (Cruaud et al. 2017), 6 µl of 10 % DMSO (Sigma), 0.15 µl of 20 mg/ml BSA (NEB) and ultrapure water. Thermal cycling condition was 95 °C for 2 min; 25 cycles of 95 °C for 30 sec, 50 °C for 30 sec and 72 °C for 30 sec; and final extension at 72 °C for 5 min.

For COI libraries, the 1st step PCRs were carried out in 6 µl of 5X GoTaq Flexi Buffer, 0.6 µl of 10 mM dNTP Mix, 3.6 µl of 25 mM $MgCl_2$, 0.15 µl of 5 U/µl GoTaq G2 Flexi DNA Polymerase, 1 µl of extracted DNA, 1.5 µl of each assigned 10 µM forward and reverse primer uniquely tagged with heterogeneity spacer (Cruaud et al. 2017), 6 µl of 10 % DMSO (Sigma), 0.15 µl of 20 mg/ml BSA (NEB) and ultrapure water. Thermal cycling condition was 95 °C for 2 min; 25 cycles of 95 °C for 30 sec, 48 °C for 30 sec and 72 °C for 30 sec; and final extension at 72 °C for 5 min. Target PCR products generated from COI primers were gel extracted to eliminate non-targets.

For 12S rDNA libraries, 1st step PCRs were carried out in 30 µl reactions containing 6 µl of 5X GoTaq Flexi Buffer, 0.6 µl of 10 mM dNTP Mix, 3.6 µl of 25 mM $MgCl_2$, 0.15 µl of 5 U/µl GoTaq G2 Flexi DNA Polymerase, 1 µl of extracted DNA, 0.6 µl of each assigned 10 µM forward and reverse primer uniquely tagged with heterogeneity spacer (Cruaud et al. 2017), 6 µl of 10 % DMSO (Sigma), 0.15 µl of 20 mg/ml BSA (NEB) and ultrapure water. Each of the 1st step PCR products was eluted in 20 µl elution buffer and used as the templates for the 2nd step PCRs.

The 2nd step PCRs of both markers were performed with the same conditions: 2nd step PCRs were performed in 45 µl reactions comprising 9 µl of 5X GoTaq Flexi Buffer, 0.9 µl of 10 mM dNTP Mix, 5.4 µl of 25 mM $MgCl_2$, 0.225 µl of 5 U/µl GoTaq G2 Flexi DNA Polymerase, 20 µl of 1st step PCR products, 0.9 µl of each assigned 10 µM forward and reverse index primers, 4.5 µl of 10 % DMSO (Sigma) and ultrapure water. PCR condition was 95 °C for 2 min; 15 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec; and final extension at 72 °C for 5 min. We used the PureLink PCR Purification Kit (Invitrogen, Carlsbad, CA) to clean up all PCR products. The band sizes of all 1st and 2nd PCR products were checked on 1.5% agarose gels by gel electrophoresis.

Reference

Cruaud P, Rasplus J-Y, Rodriguez LJ, Cruaud A (2017) High-throughput sequencing of multiple amplicons for barcoding and integrative taxonomy Scientific reports 7:41948

Supplementary Figures

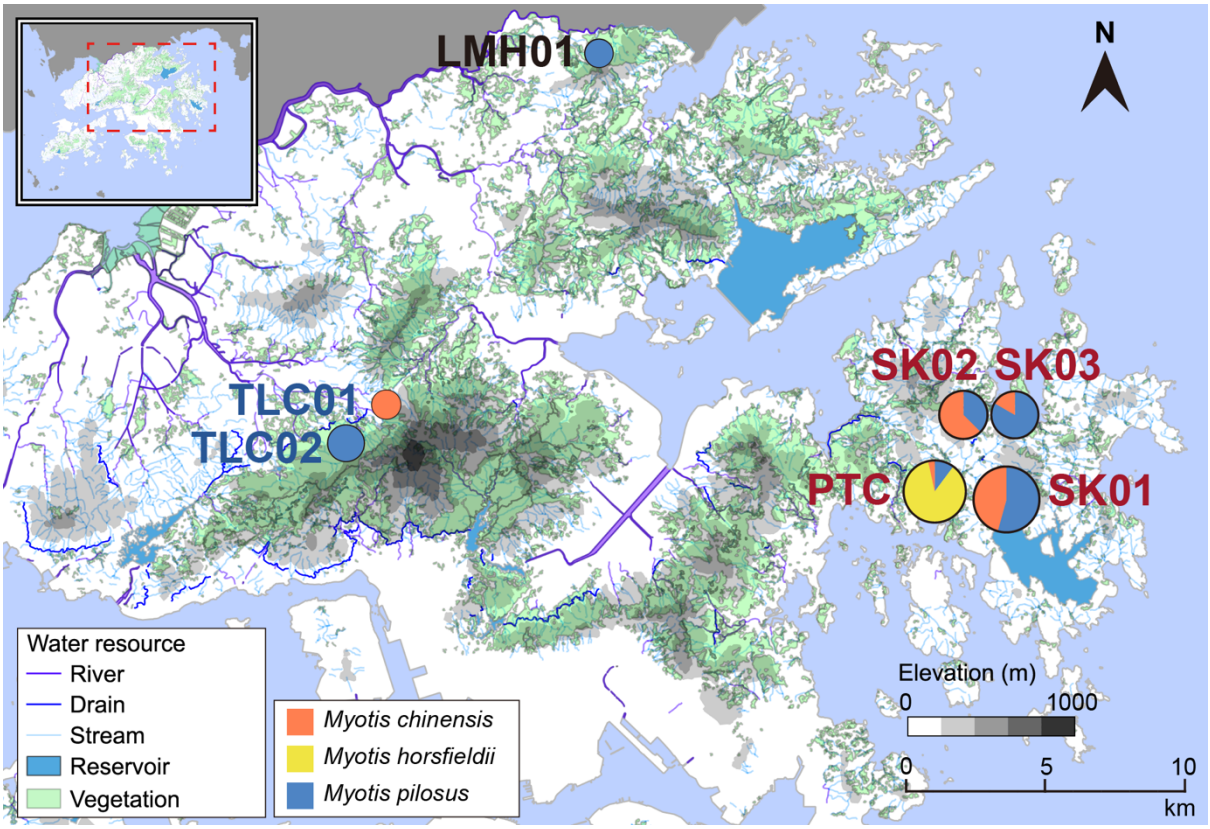

Figure S1.
The fecal samples of *Myotis* bats were collected from seven sites in Hong Kong. The small map at the upper left corner represents Hong Kong at the south coast of China, with the area enclosed in a red box magnified for ease of viewing. The pie charts in the magnified map represent the relative quantity (not to scale) of samples collected at each location, with larger pie charts indicating a larger sample size. Sites in close proximity were grouped as sampling locations for analyses (see Table S1). The locations include LMH (indicated in black), TLC (indicated in dark blue, including TLC01 and TLC02), and SK (indicated in dark red, including PTC, SK01, SK02, and SK03). LMH, Lin Ma Hang. TLC, Tai Lam Chung. PTC, Pak Tam Chung Camp. SK, Sai Kung.

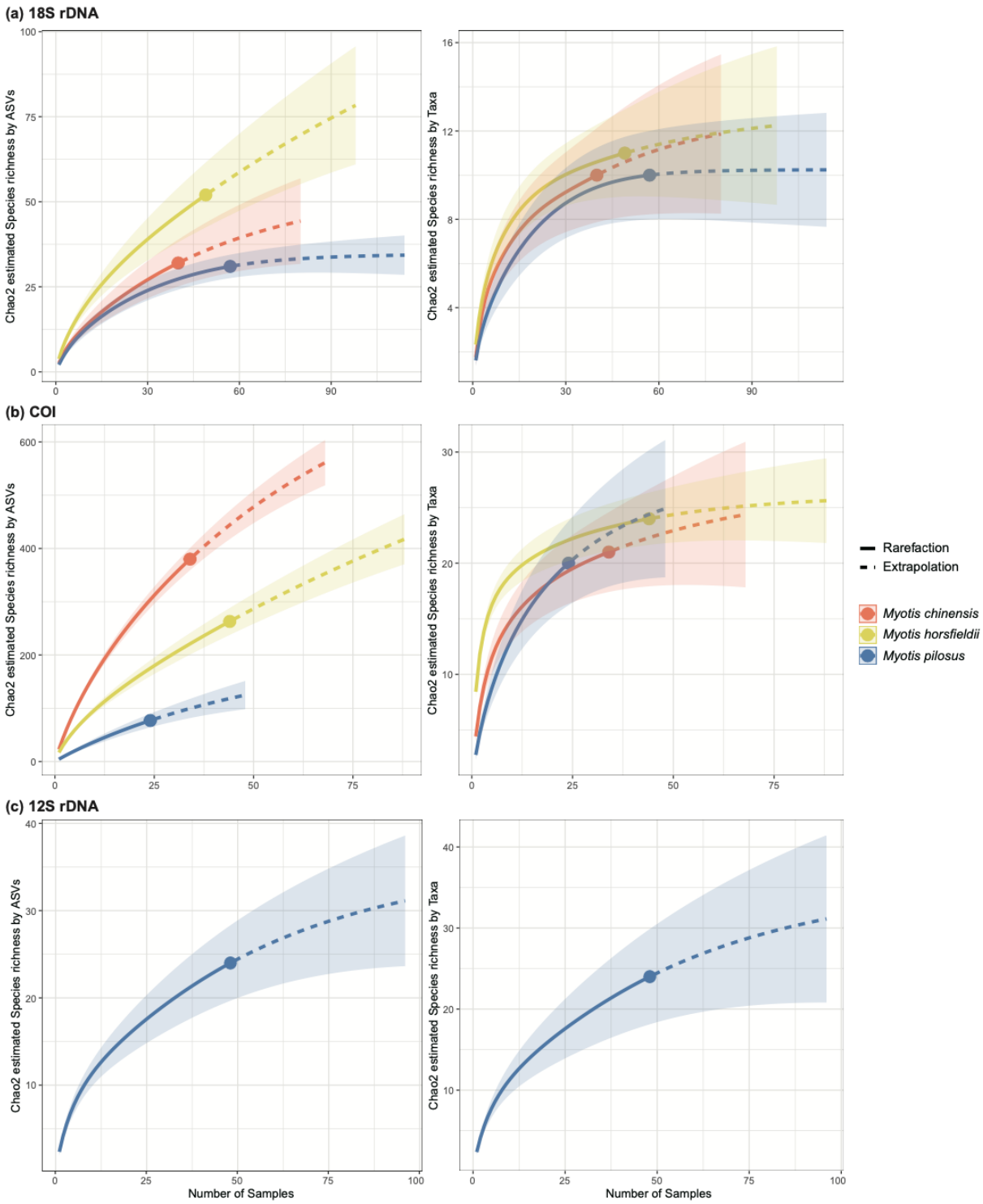

Figure S2.
Rarefaction curves based on Chao2 species richness. The Chao2 species richness was estimated from hill numbers of diversity order q=0, which were evaluated based on ASVs (left panel) or taxa (b) in each *Myotis* species. Dietary ASVs or taxa were identified by (a) 18S rDNA, (b) COI, and (c) 12S rDNA marker gene. The shading area represent the 95% confidence intervals, which were estimated using a bootstrap method based on 100 replicates.

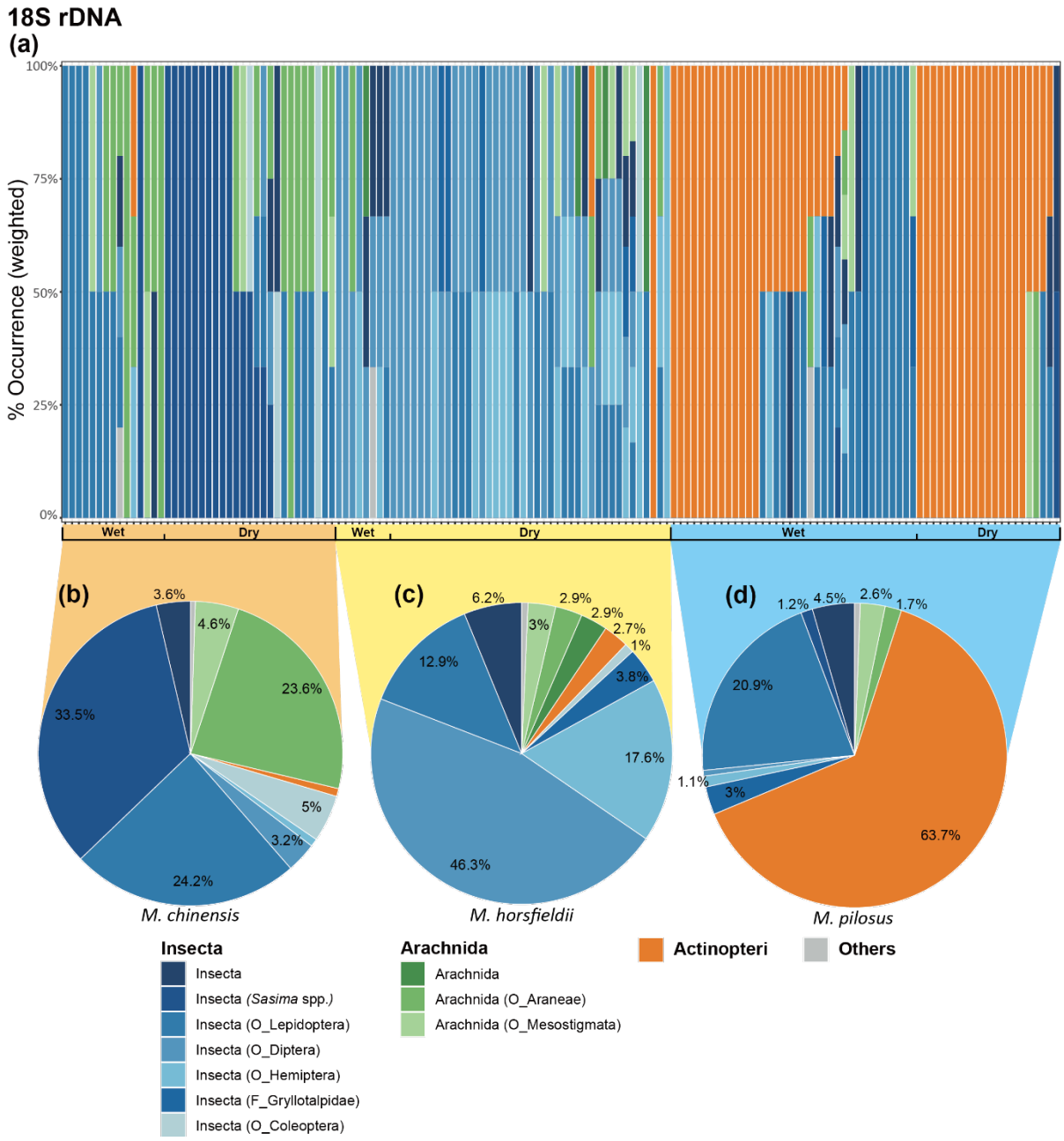

Figure S3.
Dietary composition of three *Myotis* bat species based on taxa detected in fecal samples using the 18S rDNA marker. The weighted percentages of occurrence of each taxon were visualized at both (a) bat individual level (n=146) and (b-d) bat species level for (b) *M. chinensis* (n=40), (c) *M. horsfieldii* (n=49), and (d) *M. pilosus* (n=57). Prey in classes with read abundance lower than 0.1% of all taxa detected were grouped into "Others". Only taxa with abundance higher than 1% in relative read abundance are shown. F, Family. O, Order.

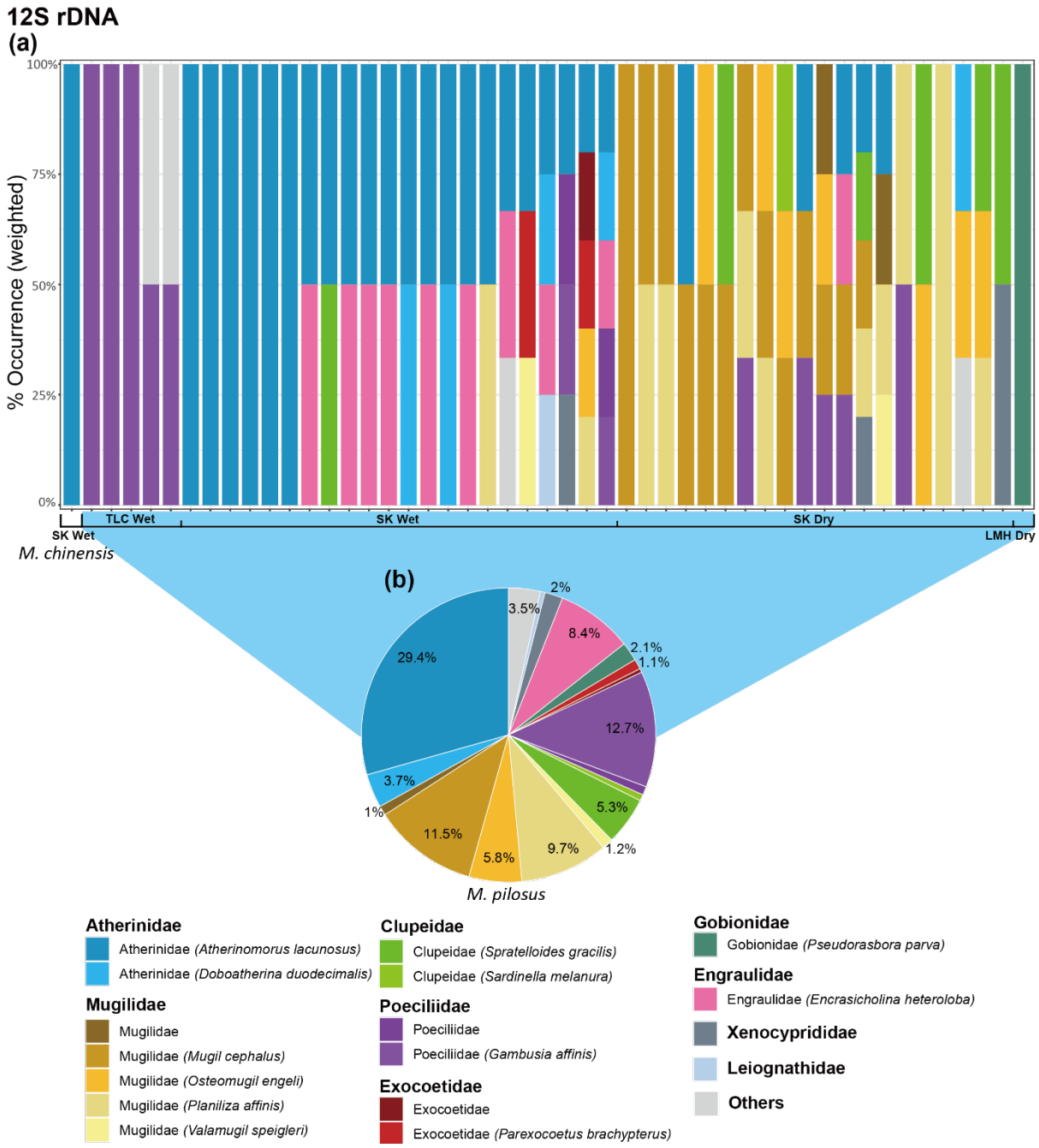

Figure S4.
Dietary composition of two *Myotis* bat species based on taxa detected in fecal samples using the 12S rDNA marker. The weighted percentages of occurrence of each taxon were visualized at both (a) individual level [*M. chinensis* (n=1) and *M. pilosus* (n=48)] and (b) species level (*M. pilosus*, n=48). In the chart, bars representing bat samples collected from the same location in the same season were clustered. Prey in classes with read abundance lower than 0.1% of all taxa detected were grouped into "Others". Only taxa with abundance higher than 1% in relative read abundance are shown. SK, Sai Kung (including Pak Tam Chung); TLC, Tai Lam Chung; LMH, Lin Ma Hang. Refer to Fig. S1 for locations.

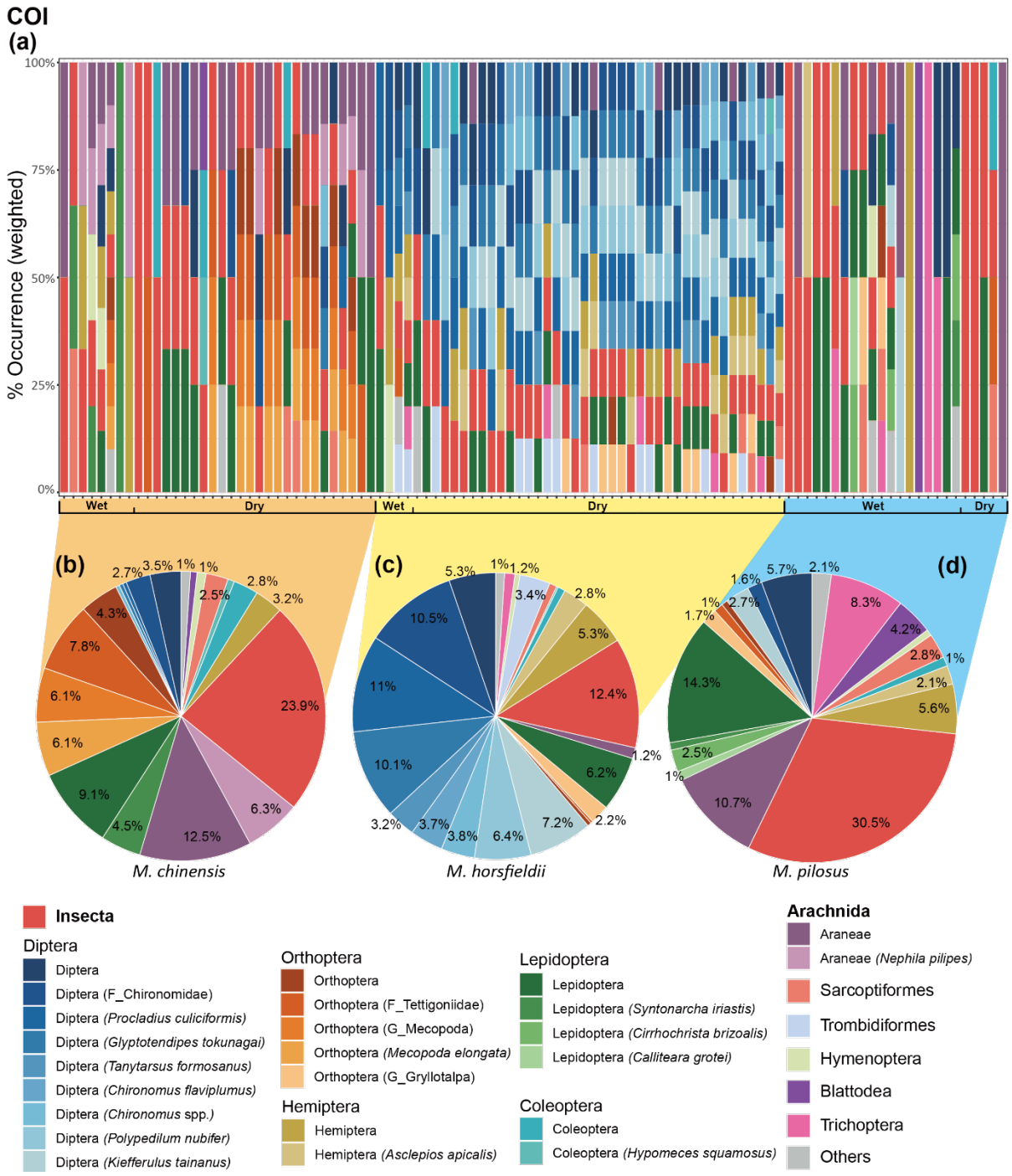

Figure S5.
Dietary composition of three *Myotis* bat species based on taxa detected in fecal samples using the COI marker. The weighted percentages of occurrence of each taxon were visualized at both (a) individual level (n=102) and (b-d) species level for (b) *M. chinensis* (n=34), (c) *M. horsfieldii* (n=44), and (d) *M. pilosus* (n=24). Prey in classes with read abundance lower than 0.1% of all taxa detected were grouped into "Others". Only taxa with abundance higher than 1% in relative read abundance are shown. G, Genus. F, Family.