High-throughput sequencing-based waterbirds' diet analysis: application to wintering herbivorous geese

Yuzhan Yang, Aibin Zhan, Lei Cao, Fanjuan Meng, Wenbin Xu

Food availability and diet selection are important factors influencing the abundance and distribution of wild waterbirds. In order to deeply understand factors influencing demographic change of waterbird population, it is essential to assess their diet. However, the use of traditional methods such as microhistologic observation could be difficult and sometimes inaccurate. Alternatively, molecular methods such as DNA metabarcoding have been proved useful in recent years. Here, we analyzed diets of greater white-fronted goose *Anser albifrons* and bean goose *Anser fabalis*, which are obligate herbivores wintering mostly in Middle and Lower Yangtze River Floodplain. We firstly prepared a local plant reference library by selecting optimal gene (P6 loop of chloroplast *trn*L intron) and amplifying the most common plants that these geese would potentially consume. By using DNA metabarcoding, we discovered 15 food items in total from feces of these two waterbirds. Of the 15 unique food items, 10 could be identified to the specie-level. For the greater white-fronted goose, 73.10% and 26.52% of sequences belonged to *Poaceae* spp. and *Carex* spp., respectively. In contrast, almost all sequences of the bean goose belonged to *Carex* spp. (99.72%). Using the same samples, microhistology provided consistent food composition with metabarcoding results for greater white-fronted goose, while 13% of *Poaceae* spp. was recovered for bean goose. In addition, two other taxa were discovered only through microhistologic analyses. Although most of the identified taxa matched relatively well between the two methods, DNA metabarcoding gave taxonomically more detailed information. The discrepancy was likely due to biased PCR amplification in metabarcoding, low discriminating power of selected genes for monocots, and biases in microhistologic analysis. The diet discrepancy between two geese species might indicate deeper ecology significance beyond the scope of this study. We concluded that DNA metabarcoding provided new perspectives for studies of herbivorous waterbirds diets and inter-specific interactions, as well as new possibilities to investigate interactions between herbivores and plants. In addition, microhistologic analysis or other approaches should be used together with metabarcoding methods to bring integrative information.

High-throughput sequencing-based waterbirds' diet analysis: application to wintering herbivorous geese

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Abstract

Food availability and diet selection are important factors influencing the abundance and distribution of wild waterbirds. In order to understand better about changes of waterbird population, it is essential to figure out what they feed on. However, analyzing diet could be difficult and inefficient using traditional methods, such as microhistologic observation. Here, we addressed this gap of knowledge by investigating diet of greater white-fronted goose *Anser albifrons* and bean goose *Anser fabalis*, which are obligate herbivores wintering mostly in Middle and Lower Yangtze River Floodplain. We firstly prepared a local plant reference library by selecting optimal marker gene (P6 loop of chloroplast *trn*L intron) and amplifying the most common plants that these geese would consume. Then utilizing DNA metabarcoding, we discovered 15 food items in total from feces of these birds. Of the 15 unique dietary sequences, 10 could be identified at specie-level. As for greater white-fronted goose, 73.10% of sequences belonged to *Poaceae* spp., and 26.52% belonged to *Carex* spp. In contrast, almost all sequences of bean goose belonged to *Carex* spp. (99.72%). Using the same samples, microhistology provided consistent food composition with metabarcoding results for greater white-fronted goose, while 13% of *Poaceae* was recovered for bean goose. Besides, two other taxa were discovered only through microhistologic analysis. Although most of the identified taxa matched relatively well between the two methods, DNA metabarcoding gave taxonomically more detailed information. The discrepancy were likely due to biased PCR amplification in metabarcoding, low discriminating power of current marker genes for monocots, and biases in microhistologic analysis. The diet discrepancy between two geese species might indicate deeper ecology

significance beyond the scope of this study. We concluded that DNA metabarcoding provided new perspectives for studies of herbivorous waterbirds diets and inter-specific interactions, as well as new possibilities to investigate interactions between herbivores and plants. In addition, microhistologic analysis should be used together with metabarcoding methods to bring integrative information.

Introduction

Wetlands are one of the most important ecosystems in nature, and they harbor a variety of ecosystem services such as protection against floods, water purification, climate regulation and recreational opportunities (Brander, Flora & Vermaat, 2006). Waterbirds are typically wetland-dependent animals upon which they could get abundant food and suitable habitats (Ma *et al.*, 2010). Waterbirds' abundance and distribution could reflect the status of wetland structure and functions, making them important bio-indicators for wetland health (Fox *et al.*, 2011). Among all factors affecting waterbird community dynamics, food availability is frequently considered to play one of the most important roles (Wang *et al.*, 2013). However, recently suitable food resources tend to decrease or even disappear due to deterioration and loss of natural wetlands (Fox *et al.*, 2011). As a result, waterbirds are forced to discard previous habitats and sometimes even feed in agricultural lands (Zhang *et al.*, 2011). In addition, migratory waterbirds may aid the dispersal of aquatic plants or invertebrates by carrying and transporting them between water bodies at various spatial scales (Reynolds, Miranda & Cumming, 2015). Consequently, long-time monitoring and systematic studies of waterbird diets are essential to understand population dynamics of waterbirds, as well as to establish effective management programs for them (Wang *et al.*, 2012).

As for waterbird diet analysis, traditional method was direct observation in the field (Swennen & Yu, 2005) or microhistologic analysis of remnants in feces and/or gut contents (James & Burney, 1997; Fox *et al.*, 2007). While these approaches have been proved useful in some cases, they are relatively labor-intensive and greatly skill-dependent (Fox *et al.*, 2007;

Samelius & Alisauskas, 1999; Symondson, 2002). Applications of other methods analyzing gut contents or feces were also restricted due to inherent limitations, as reviewed by Pompanon *et al.* (Pompanon *et al.*, 2012). Recently, based on high-throughput sequencing, metabarcoding methods provide new perspectives for diet analysis and biodiversity assessment (Taberlet *et al.*, 2007; Creer *et al.*, 2010). These methods provide higher taxonomy resolution and enormous sequence output simultaneously from large-scale environmental samples, such as soil, water and feces (Shokralla, Spall & Gibson, 2012; Bohmann *et al.*, 2014). Owing to these advantages, metabarcoding has been widely employed in diet analysis of herbivores (Taberlet *et al.*, 2012; Ando *et al.*, 2013; Hibert *et al.*, 2013), carnivores (Deagle, Kirkwood & Jarman, 2009; Shehzad *et al.*, 2012) and omnivores (De Barba *et al*., 2014). But pitfalls of metabarcoding should not be ignored when choosing suitable technique(s) for new studies. For instance, many researches have shown that it is difficult to obtain quantitative data using metabarcoding (Sun *et al.*, 2015). This drawback might result from both technical issues of this method and relevant biological features of samples (Pompanon *et al.*, 2012).

One paramount prerequisite of metabarcoding methods is to select robust genetic markers and corresponding primers (Zhan *et al.*, 2014; Zhan & MacIsaac, 2015). For diet study of herbivores, at least eight chloroplast genes and two nuclear genes are used as potential markers for land plants (Hollingsworth, Graham & Little, 2001). Although mitochondrial cytochrome *c* oxidase I (COI) is extensively recommended as a standard barcode for animals, relatively low rate of evolution in botanical genome precludes it being an optimum for plants (Wolfe, Li & Sharp, 1987; Fazekas *et al.*, 2008). The internal transcribed spacer (ITS) is excluded due to

82 divergence discrepancies of individuals and low reproducibility (Álvarez & Wendel, 2003). A
83 variety of combinations and comparisons have been performed for the eight candidate genes,
84 however, none proved equally powerful for all cases (Fazekas *et al.*, 2008). Consequently, it is
85 more effective to choose barcodes for a circumscribed set of species occurring in a regional
86 community (Kress *et al.*, 2009). Another equally important aspect of metabarcoding application
87 is the constructing of reference libraries which can dramatically assist taxonomic assignment
88 (Rayé *et al.*, 2011; Xu *et al.*, 2015). It is difficult to accurately interpret sequence reads without a
89 well-recovered reference library (Elliott & Jonathan Davies, 2014).
90 Diet analysis is the central issue in waterbird research, both for deciphering waterfowl
91 population dynamics and interpreting inter- or intra-specific interactions of cohabitating species
92 (Zhao *et al.*, 2015). For instance, there are more than 60% of bean goose *Anser fabalis*
93 population and almost 40% of greater white-fronted goose *Anser albifrons* population along the
94 East Asian – Australian Flyway Route wintering at the Shengjin Lake National Nature Reserve
95 (Zhao *et al.*, 2015). Previous studies based on microhistologic observation illustrated that the
96 dominant composition of their diets were monocotyledons, such as *Carex* spp. (Zhao *et al.*,
97 2012), *Poaceae* (Zhang *et al.*, 2011), and a relatively small proportion of non-monocots (referred
98 to as dicotyledons in study of "Zhao, Cao & Fox, 2013"). However, few food items could be
99 identified to species-level, mainly owing to variable tissue structures within plants, similar
100 morphology between relative species, and a high level of degradation after digestion (Zhang *et*
101 *al.*, 2011; Zhao *et al.*, 2012; Zhao, Cao & Fox, 2013). Ambiguous identification hindered
102 understanding of waterbirds population dynamics and abilities to establish effective conservation

plans for them in the future.

In this study, we tried to improve this situation using metabarcoding method to analyze diets of these species (see flowchart in Fig. 1). By examining the efficiency of eight candidate genes (*rbc*L, *rpo*C1, *rpo*B, *mat*K, *trn*H-*psb*A, *trn*L (UAA), *atp*F-*atp*H, and *psb*K-*psb*I), we selected robust genes and corresponding primers for reference library constructing and high-throughput sequencing. Subsequently, we used the metabarcoding method to investigate diet composition of these two species based on faeces collected from the Shengjin Lake. Finally, we discussed and compared results from microhistology and DNA metabarcoding using the same samples to assess the utility and efficiency of two methods.

Materials and Methods

Ethics Statement

There is no need for an ethics statement, because our research work did not involve capture or any direct manipulation or disturbances of animals. We only collected samples of plants and feces for molecular analyses. We got access to the reserve under the permission of Shengjin Lake National Nature Reserve Administration (Chizhou, Anhui, China), which is responsible for the management of the protected area and wildlife. We were forbidden to capture or disturb geese in the field.

Study Area

Shengjin Lake (116º55′ - 117º15′ E, 30º15′ -30º30′ N) was established as National Nature Reserve in 1997, aiming to protect diverse waterbirds including geese, cranes and storks. The water level fluctuates greatly in this lake, with maximal water level of 17 m during summer

(flood season) but only 10 m during winter (dry season). Due to this fluctuation, receding waters expose two large *Carex* spp. meadows and provide suitable habitats for waterbirds. This makes Shengjin Lake one of the most important wintering sites for migratory waterbirds (Zhao *et al.*, 2015). Greater white-fronted goose and bean goose are dominant herbivores wintering (from October to next April) in this area, accounting for 40% and 60% of population along the East Asian – Australian Flyway Route, respectively (Zhao *et al.*, 2015).

Field Sampling

The most common plant species that these two geese possibly consume were collected in May 2014 and January 2015, especially species belonging to *Carex* and *Poaceae*. Fresh and intact leaves were carefully picked, tin-packaged in the field and stored at -80 °C in the laboratory before further treatment. Morphological identification was carried out with the assistance of two botanists (Profs Zhenyu Li and Shuren Zhang from Institute of Botany, Chinese Academy of Sciences). In total, 87 specimens were collected, belonging to 25 families, 53 genera and 70 species (Table S1).

All feces were collected at the reserve (Fig. 2) in January 2015. Based on previous studies and latest waterbird survey, sites with big flocks of geese (i.e. more than 200 individuals) were chosen (Zhang *et al.*, 2011). As soon as geese finished feeding and feces were defecated, fresh droppings were picked and stored in dry ice. Droppings of bean geese were generally thicker than those of smaller greater white-fronted goose to the degree that these could be reliably distinguished in the field (Zhao *et al.*, 2015). Disposal gloves were changed for each sample to avoid cross contamination. To avoid repeated sampling and make sure samples were from

different individuals, each sample was collected with a separation of more than two meters. In total, 21 feces were collected, including 11 for greater white-fronted goose and 10 for bean goose. All samples were transported to laboratory in dry ice and then stored at -80 °C until further analysis.

Selection of Molecular Markers and Corresponding Primers

In this part, we aimed to select gene markers with adequate discriminating power for our study. We included eight chloroplast genes - *rbc*L, *rpo*C1, *rpo*B, *mat*K, *trn*H-*psb*A, *trn*L (UAA), *atp*F-*atp*H, and *psb*K-*psb*I for estimation. Although Shengjin Lake consisted an array of plant species, we focused mainly on the most potential food resources (Xue *et al.*, 2008; Zhao *et al.*, 2015) that geese would consume for candidate gene tests. These covered eleven genera and one family (Table S2). For tests of all candidate genes, we recovered sequences of representative species in the selected groups from GenBank (http://www.ncbi.nlm.nih.gov/nuccore). We calculated inter-specific divergence within every genus or family based on Kiruma 2-paramter model (K2P) using MEGA version 6 (Tamura *et al.*, 2013). We also constructed molecular trees based on UPGMA using MEGA and characterized the resolution of species by calculating the percentage of species recovered as monophyletic based on phylogenetic trees. Secondly, primers of candidate genes selected out of eight were used to amplify all 87 specimens to check their amplification efficiency and universality. Thirdly, we calculated inter-specific divergence based on sequences that we obtained from last step. Generally, a robust barcode gene is recommended when the minimal inter-specific distance exceeds the maximal intra-specific distance (e.g. existence of barcoding gaps). For reference database building, we calculated the rate of

discrimination for the species in each family (Rf) by dividing the number of unique sequences per family by the number of species in each family. At last, to allow the recognition of sequences after high-throughput sequencing, both of the forward and reverse primers of the selected marker gene were tagged specifically for each sample with 8nt nucleotide codes at the 5' end (Parameswaran *et al.*, 2007).

DNA Extraction, Amplification and Sequencing

Two hundred milligrams of leaf was used to extract the total DNA from each plant sample using a modified CTAB protocol (Cota-Sanchez, Remarchuk & Ubayasena, 2006). DNA extraction of feces was carried out using the same protocol with minor modification in incubation time (elongate to 12 h). Each fecal sample was crushed thoroughly and divided into four quarters. All quarters of DNA extracts were then pooled together. DNA extraction was carried out in a clean room used particularly for this study. For each batch of DNA extraction, negative controls (i.e. extraction without feces) were included to monitor possible contamination.

For plant DNA extracts, PCR amplifications were carried out in a volume of 25μl with ~100 ng total DNA as template, 1U of *Taq* Polymerase (Takara, Dalian, Liaoning Prov., China), 1× PCR buffer, 2 mM of Mg^{2+}, 0.25 mM of dNTPs, 0.1 μM of forward primer and 0.1 μM of reverse primer. After 4 min at 94 °C, the PCR cycles were as follows: 35 cycles of 30 s at 94 °C, 30 s at 56 °C and 45 s at 72 °C, and the final extension was 10 min at 72 °C. We applied the same PCR conditions for all primers. All the successful PCR products were sequenced in Genewiz (Suzhou, Jiangsu Prov., China).

For fecal DNA extracts, PCR mixtures (25μl) were prepared in six replicates for each sample to reduce biased amplification. Each replicate consisted of ~100 ng total DNA of DNA extract as template, 1U of *Taq* Polymerase (Takara, Dalian, Liaoning Prov., China), 1× buffer, 2 mM of $MgCl_2$, 0.25 mM of each dNTPs, 0.1 μM of foward primer and 0.1 μM of reverse primer. After 4 min of denaturation at 94 °C, the PCR cycles were as follows: 35 cycles of 30 s at 94 °C, 30 s at 56 °C, 45 s at 72 °C, and the final elongation was 10 min at 72 °C. Each set of six replicates was pooled and purified using the Sangon PCR product purification kit (Sangon Biotech, Shanghai, China). Quantification was carried out to ensure equilibrium of contribution of each sample using the NanoDrop ND-2000 UV-Vis Spectrophotometer (NanoDrop Technologies, Delaware, United States of America). High-throughput sequencing was performed using Illumina MiSeq platform following manufacturer's instructions by BGI (Shenzhen, Guangdong Prov., China). Reads of high-throughput sequencing could be found at NCBI's Sequence Read Archive (Accession number: SRP070470).

Data Analysis for Estimating Diet Composition

After high-throughput sequencing, pair-ended reads were merged with using UPARSE pipeline (http://drive5.com/usearch, Edgar, 2010). Reads were then split into independent files according to unique tags using RDP pipeline (http://rdp.cme.msu.edu/). We removed sequences i) that didn't perfectly match tags and primer sequences; ii) that contained ambiguous nucleotide (N's). Tags and primers were then trimmed with RDP pipeline. Further quality filtering based on UPARSE pipeline discarded sequences with i) quality score less than 30 (<Q30) and ii) shorter than 100 bp and longer than 200 bp. Unique sequences were clustered to operational taxonomy

units (OTUs) at the similarity threshold of 98% (Edgar, 2013). All OTUs were assigned to unique taxonomy with local blast 2.2.30+ (Altschul *et al.*, 1990). We detected a plant within the reference library for each sequence with the threshold of length coverage > 98%, identity > 98% and e-value < 1.0 e^{-50}. If a query sequence matched two or more taxa, it was assigned to a higher taxonomic level which included all taxa.

Microhistology analysis

We used the method described by Zhang *et al.* (2011) to perform microhistologic examination of fecal samples. Each sample was first washed with pure water and filtered with a 25-μm filter. Subsequently, the suspension was examined under a light microscope at 10× magnification for quantification statistics and at 40× magnification for species identification. We compared photos of visible fragments with epidermis database of the Shengjin Lake to identify food items.

Results

Selection of Genes and Corresponding Primers and Reference Library Constructing

A total of 3,296 representative sequences were recovered from GenBank and the sequence number per gene ranged from 0 to 345 (Table S2). Among the eight candidate genes, *trn*L, *trn*H-*psb*A, *mat*K and *rbc*L showed largest inter-specific divergence in seven, three, one and one taxonomic groups, respectively. These four genes also displayed relatively high resolution of species (Table S2). For example, with *mat*K gene, 77% of *Carex* could be identified to species-level. However, our results indicated that none of these eight genes could simultaneously differentiate all 12 genera or families to species-level (Table S2). Considering the inter-specific divergence and resolution of species, we chose the most commonly used chloroplast genes *rbc*L,

228 *mat*K, *trn*H-*psb*A and *trn*L for further tests.

229 Primers of these four genes (Table 1) were used to amplify the plants that we collected in
230 the field. The selected primers for *trn*L and *rbc*L successfully amplified 100% and 91% of all
231 species, respectively, while primers for *trn*H-*psb*A and *mat*K amplified only 71% and 43%,
232 respectively. Therefore, we chose *trn*L and *rbc*L to test their discriminating power in our target
233 plants.

234 We calculated the inter-specific divergence within genera and families with at least two
235 species to compare their discriminating power. Maximal, minimal and mean inter-specific
236 distances were calculated for seven dominant genera and six dominant families (Table 2). Both
237 genes could not differentiate species of *Vallisneria Linn.* (mean=0.000±0.000%) and *Artemisia*
238 *Linn.* (mean=0.000±0.000%). But *trnL* showed larger divergence range for other six genera and
239 five families. Hence, we chose *trn*L as the barcoding gene for reference library constructing and
240 high-throughput sequencing for our study. The discriminating power of *trn*L was strong for most
241 species (Table 3). However, some species could only be identified at genus-level or family-level
242 with *trn*L. For instance, five species of *Potamogetonaceae* shared the same sequences and this
243 made them to be identified at family-level. Species could be identified easily for genera or family
244 containing one species, except for *Beckmannia syzigachne, Phalaris arundinacea L.*, and
245 *Polypogon fugax* which shared identical sequences.

246 **Data Processing for Estimating Diet Composition**

247 In total, 0.21 and 0.18 million reads were generated for greater white-fronted goose (GWFG) and
248 bean goose (BG), respectively (Table 4). The number of recovered OTUs ranged from 8 to 123

for GWFG and BG samples. We used local BLAST to compare these sequences with Shengjin Lake reference database. Finally, with DNA metabarocoding, 12 items were discovered in the feces of GWFG, including one at family-level, three at genus-level and eight at species-level (Table 5). Four items were discovered in the faeces of BG, including one at genus-level and three at species-level. This method identified 15 taxa in feces of these geese.

However, the sequence percentage of each food item varied greatly (Table 5). For GWFG, the majority of sequences (96.36%) were composed of only five items - *Poaceae* spp. (47.98%), *Poa annua* (21.86%), *Carex heterolepis* (17.51%), *Carex* spp. (9.01%), and *Alopecurus aequalis* (3.21%). For BG, almost all the sequences belonged to *Carex heterolepis* (99.49%). Other items only occupied a relatively small proportion of sequences. In addition, the presence of each item per sample was also unequal (Table S3). For example, *Carex heterolepis*, *Carex* spp., *Poa annua* and *Potentilla supina* were present in almost all the samples, while *Stellaria media (L.) Cyr.*, *Asteraceae* sp. and *Lapsana apogonoldes* occurred in only about one third of samples of greater white-fronted goose.

When microhistologic examination were performed using the same samples, eight items were found in the feces of greater white-fronted goose, including one at family-level, four at genus-level and three at species-level (Table 5). Dominant items were *Poaceae* spp. (45.68%), *Alopecurus Linn.* (30.93%) and *Carex heterolepis* (16.39%). Seven items were found in the feces of bean goose, including four at genus-level and three at species-level (Table 5). Dominant items were *Carex heterolepis* (62.85%), *Asteraceae* sp. (14.55%), and *Alopecurus Linn.* (13.18%).

Discussion

Marker Selection and Reference Library Constructing for Diet Analysis

With greatly reduced cost, extremely high throughput and explosive information content, metabarcoding revolutionized the exploration and quantification of dietary analysis with noninvasive samples containing degraded DNA (Fonseca *et al.*, 2010; Shokralla *et al.*, 2014). Despite enormous potential to boost data acquisition, successful application of this technology relies greatly on the power and efficiency of genetic markers and corresponding primers (Bik *et al.*, 2012; Zhan *et al.*, 2014). In order to select the most appropriate marker gene for our study, we compared the performance of eight commonly used chloroplast genes, *rbc*L, *rpo*B, *rpo*C1, *mat*K, *trn*L, *trn*H-*psb*A, *atp*F-*atp*H, and *psb*K-*psb*I and their corresponding primers. Although a higher level of discriminating power was shown in several studies, *atp*F-*atp*H, *psb*K-*psb*I, *rpo*B and *rpo*C1 were not as commonly used as other barcoding genes (Hollingsworth, Graham & Little, 2001). As one of the most rapidly evolving coding genes of plastid genomes, *mat*K was considered as the closest plant analogue to the animal barcode *COI* (Hilu & Liang, 1997). However, *mat*K was difficult to amplify using available primer sets, with only 43% of successful amplification in this study. In spite of the higher species discrimination success of *trn*H-*psb*A than *rbc*L+*mat*K in some groups, the presence of duplicated loci, microinversions and premature termination of reads by mononucleotide repeats lead to considerable proportion (30% in this study) of low-quality sequences and over-estimation of genetic difference when using *trn*H-*psb*A (Graham *et al.*, 2000; Whitlock Hale & Groff, 2010). In contrast, the barcode region of *rbc*L is easy to amplify, sequence, and align in most plants and was recommended as the standard barcode for land plants (Chase *et al.*, 2007). The relatively modest discriminating power

(compared to *trn*L) precludes its application for our study aiming to recover high resolution of food items. Consequently, *trn*L was selected out of eight candidate markers, with 100% amplification success, more than 90% of high quality sequences, and relatively large inter-specific divergence.

One of the biggest obstacles in biodiversity assessment and dietary analysis is the lack of a comprehensive reference library, without which it is impossible to accurately interpret and assign the enormous sequences generated from high-throughput sequencing (Valentini, Pompanon & Taberlet 2009; Barco *et al.*, 2015). In this study, we constructed a local reference library by amplifying the most common species (70 morpho-species in total) during the wintering period with *trn*L gene. Although not all of them could be identified at species-level with *trn*L due to relatively low inter-specific divergence, many species could be separated with distinctive sequences. Previous studies have recommended group-specific barcodes to differentiate closely related plants at the species level (Li *et al.*, 2015). For instance, *mat*K has been proved to be more efficient for the discrimination of *Carex* spp. (Starr, Naczi & Chouinard, 2009). However, the primer set of *mat*K failed to amplify species of *Carex* spp. in our study, suggesting the universality of selected primer pairs should be tested in each study (Zhan *et al.*, 2014).

Applications of Metabarcoding for Geese Diet Analysis

A variety of recent studies have demonstrated the great potential of metabarcoding for dietary analysis, mainly owing to the high throughput, high discriminating power, and the ability to process large-scale samples simultaneously (Creer *et al.*, 2010; Taberlet *et al.*, 2012; Shehzad *et al.*, 2012). In this study, we applied this method to recover diets of herbivorous geese and

312 provided standard protocols for dietary analysis of these two ecologically important waterbirds.
313 Our results further proved the more objective, less experience-dependent and more time-efficient
314 character of DNA metabarcoding. However, not all the species in the reference library could be
315 identified at species-level, owing to low inter-specific divergence. We suggested multiple group-
316 specific markers to be incorporated in the future, such as studies of De Barba *et al.*(2014).
317 Besides, *Carex thunbergii* and *Fabaceae* sp., were only discovered via microhistologic analysis
318 rather than metabarcoding. This failure might reflect the biased proliferation of current
319 technology, of which dominant templates could act as inhibitors of less dominant species (Piñol
320 *et al.*, 2015). However, three species of *Poaceae* were only discovered using metabarcoding. In
321 total, more taxa and higher resolution were attained using metabarcoding. But microhistology
322 still proved a powerful supplementary. Previous studies using metabarcoding usually detected
323 dozens of food items, even as many as more than one hundred species. For instance, 18 taxa prey
324 were identified for leopard cat (Shehzad *et al.*, 2012); 44 plant taxa were recovered in faeces of
325 red-headed wood pigeon (Ando *et al.*, 2013); while more than 100 taxa were found in diet
326 studies of brown bear (De Barba *et al.*, 2014). The relatively narrow diet spectrum of
327 herbivorous geese may lead to misunderstanding that this result of our study is merely an artefact
328 due to small sampling effort. However, this result is credible since these two geese species only
329 feed on *Carex* meadow, where the dominant vegetation is *Carex* spp., with other species such as
330 *Poaceae* and dicots (Zhao *et al.*, 2015). Even though other wetland plants exist, they usually
331 composed only a small proportion of the geese diets.
332 Quantification of food composition is another key concern in dietary analysis. Although the

relative percentage of sequences were not truly quantitative, taxa of the majority sequences in this study were in accord with microhistologic observations, which was granted as an efficient way to provide quantitative results (Wang *et al.*, 2013), while discrepancy also existed. This might come from the semi-quantitative nature of metabarcoding methods (Sun *et al.*, 2015). This is likely derived from PCR amplification, which always entails biases caused by universal primer-template mismatches, annealing temperature or number of PCR cycles (Zhan *et al.*, 2014; Piñol *et al.*, 2015). Other methods such as shot-gun sequencing or metagenomic sequencing could be incorporated in the future to bring integrative information (Srivathsan *et al.*, 2015).

Implications for Waterbird Conservation and Wetland Management

For long-distance migratory waterbirds, such as wild geese in this study, their abundance and distribution were greatly influenced by diet availability and habitat uses (Wang *et al.*, 2013). For example, waterbirds may be restricted at (forced to leave) certain areas due to favoring (loss) of particular food (Wang *et al.*, 2013), while the recovery of such food may contribute to return of bird populations (Noordhuis *et al.*, 2002). Results of both metabarcoding and microhistologic analysis in this study revealed that *Carex* and *Poaceae* were dominant food components which is in accordant with previous studies. The increasing number of these two geese wintering at the Shengjin Lake may be attributed to the expansion of *Carex* meadow, which offers access to abundant food resources (Zhao *et al.*, 2015). Besides, considering the long-distance migratory character of these birds, it is important to maintain energy balances and good body conditions in wintering areas because this might further influence their departure dates and reproductive success after arriving at breeding areas (Prop, Black & Shimmings, 2003). Based on this, it is

354 important for wetland managers to maintain the suitable habitats and food resources to perform
355 sustainable conservation of waterbirds, and all of which highlighted the significance of diet
356 information. Our study also indicated that overlap and dissimilarity existed between the diets of
357 these two geese. As we all know, animals foraging in the same habitats may compete for limited
358 food resources (Madsen & Mortensen, 1987). This discrepancy of food composition may arise
359 from the avoidance of inter-specific competition (Zhao *et al.*, 2015). However, with the increase
360 of these two species in Shengjin Lake, further researches are needed to investigate the
361 mechanisms of food resources partitioning and spatial distribution.

362 Shengjin Lake is one of the most important wintering sites for tens of thousands of
363 migratory watebirds, while annual life cycles of these birds depend on the whole migratory route,
364 including breeding sites, stop-over sites and wintering sites (Kear, 2006). Thus, a molecular
365 reference library covering all the potential food items along the whole migratory route will be
366 useful both for understanding of wetland connections and waterbird conservation. Besides, the
367 ability of DNA metabarcoding to process lots of samples simultaneously enables the quick
368 treatment and makes this method helpful for waterbird studies.

369 **Acknowledgements**

370 We are very grateful to the stuff of the Shengjin Lake National Nature Reserve for their excellent
371 assistance during the field work. Great thanks to Zhujun Wang and An An for faeces collection
372 in the field. We thank Song Yang for collecting plants in the Shengjin Lake Reserve. We also
373 thank Profs. Zhenyu Li and Shuren Zhang for plant identification. Special thanks to Drs. Meijuan
374 Zhao, Xin Wang, Fanjuan Meng and Peihao Cong for preparing the epidermis database and

375 guiding microhistologic analysis.

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Table 1(on next page)

Primers of candidate genes and reference library constructing.

Only the c *and h* were used for high-throughput sequencing in fusion primer mode (primer + tags). The unique tags were used to differentiate PCR products pooled together for high-throughput sequencing (Parameswaran *et al.*, 2007).

Gene	Primer	Sequence(5'-3')
*mat*K	matK-XF[a]	TAATTTACGATCAATTCATTC
	matK-MALP[b]	ACAAGAAAGTCGAAGTAT
*rbc*L	rbcLa-F[c]	ATGTCACCACAAACAGAGACTAAAGC
	rbcLa-R[d]	GTAAAATCAAGTCCACCRCG
*trn*H-*psb*A	pasbA3_f[e]	CGCGCATGGTGGATTCACAATCC
	trnHf_05[f]	GTTATGCATGAACGTAATGCTC
*trn*L	c[g]	CGAAATCGGTAGACGCTACG
	h[g]	CCATTGAGTCTCTGCACCTATC

Footnotes: “a” referred to “Ford *et al.*, 2009”; “b” referred to “Dunning & Savolainen, 2010”; “c” referred to “Hasebe *et al.*, 1994”; “d” referred to “Kress *et al.*, 2009”; “e” referred to “Tate & Simpson, 2003”; “f” referred to “Sang, Crawfor & Stuessy, 1997”; “g” referred to “Taberlet *et al.*, 2007”.

Table 2(on next page)

Inter-specific divergences within dominant genera and families of *rbc*L gene and *trnL* gene with Kiruma 2-Parameter model.

Underscores indicate the most common food composition based on earlier microhistologic analysis (Zhao *et al.*, 2012 & 2015).

Inter-specific divergence	Taxa	*rbc*L			*trn*L		
		maximal	minimal	mean	maximal	minimal	mean
Within genera	*Artemisia Linn.*	0.000	0.000	0.000±0.000	0.000	0.000	0.000±0.000
	Carex	0.013	0.000	0.008±0.006	0.058	0.000	0.027±0.021
	Polygonum L.	0.027	0.000	0.010±0.006	0.076	0.000	0.033±0.022
	Potamogeton Linn.	0.012	0.000	0.005±0.0034	0.016	0.000	0.005±0.005
	Ranunculus L.	0.031	0.000	0.020±0.009	0.042	0.021	0.024±0.022
	Trapa L.	0.000	0.000	0.000±0.000	0.081	0.000	0.049±0.030
	Vallisneria Linn.	0.000	0.000	0.000±0.000	0.000	0.000	0.000±0.000
Within families	*Cyperaceae*	0.043	0.000	0.018±0.010	0.178	0.000	0.084±0.046
	Asteraceae	0.120	0.000	0.049±0.017	0.087	0.000	0.023±0.018
	Poaceae	0.025	0.000	0.016±0.0009	0.166	0.000	0.074±0.039
	Hydrocharitaceae	0.122	0.000	0.078±0.020	0.159	0.000	0.100±0.054
	Polygonaceae	0.043	0.000	0.020±0.009	0.129	0.000	0.031±0.022
	Ranunculaceae	0.033	0.016	0.017±0.015	0.045	0.000	0.018±0.013

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Table 3(on next page)

Species discrimination rate (Rf) for families with more than one species in Shengjin Lake plant database.

Family	Species No.	Sequence No.	Rf (%)
Asteraceae	8	7	88
Caryophyllaceae	2	2	100
Cyperaceae	7	5	71
Fabaceae	2	2	100
Hydrocharitaceae	4	3	75
Lamiaceae	2	2	100
Poaceae	10	8	80
Polygonaceae	5	5	100
Potamogetonaceae	5	1	20
Ranunculaceae	3	3	100
Scrophulariaceae	3	3	100
Trapaceae	4	3	75
Umbelliferae	2	2	100

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Table 4(on next page)

Summary of the process and results of high-throughput sequencing analysis.

GWFG=Greater white-fronted goose; BG=Bean goose.

Sample	Pair-end sequences	Sequences for which primers and tags were identified and Length > 100 bp	Unique sequences	OTUs	Food items
GWFG1	16303	8627	1288	78	8
GWFG2	25482	13449	1091	102	8
GWFG3	19063	10056	1277	48	10
GWFG4	23856	12548	1419	114	8
GWFG5	20955	11249	1720	123	9
GWFG6	11677	7205	973	52	9
GWFG7	13377	6782	1328	59	9
GWFG8	7749	3959	774	89	9
GWFG9	16833	8799	1436	90	6
GWFG10	18474	9819	449	32	9
GWFG11	19648	10458	617	31	6
BG1	20225	10254	784	23	4
BG2	14195	7161	564	16	2
BG3	2229	1149	255	12	4
BG4	517	268	77	8	3
BG5	28152	14033	1000	15	3
BG6	16723	8484	740	17	4
BG7	30166	15403	974	15	4
BG8	30928	15706	1028	15	3
BG9	8382	4489	446	13	4
BG10	10714	5526	537	13	4

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Table 5(on next page)

List of the lowest taxonomic food items in the diet of geese.

GWFG=Greater white-fronted goose; BG=Bean goose. F_s, percentage of sequences in DNA metabarcoding; F_m, percentage of epidermis squares in microhistological analysis.

Food items	Level of identification	GWFG			BG		
		N reads	F_s(%)	F_m(%)	N reads	F_s(%)	F_m(%)
Poaceae spp.	Family	51705	47.98	45.68	0	0.00	0.00
Poa annua	Species	23554	21.86	0.00	167	0.20	0.00
Carex heterolepis	Species	18867	17.51	16.39	81457	99.49	62.85
Carex spp.	Genus	9706	9.01	2.31	191	0.23	3.49
Alopecurus aequalis	Species	3458	3.21	0.00	0	0.00	0.00
Potentilla chinensis Ser.	Species	184	0.17	1.18	65	0.08	2.06
Cynodon dactylon	Species	155	0.14	0.00	0	0.00	0.00
Polygonum spp.	Genus	56	0.05	0.00	0	0.00	0.00
Stellaria media (L.) Cyr.	Species	26	0.02	0.00	0	0.00	0.00
Ranunculus chinensis	Species	14	0.02	0.00	0	0.00	0.00
Lapsana apogonoides Maxim.	Species	11	0.02	0.00	0	0.00	0.00
Asteraceae sp.	Genus	16	0.01	2.33	0	0.00	14.55
Alopecurus Linn.	Genus	0	0.00	30.93	0	0.00	13.18
Carex thunbergii	Species	0	0.00	0.54	0	0.00	2.79
Fabaceae sp.	Genus	0	0.00	0.64	0	0.00	1.08

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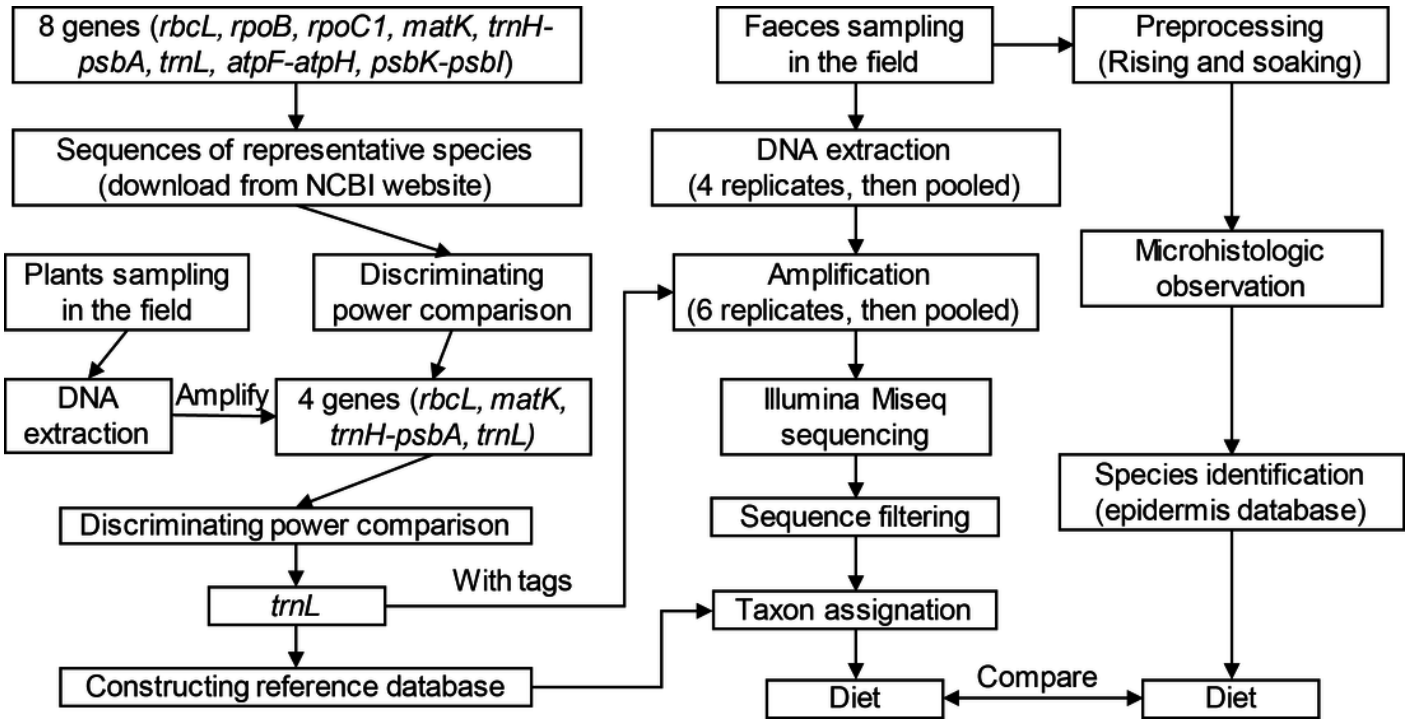
Technical flowchart of this study.



2

The location of our study area, Shengjin Lake National Nature Reserve and our sampling sites. (Source: http://eros.usgs.gov/#).