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Cross-Validation of Diet Determination Methods for Seabird Conservation

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ABSTRACT

Seabirds are recognized as one of the most vulnerable groups of birds, with around a third of species identified as globally threatened. The conservation of seabirds is often linked with their feeding and diet, due to undesirable interactions with human-related fishing activities and fisheries depletion/climate change impacting food resources. Therefore, understanding the diet of seabirds is often a critical first step towards identifying effective conservation measures. DNA metabarcoding and hard parts analyses provide a foundation for assessing the diet of a predatory seabird species, giving insight into predator–prey relationships and ecosystem-wide food webs. Congruency between these two methods would increase confidence, providing validation that either method provides a reliable representation of the diet. This study on the diet of the endangered New Zealand king shag (*Leucocarbo carunculatus*) compared the frequency of occurrence of fishes detected from the same regurgitated pellets ($n = 191$) using both hard parts and DNA metabarcoding methodologies. The number of pellets with overlapping fish families between methods showed a significant positive correlation between methods ($r = 0.96$; $p < 0.001$), with 50 out of 191 pellets showing complete alignment and only two pellets without any alignment. Both methods confirmed the predominance of Bothidae (DNA: 71% of pellets, hard parts: 77%, total: 80%) and Rhombosoleidae (DNA: 45%, hard parts: 51%, total: 59%) taxa in the diet of king shag, while also revealing the consumption of a diversity of other fish species. Overall, this study demonstrates that the two methods provide a complementary approach for revealing the dominant fish prey species in the diet, as well as providing an overview of the diversity of prey species (DNA: 14 unique species, hard parts: 8, total: 28). However, the inexact alignment between the two methods for detecting every taxon in every pellet suggests differences in detection, especially for less common taxa and for DNA metabarcoding, where species-level resolution is dependent on adequate DNA database sequence entries of taxa in the local area. In addition, filtering thresholds for DNA metabarcoding further influenced alignment. Overall, the results indicate that both methods provide consistent detection of major prey items; however, reliably capturing the overall full diversity of prey species with either method is reliant on a sufficient sample size.

1 | Introduction

Seabirds are recognized as one of the most vulnerable groups of birds, with 41% of species listed as Threatened or Near Threatened (BirdLife International 2022). The dominant threats to seabirds are related to interactions with human fishing activities both directly (e.g., incidental capture whilst feeding on bait and discards) and indirectly through the reduction in the abundance of key prey species (Dasnon et al. 2022; Dias et al. 2019; Melvin et al. 2023). Thus, knowledge of the

diet composition of seabirds has become increasingly important (Parsons et al. 2008; Piatt et al. 2007; Suryan et al. 2002). Dietary changes in seabirds caused by natural fluctuations in prey population, or from anthropogenic impacts on the environment (e.g., fisheries and climate change), can cause shifts in the availability of food sources that lead to a decline in fish populations (Cianchetti-Benedetti et al. 2018; Padró 2024; Tasker et al. 2000).

For piscivorous birds that consume prey whole, hard parts analysis of undigested material (e.g., jaw bones, vertebrae and otoliths) has traditionally been the popular method to determine diet and assumes these remains are representative of their diet. However, the emergence of DNA analysis to detect multiple taxa simultaneously (DNA metabarcoding), is increasingly providing a complementary approach for identifying dietary composition and similarly assumes that DNA remains are representative of the diet (Nimz et al. 2022). While both methods have these underlying assumptions, direct comparisons of results from both methods using the same samples for analyses is infrequent but provides the opportunity for cross-validation (Correia et al. 2023; da Silva et al. 2019; Oehm et al. 2017).

Research suggests that a highly suitable source of samples for direct comparison of these methods is regurgitated material, which can be collected non-invasively, although fecal matter may be a viable alternative if collecting regurgitated material is not possible (Correia et al. 2023; Oehm et al. 2017). In some seabirds, regurgitated pellets of undigested material contain hard parts of prey as well as the remains of prey flesh, allowing for DNA metabarcoding and hard part methodologies to be directly compared, and thus for the mutual validation of the two methodologies. Regurgitated pellets also provide the greatest opportunity to detect the widest spectrum of prey with the best possible resolution, irrespective of the chosen method, although the results may vary between methods (Oehm et al. 2017).

Both DNA metabarcoding and hard part analyses provide a foundation for assessing the diet of predatory seabirds and deliver a mechanism for understanding predator–prey relationships, and in turn ecosystem-wide food webs (Carroll et al. 2019; Kartzinel et al. 2015; van der Reis et al. 2020). DNA metabarcoding is one method increasingly being used in avian dietary analyses (e.g., Nimz et al. 2022; Snider et al. 2021; Volpe et al. 2021). This method relies upon gene regions that are useful for taxonomic assignments as they are typically unique to a species ('DNA barcode') and as such is not dependent on morphological preservation (Clare 2014; Taberlet et al. 2012). Advantages of DNA metabarcoding include prey detection in the absence of hard parts, processing time efficiency, and specialist taxonomic expertise is not needed for the identification of prey remains to genus/species level (Berry et al. 2015). However, DNA-based methods for prey species identification from regurgitated pellets are wholly reliant on sufficient quality of residual DNA after digestive processes. The DNA samples are also subject to further degradation caused by the natural elements prior to collection and preservation. Furthermore, those sources of DNA that may be persistent in digestive processing have the potential to unduly bias the resulting dietary analyses as there may be differential digestion rates among the consumed prey (Clare 2014; Clarke et al. 2020; Young et al. 2015). Similarly, restrictions exist for indigestible material used in hard parts analysis, such as small material being passed through the gut and not being detected in regurgitated pellets (Barrett et al. 2007). Hard parts may also be damaged thereby compromising the morphological traits vital for accurate species identification. Regardless, a major advantage of utilizing indigestible material is that minimum prey numbers can be determined for the taxa detected and broad categories (e.g., class or family) can typically be assigned with high confidence relatively quickly. Thus, both methods can potentially over- or under-estimate prey diversity and abundance.

The New Zealand king shag (*Leucocarbo carunculatus*; kawau pāteketeke) is a nationally endangered endemic seabird (Robertson et al. 2021). The population is confined to a small number of colonies in the Marlborough Sounds (Schuckard et al. 2018). This coastal region is subject to intensive commercial and recreational fishing which has led to the marked depletion of a number of coastal taxa, including fish (e.g., cod and flat fish species) and non-fish taxa (Handley 2016; Ministry for Primary Industries 2015; Toone et al. 2023), and in the last 50 years has seen a proliferation of coastal mussel aquaculture with more 570 farms covering nearly 3000 ha (Toone et al. 2022). These changes in fisheries resources and potential reduction in the availability of natural foraging habitat have been creating concerns over potential impacts on successful foraging by king shag (Taylor 2020).

The small islands and rocky outcrops that host colonies of king shags provide relatively easy access for sample collection. These seabirds are known to prey largely on fish, regurgitating pellets in a mucous sac which typically contain mostly indigestible material, such as fish otoliths and skeletal remains, and exoskeletal remains of invertebrates (Lalas and Brown 1998). Regurgitated pellets, and their contents, provide a record of the composition of prey items and therefore insight into the diet of these seabirds. Using samples recovered from king shag colonies, this study aimed to compare the detection of fish taxa (presence-absence), which are predominant prey species, using hard part and DNA metabarcoding analyses.

2 | Material and Methods

2.1 | Collection of King Shag Pellets

Regurgitated pellets ($n = 217$) from king shags were collected between March 2019 and March 2020 from seven colonies in the Marlborough Sounds (South Island, New Zealand). Pellets were collected from colonies at Blumine ($n = 28$; 1× sampling event), Duffers Reef (43; 2×), North Trio (47; 2×), Tawhitinui (42; 2×), The Haystack (10; 1×), The Twins (24; 1×) and White Rocks (23; 2×) (Figure 1; Table S1). Immediately upon collection, the samples were preserved in 90% ethanol. Pellets that were considered recently yielded based on moisture content and undisturbed since regurgitation (i.e., complete/fully intact) were collected, avoiding any that may have been partly scavenged by other seabirds.

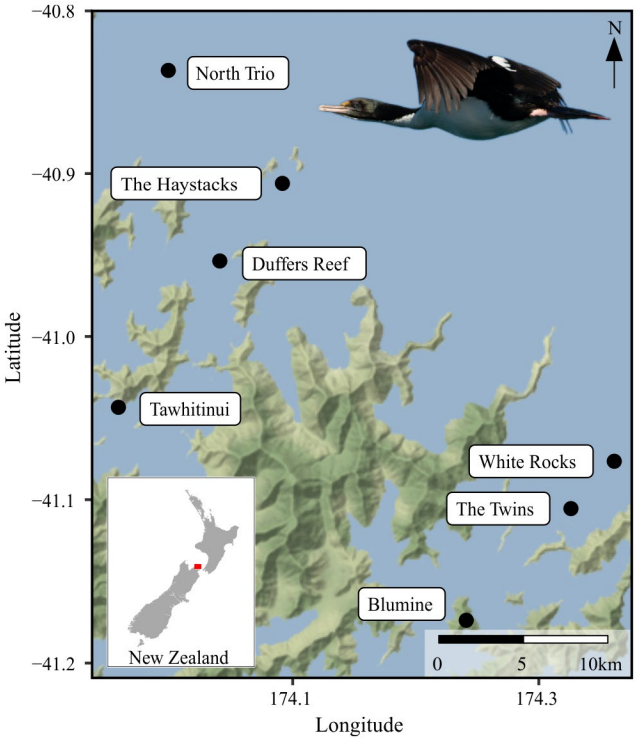

FIGURE 1 | Regurgitated pellets were collected from seven New Zealand king shag colonies in the Marlborough Sounds, South Island, New Zealand. Adult king shag photographed flying near Duffers Reef colony during research undertaken in July 2019 by Dan Burgin from Wildlife Management International Ltd.

2.2 | Fish Identification Using DNA Metabarcoding

2.2.1 | DNA Extraction and Amplification

Each pellet was dissected, and the hard items and any visible tissues were separated (including any tissue that remained attached to bones). The tissues were homogenized allowing for a subset of gut digesta tissue to be used for DNA extractions and any remaining content was stored for morphological examination. All dissection instruments were flame-sterilized between pellet samples. An E.Z.N.A. Mollusk DNA Kit (Omega Bio-Tek) was used for the extractions, following the manufacturer's instructions. The E.Z.N.A. kit was chosen as a preventative measure to mitigate possible polymerase chain reactions (PCR) inhibition caused by mucopolysaccharides associated with marine invertebrates which may have been consumed (Palmer 2008). Subsequently, PCRs were done using MyTaq Red Mix (Bioline; Meridian Bioscience) master mix; 6.25 μL MyTaq Red Mix, 0.25 μL of each primer (10 μM), 4.75 μL UltraPure DNase/RNase-Free Distilled Water (Invitrogen; Thermo Fisher Scientific), 0.5 μL DNA and 1 μL bovine serum albumin (1%) when necessary for optimal DNA amplification per reaction. Negative controls were included in every set of DNA extractions (DNA extraction blank—no tissue added) and every PCR run (PCR blank—no DNA added) to check for possible contamination. The PCRs were performed in triplicates to mitigate possible PCR stochasticity.

The mitochondrial cytochrome oxidase subunit 1 (COI) region (313 base pairs; mlCOIintF: GGWACWGGWTGAACWGTWTAYCCYCC—Leray et al. 2013 and jgHCO2198: TAIACYTCIGGRTGICCRAARAAYCA—Geller et al. 2013) was targeted. This primer pair is a universal primer set, and captures a wide range of metazoan taxa and has enough sequence variation for confident species-level taxonomic assignment (Leray et al. 2013). Moreover, this primer set was also chosen as it had the greatest number of sequence entries of fish species in the DNA reference database, that are dominant in the king shag diet (Lalas and Brown 1998), compared to other commonly used fish primers at the time of the study's design (e.g., 12S; Miya et al. 2015). The primer pair had Illumina Nextera library adapters added (forward: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG; reverse: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG). PCR protocol: 94°C for 4 min, 30× [94°C for 30 s, 45°C for 30 s, 72°C for 1 min], 72°C for 5 min. The PCR products were run on a 1.6% agarose gel, and visualized using Gel Red (Biotium), in a Gel Doc XR+ (Bio-Rad).

2.2.2 | DNA Clean-Up and Pooling

The PCR triplicates were pooled together before clean-up proceeded by sample. Agencourt AMPure XP (Beckman Coulter) was used following the Illumina protocol for PCR clean-up (Illumina 2013). The concentration of the purified PCR products was determined using Qubit dsDNA HS Assay Kit (Invitrogen) following the manufacturer's instructions. The PCR products were brought to equal molarity, 2 ng μL^{-1} where possible. Sequencing was done through Auckland Genomics (Auckland, New Zealand) where indexing, using the Nextera DNA library Prep Kit and the second round of PCR clean-up occurred before sequencing on an Illumina MiSeq System (2 × 300 pair-end).

2.2.3 | Bioinformatics and Quality Control

Cutadapt v4.1 (Martin 2011) was used to trim the forward and reverse primers from the raw demultiplexed sequencing data. Primers were removed if an exact sequence match could be found anchored at the beginning of the sequence. All untrimmed sequences were discarded. Qiime 2 (v2023.5; Bolyen et al. 2019) was used to visualize the initial sequence quality. DADA2 (within Qiime 2; Callahan et al. 2016) was used for sequence filtering based on quality scores, denoising, merging and chimera formation to ensure only high quality paired-end sequences were retained (amplicon sequence variants—ASVs; Callahan et al. 2017). The ASVs were assigned taxonomy using MIDORI 2 (v259; COI unique sequences database; hereon referred to as MIDORI) with the RDP classifier (97% confidence threshold; within QIIME; Wang et al. 2007). GenBank's MegaBLAST option in the BLASTn suite (v2.13.0; Morgulis et al. 2008) was also used for taxonomic assignments of ASVs using the BLAST v2024-01 database (Benson et al. 2013) for comparison (filter parameters: e-value < 0.001, percentage identity $\geq 90\%$, percentage query coverage $\geq 95\%$). The MegaBLAST percentage threshold was set at 90% to be inclusive of assignment to higher taxonomic levels that could be derived from the assigned species if need be, whereas the

RDP classifier is designed to assign taxonomy at higher levels at the confidence set. Lineage was pulled using the resulting MegaBLAST assigned staxids from the GenBank database using Entrez Direct v13.3 (Kans 2013).

The resulting sequences were further filtered and analyzed in R Studio (R Studio v2024.09.0+375; R base v4.4.1; R Core Team 2024). The results from the databases were consolidated to create an ASV taxonomic assignment table. Host assigned ASVs ("*Leucocarbo*") were removed. Proportional subtraction of reads took place for any ASVs that had been identified in the negative controls, i.e., subtracting reads in proportion to their presence in the negative controls. Thereafter, the minimum read number (100) was set based on the highest read count identified in the negative control ASVs.

2.3 | Fish Identification Using Hard Part Analyses

The retained hard part materials from each pellet were teased apart in water, and all the retained diagnostic prey remains were identified from a comprehensive reference collection (C. Lalas, unpublished data). Diagnostic remains differ among taxa and were used for identification: mouthparts from hagfish and lamprey; teeth, body thorns, dorsal spine sheaths, and (cartilaginous) vertebrae from sharks and rays; and otoliths (sagittal otoliths), jaws, and other dentition, caudal vertebrae, and some other species-specific bones for teleost fishes. Specifically, the caudal vertebrae were selected for species identification in this study, as they offered more robust identification (especially among flatfish genera) in comparison to other vertebrae. Otoliths for all the genera and most of the teleosts were identified according to comprehensive catalogues (Furlani et al. 2007; Schwarzhans 1984, 1999; Smale et al. 1995), and jaws and some other fish bones were identified in a similar manner (Leach 1997).

2.4 | Comparison of Methods

Only pellets that passed both (1) filtering for DNA metabarcoding and completeness assessment for hard parts (DNA metabarcoding—pellet passed DADA2 sequence filtering and taxonomic assignment quality measures; morphological identification—identified as being a complete pellet), and (2) were identified to contain fish taxa in both methods were retained for direct comparison ($n=191$), as fish are known to be the main dietary item (Lalas and Brown 1998). Furthermore, both hard part and DNA databases have a high number of references available, allowing for a robust comparison of taxa detection. Fish identified from the pellets were assigned to the lowest taxonomic level possible (taxonomy updated where necessary; Roberts et al. 2023) and the dataset produced was in binary form (i.e., presence-absence), however, for comparison purposes the data was compared at family level.

A permutational multivariate analysis of variance (PERMANOVA; function: adonis2; Jaccard dissimilarity index; vegan 2.5–6; Oksanen et al. 2019) was used to identify if significant differences existed within the fish families identified, between the two methods of prey detection. The data were transformed into a count summary (i.e., number of pellets identified in each fish family) and differences between methods detecting fish families was investigated using a zero-inflated regression model (function: zeroinfl; pscl v1.5.9; Jackman 2024; Zeileis et al. 2008) and estimated marginal means for post hoc comparisons (function: emmeans; emmeans v1.4.3; Lenth 2019). A Pearson's correlation test (function: cor.test; stats v4.4.1) was then used to identify possible correlation between the number of pellets identified by hard part and DNA metabarcoding analyses for the overlapping fish families. Frequency of occurrence was also calculated from the count data.

To explore the potential maximum number of pellets that overlapped for fish taxa between methods, ASVs that were either filtered out due to not passing conservative filtering thresholds or did not have taxonomy assigned (confidence <97%), were included by dropping the minimum number of reads to 10 and GenBank confidence to 80%. When there was more than one ASV with the same taxonomic assignment per pellet, the reads were summed and confidence percentage averaged. GenBank typically provides assignments at the species level, making it useful for cross-comparison with the genera/species detected in the hard part analysis.

3 | Results

3.1 | DNA Metabarcoding and Hard Part Dataset Filtering

Post DADA2 there were 1948 ASVs identified from the 217 pellets (12,898,923 reads; Table S2). The reads per sample ranged from 268 to 272,206, with a mean of 59,442. There were 32 ASVs assigned to the king shag genus ("*Leucocarbo*"), and these were filtered out (11,444,290 reads; 89%). There were five ASVs identified in the negative controls, and the greatest read count was 84. Thus, the minimum read count needed to retain any ASVs identified in the samples was set to 100, post proportional subtraction of the negatives. The resulting mean number of reads per sample was 6984 (range: 105–46,546). Of the remaining ASVs ($n=375$; 1,403,940 reads), 95 ASVs could not be taxonomically assigned by MIDORI nor GenBank and were identified in 75 pellets. Five unidentified ASVs were identified in more than 10 pellets. Thirteen pellets had 'unidentified' ASVs account for >50% of the relative read abundance (RRA), five of which were >90% RRA. There were 10 non-fish classes identified, two of which were identified as likely, but infrequently detected, prey items (Cephalopoda—*Robsonella huttoni*: $n=4$ pellets; Malacostraca—various species: $n=8$; Figure S1). Comparing MIDORI (RDP classifier) and GenBank (MegaBLAST) taxonomic assignment results, the assignments were typically congruent with species matches at ~99% and above (Figure S1). A fish dataset was created by filtering for classes Actinopteri (ray-finned) and Chondrichthyes (cartilaginous), retaining 195 (85%) pellet samples and 199 ASVs (1,168,238 reads). There were two genera assigned using the RDP classifier that were assigned at species level (within the genera) using BLAST. *Helicolenus* was assigned with RDP (100% confidence), but none to species level, whereas BLAST assigned to *H. barathri* (≥99%) and the other to *H. percoides* (100%) – both of which are in MIDORI. *Congiopodus coriaceus* was assigned by both RDP and

BLAST, but another ASV was assigned by RDP as *Congiopodus* (100%) to genus level only, but *C. coriaceus* (100%) with BLAST.

A total of seven pellets were identified from the morphological analyses as either being empty or as damaged (i.e., contents missing), and omitted from further analyses. Thus, 210 pellets were retained for hard part analyses. Other than fishes that were detected in the majority of the pellets, Cephalopoda (*Robsonella* cf. *huttoni*) were detected in 12 pellets and Malacostraca in 10 (cf. *Halicarcinus* sp. or spp. and *Nectocarcinus antarcticus*).

To directly compare the results, only the pellets that had fish identified in them and analyzed in both datasets were retained ($n = 191$) (Tables S3 and S4). This excluded one fish family identified in the hard parts only from the analysis (Trachichthyidae), which was only identified in a single pellet.

3.2 | Analyses of Fishes Identified by Hard Part and DNA Metabarcoding

There were 20 families identified in total, of which 11 were identified by both methods (Moridae, Sebastidae, Scorpaenidae, Triglidae, Anthiadidae, Labridae, Pinguipedidae, Tripterygiidae, Bothidae, Rhombosoleidae, and Monacanthidae; Table 1). There were five families only identified in the DNA metabarcoding dataset (Scyliorhinidae, Syngnathidae, Zeidae, Uranoscopidae, and Congiopodidae) in two or fewer pellets (Figure 2). There were four families identified only in the hard parts dataset (Congridae, Ophidiidae, Macrouridae, and Hemerocoetidae); all were identified in a single pellet only, except Hemerocoetidae, which was identified in 72 pellets (Figure 2). Bothidae was the most commonly identified family in the pellets (80%), with 130 identifications by both methods (i.e., identified within the same pellet), a further 18 by the hard part method only, and five by DNA only (Table 1; Figures 2 and 3). In comparison, Rhombosoleidae was the second-most frequently identified (59%) with 69 identifications made for the same pellets by both methods, and a further 28 and 16 made using hard part and DNA metabarcoding methods, respectively (Table 1; Figures 2 and 3). In total, there were 50 pellets in which the same fish families were identified by both methods. There were two pellets where there was no overlap, and the remaining pellets overlapped to varying degrees. For example, overlaps were found in families when using both detection methods, but one or both methods identified families that the other did not. In general, there was various taxa agreement at lower taxonomic levels, with only six matching at the species level, despite there being sequence entries in MIDORI for all genera identified by the hard part analysis (Table 2). Species in families that were identified by one method only were detected at most in two pellets.

There was a significant difference found between methods when investigating all fish families (PERMANOVA; $p < 0.001$) and three fish families were identified using the post hoc zero inflation model all of which had a greater number of pellets identified for hard parts (Hemerocoetidae: $p < 0.001$, z ratio = 8.49, standard error = 8.49; Sebastidae: $p < 0.05$, 2.00, 8.00; Tripterygiidae: $p < 0.001$, 5.43, 7.55). Overall, both methods had similar pellet counts for families in common (Table 1; Figure 3), resulting in a significant positive correlation (correlation coefficient: $r = 0.96$, $p < 0.001$, 95% confidence intervals = 0.84–0.99, df = 9; coefficient of determination: $r^2 = 0.92$, $y = 7.31 + 1.033\times$; Figure 4).

TABLE 1 | Number of regurgitated New Zealand king shag pellets ($n = 191$) identified per fish family, by both hard part and DNA metabarcoding analyses, as well as the number of pellets where the fish was only identified by one of the methods. Three families that were identified to have significantly different pellet counts between methods are indicated (*).

Family	Total	Both	DNA	Hard parts
Anthiadidae	8	2	0	6
Bothidae	153	130	5	18
Congiopodidae	2	0	2	0
Congridae	1	0	0	1
Hemerocoetidae*	72	0	0	72
Labridae	24	14	4	6
Macrouridae	1	0	0	1
Monacanthidae	54	38	5	11
Moridae	14	6	1	7
Ophidiidae	1	0	0	1
Pinguipedidae	12	4	7	1
Rhombosoleidae	113	69	16	28
Scorpaenidae	13	4	6	3
Scyliorhinidae	2	0	2	0
Sebastidae*	42	22	2	18
Syngnathidae	2	0	2	0
Triglidae	35	31	2	2
Tripterygiidae*	50	7	1	42
Uranoscopidae	1	0	1	0
Zeidae	2	0	2	0

When investigating filtering thresholds for ASVs, minimum read parameter largely influenced the retention of ASVs and thus the detection of fish families within pellets (Figure 3; Figure S2). Considering the detected overlap within pellets for fish families, between hard parts and DNA metabarcoding (i.e., confirmation of true positives), lowering the minimum read parameter to 10 there were a further 24 pellets that overlapped in detection for Bothidae, 17 for Rhombosoleidae, and 15 for Sebastidae. The lowered parameter also retained pellets previously filtered out. For example, Trachichthyidae was the only family identified in a single pellet by hard part analysis, and it was detected in the same pellet using DNA metabarcoding but had less than 100 reads (Figure S2). Therefore, the ASV was filtered out and a true positive was removed, subsequently removing the pellet from the comparison. Furthermore, decreasing taxonomic confidence percentage substantially increased the number of pellets where Labridae and

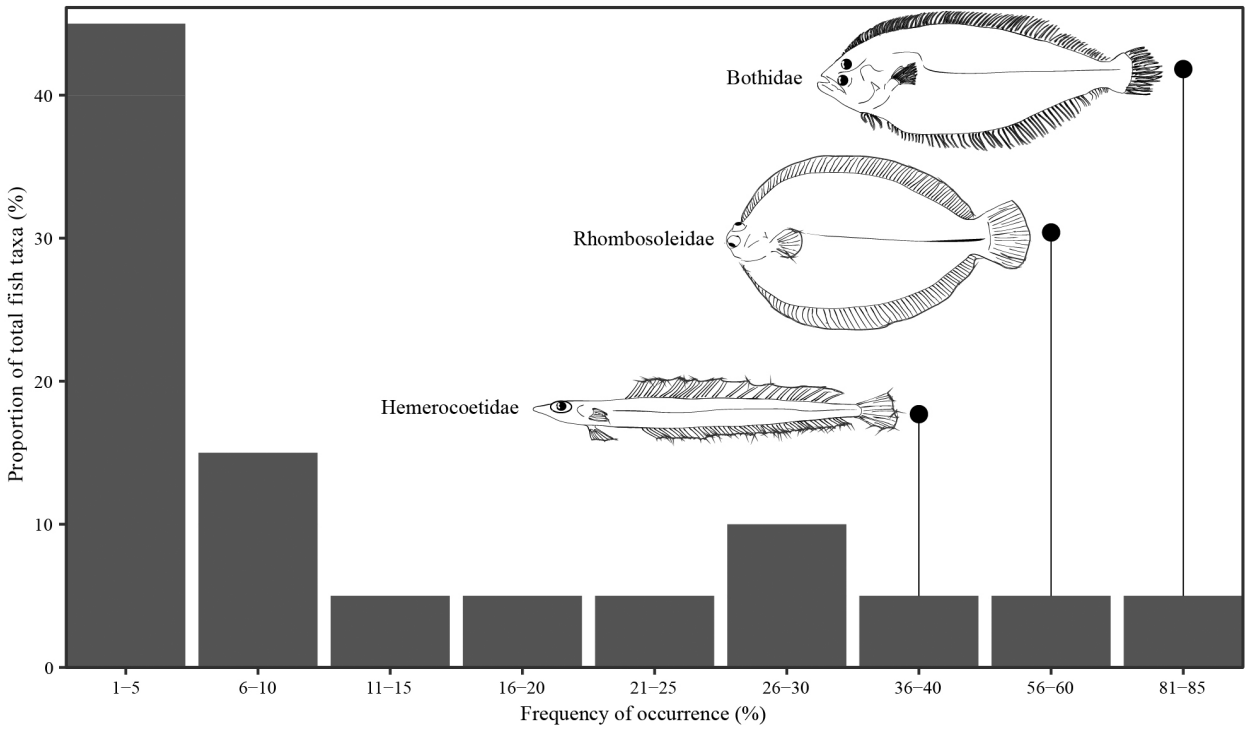

FIGURE 2 | Frequency of occurrence (%) to proportion of fish taxa (prey) identified from regurgitated pellets from king shags. Hard part and DNA metabarcoding results were combined to generate total frequency of occurrence per fish family.

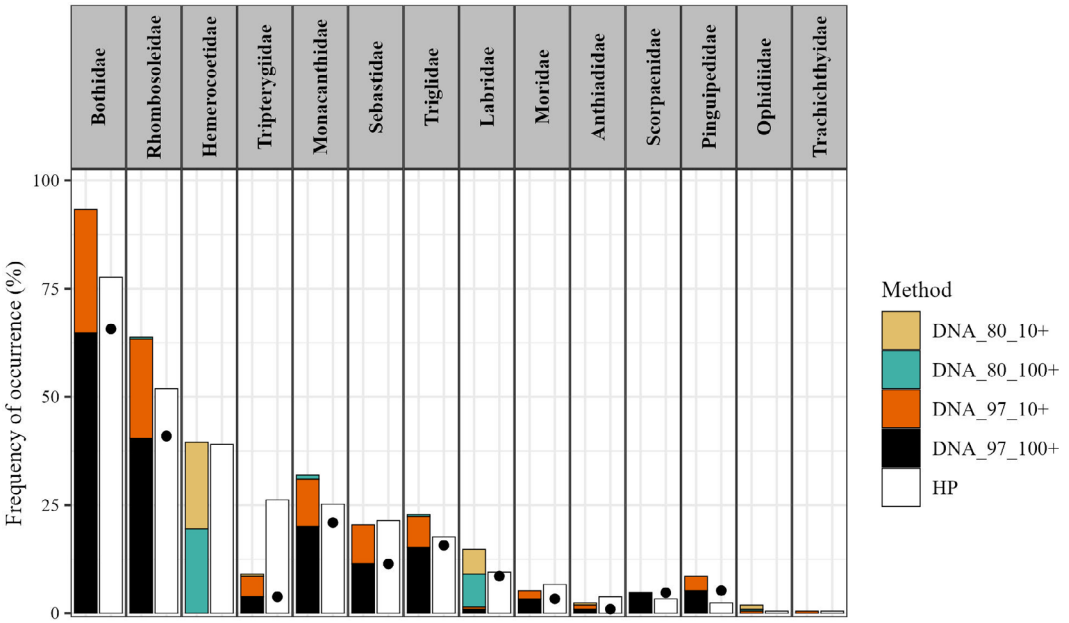

FIGURE 3 | Frequency of occurrence (FOO) of fish families among regurgitated pellets by New Zealand king shag by both hard parts (HP) and DNA metabarcoding analyses. Included for the DNA metabarcoding analysis is the percentage of pellets added when filtering thresholds were lowered ($n = 210$). When there was more than one ASV with the same taxonomic assignment per pellet, the reads were summed and confidence percentage averaged. The parameters set for a taxonomic assignment to then be retained were ≥ 100 reads after proportional subtraction of the negative controls had taken place, and a minimum average confidence of 97% needed to be met (DNA_97_100+; standard filtering). This was compared to the remaining assignments by lowering the number of reads (≥ 10) needed for an ASV to be retained (DNA_97_10+) and decreasing the confidence for assignment (DNA_80_100+; DNA_80_10+). GenBank was used for assignment, as species-level are typically given regardless of the confidence. The point on the HP bar indicates the FOO if using MIDORI assignments and standard filtering.

Hemerocoetidae were detected. The corresponding genera identified matched the hard parts results, despite having low taxonomic assignment confidence of ~90%, and largely aligned with the pellets identified to contain those genera (Figure S2). If using GenBank solely for assignments, the majority of pellets containing Labridae taxa would not have been identified.

TABLE 2 | All fishes identified by DNA metabarcoding and/or hard part analyses in 191 pellets regurgitated by New Zealand king shags compared using frequency of occurrence (FOO). The DNA taxonomic assignment mostly assigned ASVs at the species level (when an assignment was possible), however, there were some that could only be assigned at the family or genus level. This was similarly the case for the hard parts results, but differed as an estimated number of fish could usually be given. *Notolabrus* and *Pseudolabrus* could not be separated as two genera and thus were made as a single category for the hard parts analysis. All, except three species identified by hard part analysis, had entries in MIDORI. The remaining three had species entries for their respective genera (indicated by [G]).

Family	DNA (FOO %)	Hard parts (FOO %)
Anthiadidae	*Caesioperca lepidoptera* (1)	*Caesioperca lepidoptera* (4.2)
Bothidae	*Arnoglossus scapha* (60.7)	*Arnoglossus scapha* (77.5)
	Lophonectes gallus (38.2)	
Congiopodidae	*Congiopodus* sp. (0.5)	
	Congiopodus coriaceus (0.5)	
Congridae		*Gnathophis habenatus* (0.5)[G]
Hemerocoetidae		*Hemerocoetes* cf. *monopterygius* (37.7)[G]
Labridae	Labridae sp. (or spp.) (8.4)	
	Pseudolabrus miles (1)	*Notolabrus* & *Pseudolabrus* 4 spp. (9.9)
		Odax pullus (0.5)[G]
Macrouridae		cf. *Coelorinchus biclinozonalis* (0.5)
Monacanthidae	*Meuschenia scaber* (22.5)	*Meuschenia scaber* (25.7)
Moridae	*Pseudophycis bachus* (2.1)	*Pseudophycis* 1–3 spp. (5.8)
	Pseudophycis breviuscula (1.6)	
		Lotella rhacina (0.5)
		Notophycis marginata (0.5)
Ophidiidae		*Genypterus blacodes* (0.5)
Pinguipedidae	*Parapercis colias* (5.8)	*Parapercis colias* (2.6)
Rhombosoleidae	*Pelotretis flavilatus* (33.5)	*Pelotretis flavilatus* (29.3)
	Peltorhamphus latus (15.7)	*Peltorhamphus* 1–3 spp. (19.9)
	Peltorhamphus novaezeelandiae (3.1)	
	Rhombosolea plebeia (4.7)	*Rhombosolea* 1–3 spp. (22)
Scorpaenidae	*Scorpaena papillosa* (5.2)	*Scorpaena papillosa* (3.7)
Scyliorhinidae	*Cephaloscyllium laticeps* (1)	
Sebastidae	*Helicolenus* sp. (or spp.) (12.6)	
		Helicolenus percoides (20.9)
Syngnathidae	*Hippocampus abdominalis* (1)	
Triglidae	*Lepidotrigla brachyoptera* (17.3)	Triglidae 1–3 spp. (17.3)
Tripterygiidae	*Matanui profundum* (4.2)	Tripterygiidae spp. (25.7)
Uranoscopidae	*Kathetostoma giganteum* (0.5)	
Zeidae	*Zeus faber* (1)	

4 | Discussion

Effective species conservation management decisions rely in part on a good understanding of the diet of the species targeted for conservation. This study used two methods of dietary analysis for king shags namely, DNA metabarcoding and visual assessment of the hard parts in regurgitated pellets. The marine habitat and food resources utilized by king shag have been impacted over time by anthropogenic pressures including fishing, marine farm development, and climate change. Thus, these

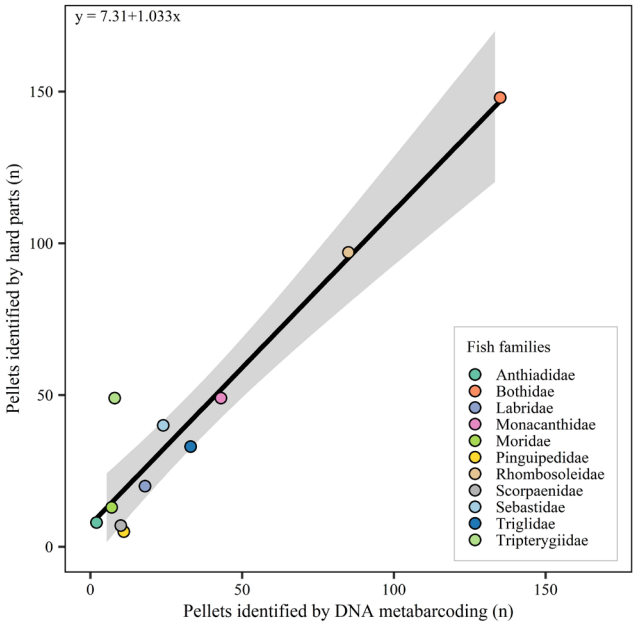

FIGURE 4 | Overall relationship between the number of pellets for the fish families identified by both hard part and DNA metabarcoding analyses (correlation coefficient: $R = 0.96$, $p < 0.001$, 95% confidence intervals = 0.84–0.99 as shown by shading, df = 9; coefficient of determination: $R^2 = 0.92$, $y = 7.31 + 1.033x$).

types of analyses can help assess possible effects on foraging and diet due to the impacts of anthropogenic activities.

Regurgitated pellets from seabirds typically contain the hard parts from prey consumed the previous day (~24 h, but may be longer) and their analysis provides a valuable snapshot of dietary information (Johnstone et al. 1990; Oehm et al. 2017). However, it is unclear whether DNA recovered from the regurgitated pellets is representative of the same dietary period as the hard parts contained in the same pellets, as DNA could persist in the digestive track and remain detectable in pellets given the high sensitivity of these methods. Congruence in composition of regurgitated pellets analyzed using both hard part and DNA metabarcoding methods would increase confidence in both methods by providing validation that either method results in robust data that can be utilized for evidence-based conservation efforts and policy changes. Overall, there was congruency between the presence of fishes identified by the hard parts and DNA metabarcoding methods used to analyze regurgitated pellets containing the remains of prey from the king shag. The taxa detected by each method had similar overall FOO, indicating that both methods are reliable in detecting fish taxa taken over a similar dietary period (Table 2).

In general, taxonomic assignment problems can arise for both methods due to limitations in reference databases. For hard parts analyses, a taxonomist is limited to a reference library (physical—often comprised of endemic species or virtual—online databases), and similarly DNA analyses are constrained by the available DNA reference database (virtual). In both cases, customization is ideal as the references can be based on taxa collected in the foraging area, but this is often impractical and costly to implement; thus, relying on both reference types to resolve diet has been proven to be beneficial.

Bioinformatic processes and quality filtering play a significant role in DNA metabarcoding, and often more conservative methods are used to ensure robust data (e.g., retaining only species that meet a certain confidence threshold). When using conservative filtering parameters for DNA metabarcoding, a greater number of pellets were detected for seven fish families, whereas eight families had more pellets detected by hard part analysis. The statistical results identified three families (Hemerocoetidae, Sebastidae, and Tripterygiidae) where the number of pellets detected by hard part analysis was significantly higher. Further investigation found that the number of pellets detected by DNA metabarcoding was largely influenced by the minimum read number parameter and removed true positives (Figure S2). For example, Sebastidae was identified in 15 pellets where the respective ASVs had been filtered out as their reads were less than 100, but these identifications all overlap with pellets identified by hard parts analysis (i.e., removal of true positives). Moreover, the reference database influenced taxonomic assignment specifically for Hemerocoetidae (Figure 3). Hard part analysis consistently detected one species of Hemerocoetidae (*Hemerocoetes monopterygius*), while the DNA metabarcoding assigned no ASVs to Hemerocoetidae at the required minimum confidence levels. Examining the unassigned ASVs revealed that the top three ASVs that had the highest number of pellets were assigned by GenBank (~90% confidence) to *Hemerocoetes* spp. Aligning the pellets identified to have *Hemerocoetes*, despite the low confidence, with the pellets identified by the hard parts analysis provided further evidence that the DNA metabarcoding results do include species from this genus (Figure S2). There are two entries for *Hemerocoetes* in MIDORI (originate from GenBank) both sampled from New Zealand, which provides evidence that the local genetic diversity is sufficiently large enough that it resulted in a 'no-confidence' match. Therefore, while MIDORI is relatively 'complete' with regards to having sequence entries for all genera listed in the hard parts results, these results suggest that smaller-scale local (i.e., foraging area) entries to the DNA database are needed to ensure assignments can be made with high confidence levels, resolving taxonomic uncertainties (Bourbour et al. 2024; Keck et al. 2023). In addition, Tripterygiidae has 230 MIDORI entries; however, *Matanui profundum* was the only species assigned to six ASVs, identified in eight pellets. When changing the filtering thresholds (> 10 reads), seven more pellets were identified that overlapped with the hard part results for this taxon. This overlap is still < 50% of the total number of pellets identified by the hard parts analysis for Tripterygiidae (Figure 3). The lack in ASV Tripterygiidae assignments may be due to DNA database limitations, with assignments not meeting the required confidence thresholds (i.e., the need for local foraging area entries), or perhaps primer biases. Regardless, the high number of ASVs detected for *M. profundum* in a small number of pellets complicates attempts to identify which unassigned ASVs, that have a low frequency of occurrence, may belong to Tripterygiidae. Other possibilities that may account for Tripterygiidae not being detected are that the DNA template may be too low to produce adequate amplification; however, a small fraction of diagnostically useful hard parts have an extended residency in the gut and thus were detected by hard part analysis only. Future studies may also consider grinding up the entire pellet for DNA extraction, which is a means for recovering DNA from fish hard parts that

may help with the detection of dietary species represented in the pellets (e.g., DNA extracted from bones; Grealy et al. 2016; Speller et al. 2012).

Low amounts of DNA template may result from secondary predation. Secondary predation (i.e., fish in the gut of predators, then consumed by seabirds) may also result in overestimates of the dietary significance of some species. Gurnards are one such group where it would be possible that secondary predation may cause an overestimation of prey, as it is not possible to directly determine secondary predation when either of the methods are used. For example, John Dory is a fish that consumes a variety of fish species that king shags also prey upon (e.g., gurnards—*Lepidotrigla* spp.; Kim et al. 2020). Thus, there may be an overlap in primary and secondary items that neither method of analyses can resolve.

Hard part analysis detected eight unique species assignments and DNA metabarcoding analysis detected 14 (Table 2). This pattern of rarer taxa only being identified by one method, and higher in DNA metabarcoding than hard parts, has been found to occur in other studies (e.g., Günther et al. 2021; Oehm et al. 2017). However, the frequency of occurrence for taxa that were only detected by one method was notably lower, typically $<1\%$, than those detected by both methods. A major finding of this study was that while *Arnoglossus scapha* (Bothidae) was the dominant species detected in the pellets by both methods, only DNA metabarcoding detected the second most consumed species, *Lophonectes gallus* (Bothidae). A subsequent closer examination determined that these species cannot be easily differentiated by the morphology of otoliths alone. Other taxa where DNA metabarcoding proves beneficial for this reason are the hard parts grouping of species in '*Notolabrus* & *Pseudolabrus*', '*Pseudophycis*', '*Peltorhamphus*', '*Rhombosolea*', 'Triglidae', and 'Tripterygiidae'. The DNA metabarcoding was also able to detect species which are known to have small otoliths, such as the big-belly seahorse (*Hippocampus abdominalis*) and John Dory (*Zeus faber*; Lombarte et al. 2006). These otoliths are more likely to pass through the gut of the king shag due to their small size and/or are unlikely to survive digestion, making them likely prey items to be under-reported using visual analyses of hard parts. In all the above cases, an inability to differentiate among morphologically indistinguishable or closely related taxa that frequently reside in the same or similar habitats would make little difference to an overall assessment of dietary intake for this seabird.

Beyond seabirds, the comparison of prey analyses methods has identified where there may potentially be an overestimation or underestimation of prey frequency (Carreon-Martinez et al. 2011; da Silva et al. 2019; Deagle et al. 2009). For DNA metabarcoding, host DNA sequences typically amplify alongside the prey when universal primers are used and can subsequently result in underestimation of prey when predator DNA outcompetes prey DNA (typically problematic when interest is in less dominant prey items; Paprocki et al. 2024). To prevent or reduce the amplification of host DNA, prey-enrichment tools like DNA blocking primers/PNA clamps could be implemented (O'Rorke et al. 2012), but effectiveness among samples can vary when the concentration of predator to prey DNA is unknown (Vestheim and Jarman 2008). Another option to avoid the host amplification would be to use prey-specific primers. For example, COI fish barcoding primers have been designed (Ward et al. 2005), but when running jgHCO2198 (Heller et al. 2018) with Fish R1/Fish R2 in silico to assess possible fish specificity, bird sequences still matched and thus amplify in vitro. More specific fish primers have been designed (Berry et al. 2017; Miya et al. 2015), but assessment of likely prey species sequence entries (if possible) for the particular gene region, and the resolution possible, should be primarily investigated as it needs to be suitable for the purpose of the study as it will impact the ability to assign taxonomy. An alternative would be to increase sequencing depth, which may be done in a more cost-effective manner when using Oxford Nanopore Technologies sequencing platforms (Bogaerts et al. 2024; Chang et al. 2024; van der Reis et al. 2023). Furthermore, tagging primers (Binladen et al. 2007) and sequencing PCR replicates would increase confidence in the presence of taxa that are identified, but increasing the number of DNA extractions would be more beneficial in identifying false negatives versus possible contamination induced true negatives—although time and resource costs are often limiting factors for implementation. Targeting shorter regions within genes may also be beneficial to mitigate potential taxa underestimation cause by DNA degradation (e.g., 18S V9; Clarke et al. 2020), but should be used in combination with primers that can provide species level resolution for a more comprehensive picture (Choi and Park 2020; van der Reis et al. 2024). If sampling design allows predator capture, swabbing of beaks, talons, and claws (e.g., keratin composition avoiding host DNA), would likely help minimize host DNA (Paprocki et al. 2024), but non-invasive methods are often needed to minimize disturbance when dealing with protected species. DNA metabarcoding may also distort the apparent diet by over-representing less digested (e.g., larger or more recently ingested) specimens and masking (i.e., under-representing) smaller or faster-digesting prey items. Specifically, using occurrence-based data can overestimate the importance of prey eaten in low proportion and underestimate prey eaten in higher proportions (Bourbour et al. 2024; Thomas et al. 2022), which is generally less likely to occur with hard part analyses. However, the higher sensitivity of DNA-based methodologies have shown the ability to provide significantly higher species diversity and richness estimates (Evans et al. 2021). There are also other possible biological and technical biases that can contribute to underestimation in dietary determination in these methods, such as PCR inhibition (van der Reis et al. 2018) and as demonstrated in this study, confidence filtering parameters can result in filtering out false negatives.

Likewise, for hard part analyses the fragile nature of otoliths and the highly acidic gastric acid (Van Dobben 1952) may have led to an underestimation if otoliths are sufficiently broken, degraded or eroded during consumption and digestion (Harris and Wanless 1993; Johnstone et al. 1990), or being sufficiently small or eroded to readily pass through the digestive tract (Barrett et al. 2007; Lumsden and Haddow 1946; Radhakrishnan et al. 2010), impacting the prey identification when using hard part analyses. This can lead to underestimating diversity within samples, that is, lowering prey species richness. Furthermore, only prey with identifiable hard parts would be able to be recorded as being present, and moreover sufficient morphological structure is needed when identifying to species-level (i.e., as found in this study for *Arnoglossus scapha* and *Lophonectes*

gallus), which is not always possible and thus species richness is lost. Observer bias and the level of regional taxonomic expertise for the visual appraisal of physical remnants can also impact taxonomic assignment.

In this current study, the frequencies of fish families identified by both methods in the pellets of the endangered king shag are largely congruent, but it is not necessarily common for FOO of prey items to align (Oehm et al. 2017; Xirouchakis et al. 2017). The congruency is largely attributed to the application of primers well-suited to capture local prey entered into the DNA database for DNA metabarcoding and having the regional taxonomic expertise and reference material to reliably distinguish among hard parts. Three key points that need to be considered when choosing a method to detect fish, at a fine taxonomic level, from piscivorous predators are, (1) time—hard part analyses are highly time-consuming and may limit the sample size that can be processed (Pompanon et al. 2012), (2) reference databases—the local species of interest need to be included in the databases to be detected (and for hard part analyses, regional taxonomic expertise is essential) (Berry et al. 2015), and (3) quantification—whether the contribution of the total mass or nutrition provided by various prey species to the daily diet of the studied species is a requirement. In regard to this last key point, morphological analysis remains an invaluable tool as a means to provide more robust quantitative prey estimates whereas DNA metabarcoding is considered only semi-quantitative when certain precautions have been taken (Deagle et al. 2013, 2019; Lamb et al. 2019; Thomas et al. 2016). Hard part analysis has the capacity to provide further insight into diet by identifying prey size through known relationship standards between otolith length and fish length, and in turn allowing estimates of the nutritional contribution provided by the various prey items. Thus, the biomass of prey items and total biomass of all prey per pellet can then be used to establish the daily intake of the study species (Lalas and McConnell 2012). Overall, this study demonstrates the two methods provide a complementary approach for revealing the extent of the diversity of prey consumed by a piscivorous seabird, while the congruence in the data between the two methods provides validation and confidence in their respective results.

Author Contributions

There were two individual studies that took place to form this collaborative effort. A.L.R. and A.G.J. contributed to the conception of the DNA metabarcoding study, while C.L. and R.S. contributed to the hard parts study. For the DNA metabarcoding study, A.L.R. carried out laboratory work and performed data analyses. A.G.J. provided significant guidance regarding the development of the DNA metabarcoding project and advised on the analyses. A.G.J. oversaw the DNA metabarcoding project's logistics and administration. For the hard parts study, C.L. and R.S. performed the analysis and equally contributed to the data analyses. K.L.M. helped with the organization and collection of the pellet samples and facilitated the collaboration. A.L.R. lead the collaborative data analysis, prepared figures and tables, and wrote the manuscript. All authors contributed to the development of the manuscript and approved the final manuscript.

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King shags are a protected species, and the research was conducted with the permission of the Department of Conservation, which has the statutory responsibility for their management under the New Zealand Wildlife Act (1953). Animal Ethics approval was not required for this work as the samples were opportunistically collected from the environment and all guidelines were followed to minimize wildlife disturbance during sample collection. Sample collection was done at a time when other work was being carried out at the colonies by the Department of Conservation under Animal Ethics Committee approval AEC 328. We wish to thank Mike Bell, Toroa Consulting (Blenheim), for the assistance in sample collection and Dan Palmer, Department of Conservation (Picton), for providing boat transport to the colonies. We also thank Ngāti Koata for access to colonies that are not on Public Conservation Land—ka pai! We also gratefully acknowledge the permission given by landowners, facilitated by Alice Woodward, for access to the colony at North Trio (Kuru Pongi). This work was funded through the Marine Species Team, Biodiversity Group at the Department of Conservation, New Zealand under project codes BCBC2019-05 and BCBC2020-05. The funder had input into the content of the manuscript and approved the manuscript before submission, and all revisions prior to publication. Lastly, we wish to acknowledge the use of New Zealand eScience Infrastructure (NeSI; www.nesi.org.nz.) high performance computing facilities and consulting support services as part of this research. New Zealand's national facilities are provided by NeSI and funded jointly by NeSI's collaborator institutions and through the Ministry of Business, Innovation & Employment's (MBIE) Research Infrastructure programme. Open access publishing facilitated by The University of Auckland, as part of the Wiley - The University of Auckland agreement via the Council of Australian University Librarians.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Additional Supporting Information can be found in the online version of the article at the publisher's website. The raw sequence data and associated metadata are available from the National Center for Biotechnology Information's Sequence Read Archive (http://www.ncbi.nlm.nih.gov/bioproject/1280759). The code used to process the fastq files is available on GitHub, including the R code that was used for the statistical analyses (https://github.com/AvdReis/NZ_king_shag).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.