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ABSTRACT

On islands, invasive predators, particularly feral cats and rats, are key drivers of bird population decline and extinction. Diet studies can be used to assess predator impacts on prey populations. Here we first evaluated the resolution of morphological identification (Class to Species) of bird remains in cat and rat diet studies. We also analysed the effect of predator size/type (cat vs rat) and sample type (faecal vs stomach contents) on the taxonomic level of bird identification. We found that difficulty in identifying bird remains significantly increased with taxonomic resolution (from Class to Species) for both predators. Bird identification was more accurate in cat than in rat diets and no sample-type effect was detected in cat diets. Second, we developed a set of molecular resources (DNA sequence database and bird-specific primer pairs) to detect and identify bird DNA. We tested and validated primer pairs' taxonomic coverage and specificity using in silico and in vitro analyses. The performances of morphological and molecular methods were then compared in a case study of cat and rat diet samples collected on Niau Atoll (French Polynesia). Our results highlight the efficiency of the molecular method in both detection and high-resolution identification of birds in predator diet samples. As robust qualitative and quantitative diet analyses are required to accurately assess predator impacts on prey populations, we recommend combining morphological and molecular methods to maximise bird detection, identification and quantification, especially when rare or threatened birds are at stake.

1. Introduction

Invasive mammals are one of the main threats to island biodiversity (Courchamp et al., 2003; Towns et al., 2009). Rats (*Rattus norvegicus*, *Rattus rattus*, *Rattus exulans*) and cats (*Felis silvestris catus*) in particular are drivers of species decline, especially for insular bird populations (Courchamp et al., 2003; Blackburn et al., 2004). Since 1500, 53% of the extinct species were birds and 94% of these extinctions have occurred on inhabited islands (Ricketts et al., 2005). Invasive predators, still a major threat to 40% of currently endangered island birds, were implicated in 42% of recent extinctions (Ricketts et al., 2005; King, 1985).

Predator diet studies can be used to decipher trophic relationships between introduced and native species and to evaluate possible predator impacts on prey population dynamics. Such studies are usually conducted by identifying post-ingestion remains, and have been widely used to assess cat and rat impacts on island birds (Ruffino et al., 2015; Medina et al., 2011). But diet studies using visual observation of undigested remains have limitations, leading to under- or over-estimation of some prey taxa (Reynolds and Aebischer, 1991; Pires et al., 2011). First, differential degradation of soft and hard parts of prey during digestion and/or varying retention time may result in misestimates of consumed prey numbers (Pires et al., 2011; Egeter et al., 2015). Second, the accuracy of qualitative and quantitative diet analyses depends on the examiner's ability to identify prey species from degraded and fragmented remains that have lost some of their diagnostic characteristics (i.e. colour, size and shape) (Day, 1966; Tollit et al., 2003). Accuracy is also affected by the proportion of remains that disappear during mastication and digestion. Although low resolution prey identification (i.e. Order level) might provide consistent indications of predator functional niche breadth, high resolution prey identification (i.e. Genus or Species level) allows a deeper exploration and quantification of trophic

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interactions among species populations (Greene and Jaksić, 1983). Because conservation studies often focus on rare or endangered species, reliable identification of prey species is crucial to precisely evaluate predator impact. Diet studies would therefore greatly benefit from alternative or additional methods enhancing prey identification.

DNA-based methods developed over the past decade have allowed successful identification of DNA sequences either in predator diet samples or directly from prey remains (e.g. Klare et al., 2011), thereby elucidating trophic interactions (Pompanon et al., 2012). These methods are based on PCR amplification of targeted prey DNA, using group- or species-specific primer pairs, in consumer diet samples. Further sequencing provides a powerful identification of prey species by comparison to customised and/or public (e.g. BOLD, EMBL) DNA reference databases (Pompanon et al., 2012). PCR-based methods enable the specific detection of prey DNA and are more robust in taxonomic prey identification at species-level than other biomarker methods (stable isotopes, signature lipids and antigen detection) (Pompanon et al., 2012). However, DNA-based methods are still rarely used to study the diet and impact of introduced predators in conservation biology, despite their capacity to detect consumption events on rare and/or endangered native species (Zarzoso-Lacoste et al., 2013; Egeter et al., 2015).

Here, we first reviewed and analysed published diet studies on cats and rats (*R. exulans*, *R. rattus* and *R. norvegicus*) that recorded consumption of birds. We evaluated the performance of the morphological method to specifically identify bird remains in predator diet samples (stomach vs faecal contents), and discuss its qualitative and quantitative limitations. Second, we compared morphological and molecular methods through a case study dealing with diet samples collected on Niau Island (Pacific, French Polynesia) from three invasive predators (*F. s. catus*, *R. exulans* and *R. rattus*). Finally, we compared the efficiency of the methods in (i) bird predation event detection and (ii) high-resolution prey identification.

2. Materials and methods

2.1. Morphological method reviewed

2.1.1. Data compilation

Our exhaustive review was not limited to island ecosystems. Data from the literature were compiled using electronic databases (Web of Knowledge, Inist, Jstor, Mendeley, Springerlink, Science Direct, Google search and Google scholar). We used the following common/taxonomic names as keywords: invasive/introduced rat*, Norway/brown rat*, ship/roof/black rat*, kiore/Pacific/Polynesian rat*, *Rattus rattus*, *Rattus exulans*, *Rattus norvegicus*, introduced/invasive/feral cat*, *Felis silvestris catus*, combined with other key words: diet analysis, feeding ecology, food/feeding habits, bird predation, remains/prey/bird identifications, feathers, guts, scats/faeces, and stomachs.

Data was compiled up to May 2015 from papers published in scientific journals and academic theses. We only selected diet studies where bird remains were detected and quantitative data given (e.g. number of prey, frequency of occurrence), excluding those where no attempt was made to identify bird species. Three diet sample types were used; scats/faeces, stomachs and guts.

2.1.2. Bird detection and identification

The total number of birds identified to Class (i.e. unidentified birds), Order, Family, Genus or Species in each study was recorded. Since our review aimed exclusively to evaluate how efficiently the morphological method identifies bird remains, irrespective of the relevance of the diet studies themselves, only data obtained from a morphological and formal identification of bird remains were attributed to the related taxonomic level. Thus, unidentifiable remains that were suspected by the authors to belong to a bird species based only on field observations or deductions (e.g. breeding period, bird abundance, nesting species) were attributed to Class. The cumulative number of birds identified to each taxonomic level was calculated for both cats and rats.

An index of identification resolution (IR) was calculated for each diet analysis to assess how efficiently the morphological method identifies bird remains to Class, Order, Family, Genus or Species levels.

$$IR = \left[(1 \times n_{Class}) + (2 \times n_{Order}) + (3 \times n_{Family}) + (4 \times n_{Genus}) + (5 \times n_{Species})\right]/N$$

Coefficients (from 1 to 5) weighted in increasing order of difficulty in identifying bird remains from the wider to the finer taxonomic level. For each diet analysis, n_i corresponds to the number of birds identified to a particular taxonomic level and N is the total number of detected birds. For example, if 284 birds are found in a cat diet analysis and 83, 43, 11, 15 and 132 identified to Class, Order, Family, Genus and Species-level respectively, the IR will be equal to 3.25. Note the range of values is 1 to 5, with 5 the highest resolution.

Non-parametric Mann–Whitney tests were performed to compare the IR obtained for (i) each cat and rat diet study, testing for a predator size/type effect and (ii) cat stomach and scat samples, testing for a sample type effect.

Statistical analyses were performed with R 2.14.0 using the "pgirmess" package (Giraudoux, 2012; R Development Core Team, 2012). Due to low sample sizes in *R. norvegicus* and *R. exulans* diet studies, we did not compare the effect of predator size/type and sample type for rats.

2.2. Comparison of morphological and molecular methods

2.2.1. Study site and sample collection

Niau Atoll (16°10′S, 146°22′W) in the Tuamotu Archipelago, South Pacific Ocean, hosts only nine nesting and nine non-nesting bird species. Four of the nesting species are endemic to the Tuamotu Archipelago or to this island, five are legally protected by French Polynesian law (category A) and four are listed under the IUCN threatened categories (IUCN, 2015) (Appendix A). This bird community is threatened by *F. s. catus*, *R. exulans* and *R. rattus*.

Diet samples were collected in February and November 2010 and March 2011. Rats were collected along six 320 m transects with 40 snap-traps spaced 15 m apart set over five consecutive nights. Traps were baited with coconut before dusk and checked early each morning. Rats caught were identified, weighed, sexed and dissected. Their stomach and faecal contents were collected, preserved in 90% ethanol and frozen. Cat scats were collected from all the island paths, stored individually in plastic bags and frozen.

To create our reference collection of Polynesian bird feathers and DNA, feather and tissue samples were taken from frozen carcasses of Polynesian bird species previously collected by the Polynesian Society of Ornithology (SOP) and specimens at the Tahiti Museum. DNA from 10 Polynesian bird species (including five species present on Niau Atoll, Appendix B) was extracted using the Gentra Puregene Tissue Kit (Qiagen) from the collected muscle tissues, sequenced and deposited on GenBank (accession numbers JX297481–JX297489, KF938933).

2.2.2. Morphological diet analysis

All morphological diet analyses were conducted in a dedicated room. Single-use non-talc gloves, surgical mask and lab coat were worn. All instruments were successively washed with bleach, 96% alcohol and distilled water between diet samples to avoid cross-contamination.

Stomach and faecal contents of each rat were extracted, homogenised and analysed separately under a dissecting microscope to detect bird predation (Zarzoso-Lacoste et al., 2013). Before morphological analysis, each cat scat was thoroughly rubbed over a 0.5 mm sieve to obtain a powder of organic matter potentially containing all prey DNA but excluding large hard remains (e.g. bones, hairs). The scat powder was then placed in new plastic bags and re-frozen. The

sieve contents for each scat were washed over a 0.5 mm sieve under a stream of hot water, and items such as feathers or bone fragments separated (Bonnaud et al., 2007). Bird remains in rat or cat diet samples were identified to the finest taxonomic level possible using our Polynesian bird reference material.

2.2.3. Molecular diet analysis

DNA extractions, PCR preparations and post-PCR treatments were performed in separate rooms, under a laminar flow hood, using filter tips and single-use non-talc gloves.

2.2.3.1. Group-specific primer set selection and validation. We targeted the multi-copy mitochondrial cytochrome oxidase subunit I (Cox1) gene, the standard marker for (meta)barcoding studies since it usually allows species-level discrimination by comparison to reference sequence databases (Hebert et al., 2003; Deagle et al., 2014). To maximise the likelihood of detecting and identifying bird DNA, we used three independent bird-oriented primer pairs (PPs) targeting conserved regions of the Cox1 gene (Appendix C.1). The two largest, PP1: BirdF1-BirdR1 (746 bp) and PP2: BirdF1-AWCintR2 (275 bp), were selected from the literature (Kerr et al. 2007, Patel et al. 2010) (Appendix C.2). To address the highly fragmented DNA in diet samples (Zaidi et al., 1999; Deagle et al., 2006) and reduce false negative bird detections, we also designed and used the short bird-specific primer pair PP3: OSXF (5′-TTATCCGTGC AGAACTTGG-3′)–OSXR2 (5′-GACTAGTCAGTTTCCGAA-3′) (143 bp).

In silico PCRs (ecoPCR programme; Ficetola et al., 2010) were conducted on the entire EMBL database to assess the percentage of bird and non-bird species theoretically amplified using each PP. Three mismatches were allowed between each primer and the templates. From the resulting output files, we randomly kept one sequence per species, to reduce overrepresentation of a few species.

To assess the theoretical coverage (B_c) and resolution capacity estimated via the barcode specificity index (B_s) of each bird PP (Ficetola et al., 2010), we compiled a customised bird-sequence database for each PP. First, the longest Cox1 sequence of each available bird species (Aves, taxid: 8782) was retrieved from GenBank (access: May 2015). Then we filtered out those that (i) did not align correctly, (ii) were too short to cover the entire length of the corresponding amplicons (i.e partial sequences) and (iii) contained ambiguous nucleotides (e.g. N). Sequences were trimmed to contain only the amplicons (including primer binding sites). We obtained sets of 410, 780 and 3076 Cox1 sequences for PP1, PP2 and PP3 respectively. Each database was transformed to ecoPCR output format, and ecotaxstat and ecotaxspecificity (Ficetola et al., 2010) were used to calculate B_c (allowing 3 or 4 mismatches) and B_s (default parameters) respectively.

Sequences from these customised databases were aligned using MEGA5 (Tamura et al., 2011) and the entropy at each position was calculated for each PP and its amplicons. Finally, we evaluated the number of mismatches between each primer and the templates.

Because in silico PCR gives only an approximation of real PCR success, each PP's taxonomic coverage and specificity was empirically tested in vitro on a bank of 12 target and 46 non-target Polynesian species (17 plants, 8 vertebrates and 21 invertebrates) (Appendix D). The sensitivity of bird detection was also tested on a dilution series containing 10, 5, 2, 1, 0.2, 0.1 and 0.01 ng/μl of bird DNA diluted in ultra pure sterile water.

2.2.3.2. DNA extraction and PCR protocol. After morphological analysis, the stomach and faecal contents of each rat were mixed together, individually homogenised and cold-ground using steel beads for 2 × 30 s at 30 Hz (Tissuelyser, Retsch) before extraction. DNA was extracted for rats from 200 mg of ground diet samples and for cats from 500 mg of scat powder, using the DNeasy mericon food kit (Qiagen) (Zarzoso-Lacoste et al., 2013). All DNA concentrations were adjusted to a maximum of 300 ng/ml to prevent possible PCR inhibition by high concentrations of DNA and/or inhibitors (Morin et al., 2001). PCR protocols were optimised in a final volume of 25 μl containing 1 μM $MgCl_2$, 0.2 μM of dNTPs, 0.5 μM of each primer, 1.5 U of Taq DNA polymerase and 1 μl of sample DNA extraction. All PCRs were heated to 95 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 1 min at the annealing temperature given in Appendix C.2, 72 °C for 1 min, then 72 °C for 10 min. All PCR runs were replicated six times and included two positive controls (bird DNA) to confirm suitable reaction conditions, a PCR blank to check for cross-contamination, and three negative controls containing *R. rattus*, *R. exulans* and *F. s. catus* pure DNA.

2.2.3.3. Bird detection and identification in cat and rat diet samples. The migration of PCR products on agarose gel allowed bird DNA to be detected in each diet sample (Corse et al., 2010; Zarzoso-Lacoste et al., 2013). Consensus sequences were obtained by forward/reverse sequencing replicated amplicons (GATC Biotech Company, Sanger technology). When an unreadable Sanger sequence was obtained, the corresponding PCR product was cloned (pGEM®-T Easy Vector cloning kit), purified (PureYield™ Plasmid Miniprep Start-Up Kits, Promega) and sequenced.

To identify consumed bird species, we supplemented our reference collection of Polynesian bird DNA sequences with all the available GenBank COI sequences for Polynesian bird species listed on both www.manu.pf and www.oiseaux.net/oiseaux/polynesie.francaise.html. When a Polynesian species was not referenced in GenBank, we used a sister species or a set of species belonging to the same Genus. Predator and human sequences were also added to check for potential contamination generated by PCR.

The 90 reference sequences (including the 10 Polynesian bird specimens sequenced in this study, 75 Polynesian bird species or sister species and 5 mammalian sequences collected from GenBank database, see Appendix B) were aligned with the sequenced amplicons obtained from diet samples using ClustalW. Because sequence lengths were not identical (the two shortest = 84–87 bp; the two longest = 1551 bp), pairwise comparison could potentially have impacted genetic distance drastically due to the non-homogeneous pattern of substitution along the gene. To reduce this effect, positions with less than 50% site coverage were eliminated.

Hebert et al. (2003) defined the DNA barcode as a short sequence used as a standard tool to identify the species to which an organism belongs. When the correct species has not been included in the reference data set, only tree-based methods, especially the strict method, coupled with a distance threshold will protect against false positives (Ross et al., 2008). Phylogenetic trees were therefore constructed in MEGA5 (Tamura et al., 2011) in order to assign each amplicon to a bird clade. The phylogenetic relationship was inferred using the Neighbour-Joining Method (Saitou and Nei 1987), known to outperform the Maximum-Likelihood Method (PhyML) (Elias et al., 2007, Austerlitz et al., 2009). Evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980).

The degree of information redundancy in fragments compared using NJ was assessed by bootstrap re-sampling of 2000 pseudoreplicate datasets (Felsenstein, 1985). Because bootstrap values may be impacted when species numbers are greater than sequence differences, species groups with a low bootstrap value (<50%) but having a phylogenetic relationship in line with other studies were tested separately to remove this bias. To test the impact of each amplicon on tree topology, we generated 35 phylogenetic trees, taking into account only one of the 35 sequenced amplicons coupled with the reference sequences.

2.2.4. Data analysis

Non-parametric Cochran Q-tests for correlated data were used to compare frequencies of bird detection among cat and rat diet samples using the three bird-specific PPs.

Non-parametric McNemar χ^2 tests for binomial and correlated data were performed to compare morphological and molecular methods for frequency of bird detection and bird identification on both rat and cat diet samples (Hollander and Wolfe, 1973).

3. Results

3.1. Morphological method performance: a review of diet studies

In total, we reviewed 45 rat and 71 cat diet studies (Appendix E), covering mainland and island ecosystems ranging from tropical to polar biomes. Occurrence frequency (OF%) was the most common descriptor of predator diet, used in 47.6 and 98.5% of the studies, and was the only method used in 46.6 and 8.5% of rat and cat diet studies respectively (Appendix F). Minimum prey number (PN) was recorded in only 6.3 and 57.6% of rat and cat diet studies respectively. 50.7% of cat diet studies combined two methods of prey quantification (usually OF% coupled with PN), whereas most rat diet studies used only one method (66.7%), generally OF%. Biomass (%) was the commonest metric used to estimate the contribution of birds to cat diets (36.4%) whereas volume (%) was the commonest in rat diet studies (19.0%).

Of the 45 rat diet studies, five recorded minimum bird number (hereafter Rat_{BPN}), and 40 recorded bird occurrence frequency (hereafter Rat_{BOF}). In total, 552 bird detections were recorded among 6765 diet samples (8.2%). 82.2% of these diet studies used stomach samples, 2.2% used gut samples and 14.55% used faeces. A total of 413, 110 and 29 birds were detected in *R. rattus*, *R. norvegicus* and *R. exulans* diet studies respectively. Morphological identification was impossible for 62.1% (n = 343) of bird remains, and of those identified, 37.9% (n = 209) could only be identified to Order, 34.4% to Family and 24.3% to Species. All birds identified to Species (n = 134) were found in rat stomachs (Appendix G.1). Of the 209 birds identified to Order (or below) in rat diet samples, 64.1% were identified to Species: Charadriiformes, Sphenisciformes, Apterygiformes and Procellariiformes. Procellariiformes were mainly identified to Family (81.1%) and Passeriformes were all identified to Order (Appendix H.1).

Of the 71 cat diet studies, 37 provided Bird minimum prey number (hereafter Cat_{BPN}), and 34 gave bird occurrence frequency (hereafter Cat_{BOF}). In total, 4 874 bird detections were recorded from 16,036 diet samples (30.4%). 57.7% of these studies were conducted on scats, 26.7% on stomach samples, 11.3% on mixed diet samples and 4.2% on gut samples. Morphological identification was impossible for 26.6% (n = 1 298) of bird remains; of the 73.4% identified (n = 3576), 9.9%, 27.1%, 2.3% and 60.7% were to Order, Family, Genus and Species respectively (Appendix G.2). In diet studies using stomach samples, the proportions of detected birds identified to Species were 64.61% and 37.7% for Cat_{BOF} and Cat_{BPN} studies respectively, and in studies using scats, they were 37.8% and 45.9% (Appendix H.2). Of the 3576 birds identified to Order (or below) in cat diet samples, 60.6% were identified to Species. 46.2% of bird identifications were not possible beyond Family, particularly in the two most detected bird Orders (Procellariiformes and Passeriformes).

Finally, the capacity to identify bird remains decreased with increasing resolution (from Class to Species) for both predator types, and became particularly low beyond Order and/or Family levels, especially in rat diet samples (Fig. A). The mean of the identification resolution index for prey ($mean_{IR}$) was 2.75 for cats and 1.85 for rats. The IR index was significantly different between cat and rat diets (Mann Whitney test, W = 2420.5, P < 0.001). No statistical difference was observed between cat stomach and scat samples (Mann Whitney test, W = 322, P = 0.285).

3.2. Comparison of morphological and molecular methods: a case study

We analysed 268 rat digestive tracts (194 *R. exulans* and 74 *R. rattus*) and 351 cat scats using both morphological and molecular methods.

3.2.1. Morphological diet analysis

Bird remains were detected in rats and cat with an OF% of 1.35 (n = 1), 1.03 (n = 2) and 5.70 (n = 20) for *R. rattus*, *R. exulans* and *F. s. catus* respectively.

Bird identification in rat diet samples was not possible beyond Class due to the extremely small number of feathers found, coupled with their high fragmentation and degradation ($mean_{IR}$ = 0.38). Among the 20 cat scats containing bird remains, only six contained remains that could be identified to Species, four being *Ptilinopus coralensis* and two being *Gallus gallus*. The other birds were all unidentifiable ($mean_{IR}$ = 1.65) (Appendix I).

3.2.2. Molecular diet analysis

3.2.2.1. Group-specific primer set selection and validation. Entropy results showed that primer binding sites are relatively well conserved among birds. Both results of in silico PCRs and empirical in vitro tests confirmed the high specificity, wide taxonomic coverage and high resolution capacity of the three PPs for amplifying and identifying very low bird DNA concentrations. A detailed description of these results is available in Appendix J.

3.2.2.2. Bird detection and identification in cat and rat diet samples. Bird DNA was detected in rats and cats with an OF% of 4.0 (n = 3), 2.6 (n = 5) and 5.1 (n = 18) for *R. rattus*, *R. exulans* and *F. s. catus* respectively. Although the molecular method yielded a significantly higher proportion of bird detection in predator diet samples than the morphological method (McNemar χ^2 test, Q = 9.333, P value = 0.002), this

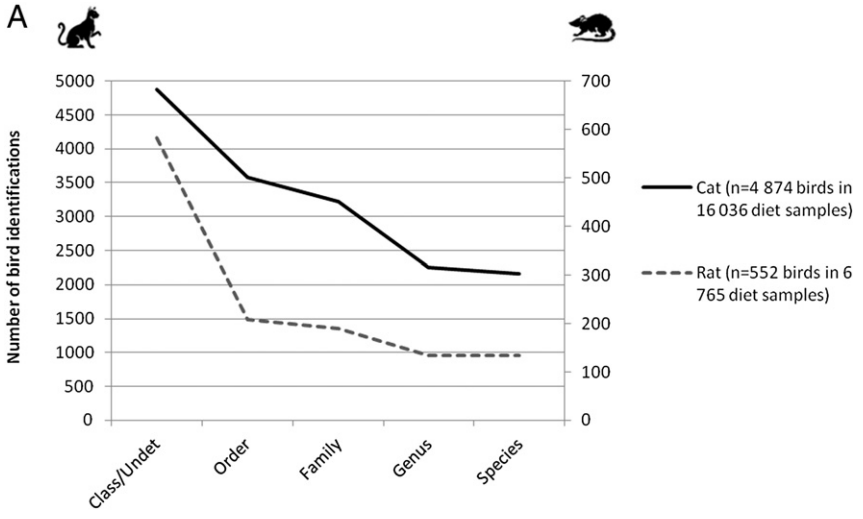

Fig. A. Evolution of the accumulated number of birds identified to each taxonomic level in the reviewed cat and rat diet studies

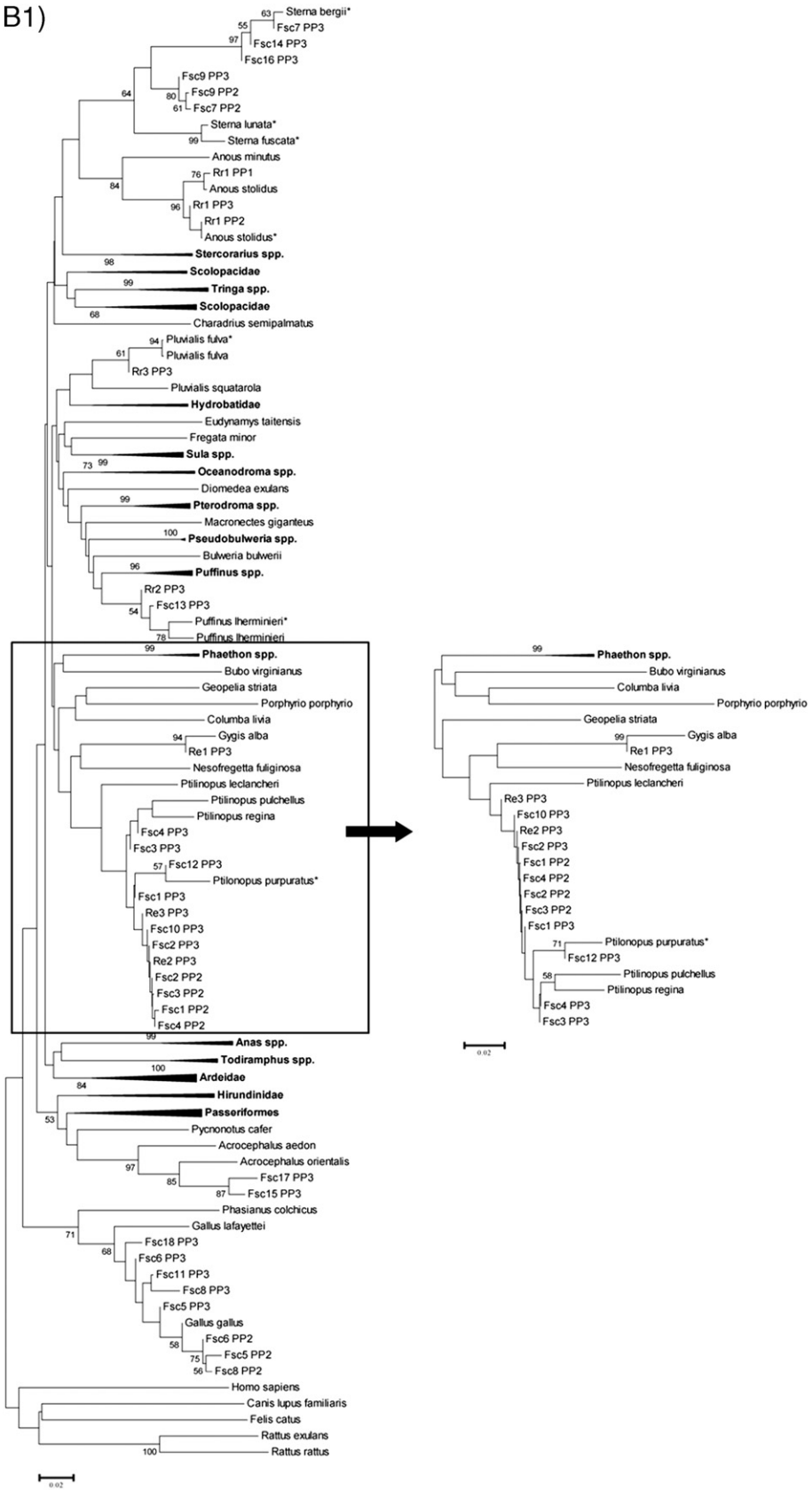

Fig. B. 1) Neighbour joining (NJ) tree including our 35 sequenced amplicons and our 101 reference sequences. Amplicons are named accordingly to predator type (Fsc = *F. s. catus*, Rr = *R. rattus* or Re = *R. exulans*) or primer pair (PP1, PP2 or PP3) and are numbered. 2) Results of sequence assignment to bird clades and species based on tree topology (theoretical assignment) and our biological knowledge of the bird species present on Niau Atoll (biological assignment). The two samples highlighted with a star were removed from further identification analysis.

B2)

Sequenced amplicons				Result of sequence taxonomic assignment	
Primer pair	Predator	Sample	Genbank accession number	Theoretical assignment	Biological assignment
BirdF1/BirdR1	*R. rattus*	Rr1_PP1	KT003542	*Anous stolidus*	*Anous stolidus*
BirdF1/AwCintR2	*R. rattus*	Rr1_PP2	KT003571	*Anous stolidus*	*Anous stolidus*
	F. s. catus	Fsc1_PP2	KT003543	Ptilinopus	*Ptilinopus coralensis*
		Fsc2_PP2	KT003545	Ptilinopus	*Ptilinopus coralensis*
		Fsc3_PP2	KT003547	Ptilinopus	*Ptilinopus coralensis*
		Fsc4_PP2	KT003549	Ptilinopus	*Ptilinopus coralensis*
		Fsc5_PP2	KT003551	Gallus (gallus)	*Gallus gallus*
		Fsc6_PP2	KT003552	Gallus (gallus)	*Gallus gallus*
		Fsc7_PP2	KT003553	Sterna (bergii)	*Sterna bergii*
		Fsc8_PP2	KT003555	Gallus (gallus)	*Gallus gallus*
		Fsc9_PP2	KT003557	Sterna (bergii)	*Sterna bergii*
OsxF/OsxR2	*R. exulans*	Re1_PP3	KT003568	Gygis (alba)	*Gygis alba*
		Re2_PP3	KT003569	Ptilinopus	*Ptilinopus coralensis*
		Re3_PP3	KT003570	Ptilinopus	*Ptilinopus coralensis*
		Re4_PP3*	-	Bird	-
		Re5_PP3*	-	Bird	-
	R. rattus	Rr1_PP3	KT003572	*Anous stolidus*	*Anous stolidus*
		Rr2_PP3	KT003573	Puffinus (lherminieri)	*Puffinus lherminieri*
		Rr3_PP3	KT003574	Pluvialis (fulva)	*Pluvialis fulva*
	F. s. catus	Fsc1_PP3	KT003544	Ptilinopus	*Ptilinopus coralensis*
		Fsc2_PP3	KT003546	Ptilinopus	*Ptilinopus coralensis*
		Fsc3_PP3	KT003548	Ptilinopus	*Ptilinopus coralensis*
		Fsc4_PP3	KT003550	Ptilinopus	*Ptilinopus coralensis*
		Fsc5_PP3	KT003540	Gallus (gallus)	*Gallus gallus*
		Fsc6_PP3	KT003541	Gallus (gallus)	*Gallus gallus*
		Fsc7_PP3	KT003554	Sterna (bergii)	*Sterna bergii*
		Fsc8_PP3	KT003556	Gallus (gallus)	*Gallus gallus*
		Fsc9_PP3	KT003558	Sterna (bergii)	*Sterna bergii*
		Fsc10_PP3	KT003559	Ptilinopus	*Ptilinopus coralensis*
		Fsc11_PP3	KT003560	Gallus (gallus)	*Gallus gallus*
		Fsc12_PP3	KT003561	Ptilinopus	*Ptilinopus coralensis*
		Fsc13_PP3	KT003562	Puffinus (lherminieri)	*Puffinus lherminieri*
		Fsc14_PP3	KT003563	Sterna (bergii)	*Sterna bergii*
		Fsc15_PP3	KT003564	Acrocephalus	*Acrocephalus atyphus*
		Fsc16_PP3	KT003565	Sterna (bergii)	*Sterna bergii*
		Fsc17_PP3	KT003566	Acrocephalus	*Acrocephalus atyphus*
		Fsc18_PP3	KT003567	Gallus (gallus)	*Gallus gallus*

Fig. B (*continued*).

pattern was more clearly pronounced in rats than cats. However, the number of molecular method bird detections significantly varied depending on the PP used (Cochran Q test, $Q = 38.48$, P value < 0.001). PP3 provided 26 bird amplicons, from which only ten and one were retrieved when using PP2 and PP1 respectively (Appendix D). Mean repeatability of bird detection among PCR replicates was at maximum for PP1 (100.0%) and high for both PP2 (85.0%) and PP3 (91.6%), which showed slight variations in band intensity patterns among the six replicates (data not shown).

Of the 37 bird amplicons obtained, 35 provided readable bird sequences (GenBank accession numbers: KT003540–KT003574). Finding no evidence of multiple sequencing (i.e. superposition of several sequences from the same diet sample), we assumed that each sample contained only one prey bird species, making any subsequent cloning step unnecessary.

The 35 phylogenetic trees obtained (from reference sequences with one of the 35 amplicons from diet samples added) were similar, indicating that the short amplicon sequences did not greatly affect tree topologies. Two phylogenetic trees are presented in Fig. B.1: the first covers the complete data set, the second the *Ptilinopus* taxon. This tree topology method identified 35 of the 37 amplified sequences to at least Genus. When topology-based identification was possible only to Genus, we took sequence identifications further based either on our biological knowledge of the different species on Niau Atoll (see Appendix A) or on high similarity to a reference sequence (Fig. B). Six amplicons were assigned to the Sterna clade and considered to belong to *Sterna bergii*, because this species was the closest molecular match (89–100% identity depending on amplicon length) and the only species from this Genus present on Niau Atoll. Three amplicons were identified as *Anous stolidus* within the Anous clade (98–100% identity). One amplicon was assigned

to the *Gygis alba* clade, the only species from this Genus present in French Polynesia, and considered to belong to this species (99% identity). Twelve and two amplicons respectively were assigned to the *Ptilinopus* (94–99% identity to the four *Ptilinopus* spp.) and *Acrocephalus* (91–93% identity to both *Acrocephalus* spp.) clades. No Cox1 reference sequence was available for *P. coralensis* and *Acrocephalus atyphus*, terrestrial and non-migrant species nesting on Niau Atoll. Birds identified to these Genera (only one species each at our study site) were therefore assumed to belong to these species. One amplicon was assigned to the *Pluvialis* clade and considered to belong to *Pluvialis fulva* (99% identity), present on the island. Two amplicons were clustered with the *Puffinus* clade and considered to be *Puffinus lherminieri* (96–98% identity). Eight amplicons were assigned to the *Gallus* clade and identified as *G. gallus* (96–100% identity), abundant on Niau Atoll. Two sequences were excluded due to shortness (86–89 bp), and the highly conserved part of the alignment that blasted with bird sequences but did not allow more precise identification (Fig. B.2). Molecular method bird identifications matched the six morphological identifications (Appendix I).

The molecular method provided more bird detections and finer taxonomic identification than the morphological approach (McNemar χ^2 test, $Q = 16.055$, P value < 0.001). The mean of the identification resolution index for prey was 3.90 for cats and 3.75 for rats. *P. coralensis* was the prey most frequently consumed by cats (6/18 identified birds in cat scats), closely followed by *G. gallus* and *S. bergii* (5 and 4/18 respectively). *A. atyphus* and *P. lherminieri* were less consumed by cats (2 and 1/18 respectively). *R. rattus* preyed upon *A. stolidus*, *P. fulva* and *P. lherminieri* (1/3 identified birds in *R. Rattus* diet for each species) and *R. exulans* preyed upon *P. coralensis* and *G. alba* (2 and 1/3 identified birds in *R. exulans* diet respectively) (Fig. B, Appendix I).

4. Discussion

While morphological diet studies have contributed substantially to our understanding of predation by invasive species, the technique is severely constrained by detection and identification of target taxa remains. Our review illustrates this, finding most (70%) bird remains in rat samples and approximately one quarter in cat samples to be reported unidentifiable beyond Class. Obviously, this limitation severely hampers efforts to accurately assess the impacts of introduced predators and inform conservation decision makers.

Our review also shows that the resolution of prey identification differs among bird Orders. Passeriformes, the largest bird Order, is the most commonly identified terrestrial bird taxon. The low resolution of their identification, mainly limited to Order, may be related to the difficulty of identifying discriminating diagnostic characteristics for such a large number of morphologically similar species. Seabirds are the most frequently detected bird group in cat and rat diet studies, especially three Orders; Procellariiformes, Charadriiformes and Sphenisciformes. Charadriiformes are almost all identified to species level, while the identification resolution of Procellariiformes and Sphenisciformes is mainly limited to Family. These long-lived seabirds are particularly targeted in conservation studies due to their vulnerability and accessibility to predators (e.g. naive behaviour, ground breeders) (Jones et al., 2008).

In terms of the morphological method alone, our review and case study yield three important findings. First, the larger predators (cats) produced samples providing finer taxonomic resolution of the prey. Presumably this is due to differences in bite volume, mastication and digestion processes between rats and cats (Tollit et al., 2003; Jarman et al., 2004; Egeter et al., 2015). Second, there was no statistical difference in resolution of bird identification between cat stomach and scat samples. Thus, scat analysis represents a reliable method of diet analysis, particularly relevant when studying rare or elusive predators (non-invasive method). However, the difficulty of finding faeces in natural settings frequently leads to use of invasive methods such as stomach analysis (e.g. for rats). Third, analysing multiple descriptive indices proved more powerful than relying on a single metric. Our review found that cat predation was quantified more rigorously than rodent predation because cat diet studies generally combined two (53%) or more than two (32%) descriptive indices. Although frequency data may be sufficient to obtain a first overview of predator diet, using multiple diet indices can significantly improve accuracy when quantifying predator impacts (Fedriani and Travaini, 2000; Klare et al., 2011). Quantitative data on prey consumption obtained from predator diet analysis are crucial to estimate predation rates and determine the number of prey consumed by a predator population (e.g. Bonnaud et al., 2012). They can also be incorporated into demographic models and used to predict prey population survival under different predation scenarios (e.g. Bonnaud et al., 2009), and/or used to rank predator species by order of impact on prey populations or communities (Greenstone et al., 2010).

Our case study clearly showed that molecular diet analysis detects and identifies prey better than the morphological method (i.e. more detections and finer taxonomic identifications in rat diet samples). This suggests that bird predation may sometimes be underestimated when only the morphological method is used. It particularly points to the efficiency of molecular methods in detecting and identifying bird DNA when no detectable hard remains are ingested (Egeter et al., 2015). For example, because rats are renowned egg predators (Stapp, 2002; Jones et al., 2008), the non-detection of egg predation could bias the estimation of rat impact on bird populations, especially for those particularly vulnerable, i.e. small birds (Zarzoso-Lacoste et al., 2011).

Our identification results confirm that the molecular method is better able to identify prey DNA to a fine taxonomic level. Of the 24 birds thus detected, 22 were identified to at least Genus level, and the six morphological identifications were confirmed. Additionally, we managed to extract prey DNA from cat scats that were several weeks old, by targeting relatively well-conserved primer binding sites amplifying a short but informative Cox1 fragment (e.g. PP3 < 150 bp). This facilitated amplification of targeted prey sequences from a matrix of multiple DNA containing small amounts of highly degraded DNA (Zaidi et al., 1999; Deagle et al., 2006). The higher number of prey amplifications obtained using PP3 could also be due to its high in silico taxonomic coverage.

Our molecular identification results were systematically congruent among the three bird-specific primer pairs, whatever their amplicon size, highlighting the potential of PP3 for future molecular diet studies. However, empirical testing is essential to ensure that a selected set of primer pairs is suitable for a particular case study, especially when targeting conserved primer binding sites (e.g. PP3). We recommend that future molecular diet studies (i) realise empirical in vitro tests to confirm both taxonomic coverage and specificity of each PP, (ii) optimise extraction and PCR (e.g. annealing T°C, salt concentration) protocols to enhance prey amplification and/or modulate PP taxonomic coverage and specificity, and (iii) include in the molecular reference database several sequences from each species potentially present at each study site, to distinguish closely-related species.

Finally, combining complementary methods of diet analysis (i.e. morphological, molecular, isotopic analyses) could provide a fuller picture of trophic food webs (Wirta et al., 2014; Kartzinel et al., 2015). However, consumption results obtained from any of these methods should be interpreted with caution since i) none of these approaches can currently distinguish between predation and scavenging and ii) these methods may underestimate predator impact on native prey populations: predators may kill individuals without consuming them, or disturb nesting birds, thus indirectly affecting their population dynamics (Ruffino et al., 2015).

4.1. Perspectives

Sanger sequencing approaches are limited to presence/absence assessments of focal prey. Because large predators may consume more

than one bird in a meal, we suggest two improvements. First, if a predator has eaten several birds from different species, prey can be distinguished by either (i) cloning the PCR amplicons before sequencing (Sanger) to isolate the DNA sequences amplified from each species or (ii) using a metabarcoding approach directly characterising many consumed species in various diet samples simultaneously through next-generation sequencing (NGS). The latter approach, or alternatively quantitative PCRs, also eliminates false negative prey detection, outperforming standard PCRs in this respect. As NGS technologies improve and costs fall, this time- and cost-effective approach will prove particularly advantageous for large-scale studies (Pompanon et al., 2012). Using prey-specific primer pairs, rather than universal primers, could considerably reduce the length of bioinformatics analyses by limiting the number of reads produced by high throughput sequencing.

The second concerns predators that have eaten several individuals from the same species. To date, prey quantification via molecular technologies has been difficult and labour- intensive. Nevertheless, recent work points to allele diversity analysis as a promising detector of the presence of more than one prey specimen in diet samples (Carreon-Martinez et al., 2014). Other studies based on NGS diet analysis have made interesting advances in estimating prey biomass from read counts, based on differences in gene copy numbers between prey species or tissues, and prey-specific digestion biases (Thomas et al., 2014). Still in its infancy, quantifying consumer diets based on prey DNA amplified from diet samples is a promising field of research.

4.2. Additional insights

The molecular method can also lead to identification of rare or transient birds not previously recorded at a study site. Here, the identification of *P. lherminieri* (Least Concern, IUCN) in two diet samples (*F. s. catus* and *R. rattus*) confirms the need to include all species potentially present at regional scale, especially those with high dispersal capacities. Unfortunately, our results did not allow us to distinguish between predation of transient birds and/or scavenging on bird carcasses. Further analyses (e.g. stable isotope analysis) and identifying *P. lherminieri* colonies at island scale will help conclude on a possible impact of introduced predators (e.g. limitation of bird establishment).

Our results also reveal that cats and rats occasionally consume two endemic and protected birds, *P. coralensis* and *A. atyphus*. This could make cats the greatest predation threat to Niau's avifauna. Accurate population size assessment of *P. coralensis* and *A. atyphus* and of our three invasive predators, with subsequent diet analysis of these predators across seasons, are required to better assess the extent of their impact on these birds.

5. Conclusion

The morphological method is the approach most widely used to assess predator impact on bird populations. This time-consuming method requires an individual protocol per predator and per sample type, which can become very expensive when large samples of different types are analysed (see Appendix K for a cost estimate on the morphological and molecular methods used in this study). Our results confirm the limitations of the morphological method: numerous identification and quantification biases affecting ecological conclusions regarding predator impact. Our results also demonstrate the superior performance of molecular methods in both prey detection and identification. Although the optimisation phase of molecular protocols can be rather time-consuming initially, these methods once optimised are time-efficient and relatively inexpensive, particularly when large sample sizes of different types are involved (Appendix K). Molecular methods can be applied simultaneously to numerous (i) target prey taxa, especially endangered and/or rare species, (ii) diet samples, (iii) predator types and (iv) ecosystem contexts. Their use should become more widespread, since the skills and equipment required for PCR are widely available and can be easily shared among research projects. Predator diet analysis can also be combined with DNA genotyping for individual identification and sexing, allowing complete analysis of a predator population and how they use the habitat and resources. Finally, the technique allows samples to be collected non-invasively (i.e. scats), which is particularly useful when studying rare or elusive predators (Waits and Paetkau, 2005). Because relevant management prioritisation should be supported by robust qualitative and quantitative data, we recommend the following: (i) calibrate and validate molecular data with morphological data before running molecular diet analyses and (ii) combine at least two morphological and DNA-based methods to enhance prey detection, identification and quantification. More accurate diet data, coupled with prey and predator population estimations, will strengthen assessment of predator impacts on prey populations.

Data accessibility

DNA sequences: GenBank accessions: KT003540–KT003574.

The list of bird species found on Niau, the references, data and results of the review, full details on the PPs used in the molecular analysis and the results of in vitro and in silico tests, the list of GenBank sequences used in our phylogenetic reconstruction, the results of the morphological and molecular bird detections and identifications, and the comparative cost estimates for the two methods were uploaded as online supplemental material (Appendix A–K).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi: http://dx.doi.org/10.1016/j.biocon.2015.11.018. These data include the Google maps of the most important areas described in this article.

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