**Title:** Quantifying wildlife conflicts with metabarcoding and traditional dietary analyses

**Running head:** Quantifying wildlife conflicts

## Abstract

Wildlife conflicts require robust quantitative data on incidence and impacts, particularly among species of conservation and cultural concern. We present a multi-assay framework to quantify predation across systems and wildlife conflict scenarios, applied in southeastern Australian scenario where complex management implications and calls for predator culling are growing despite a paucity of predation data. We apply two ecological surveillance techniques to predator diets – traditional morphometric (hard-part) and DNA metabarcoding (genetic) analyses – to provide managers with estimated predation incidence, number of species impacted and prey relative importance to the predator. We explore haplotype diversity of prey DNA obtained for a species of conservation concern as a preliminary estimate of individuals consumed. We estimate the incidence of predation on seabirds by recovering and protected long-nosed fur seals (*Arctocephalus forsteri*) ranges from 9–29% of samples and included up to 6 prey species. The most common seabird prey – the culturally valued little penguin (*Eudyptula minor*) occurred in 6–25% of samples. This is higher than previously reported from traditional morphological assays alone. DNA metabarcoding proved more sensitive in identifying additional seabird taxa and provided relative quantitative information where multiple prey species occur within a sample. Polymorphism analysis of consumed little penguin DNA identified five distinct mitochondrial haplotypes – representing a minimum of 16 individual penguins consumed across 10 fur seal scat samples. We recommend rapid uptake and development of cost-effective genetic techniques and broader spatiotemporal sampling of fur seal diets to further quantify predation incidences and hotspots of concern for wildlife conflict management.

## Introduction

New wildlife conservation and management scenarios are emerging and increasing during the Anthropocene as some species experience population increases through successful conservation efforts, while others continue to decline (Roman et al., 2015; Marshall et al., 2016; Cammen et al., 2019). Complex management scenarios arise when a species recovery results in negative interactions with other species of value, whether that reflects a trophic role in the ecosystem, conservation status, community connection or economic opportunity (Marshall et al., 2016) – for example between killer whales, sea otters and salmon (Estes et al., 1998; Williams et al., 2011); New Zealand sea lions and yellow-eyed penguins (Lalas et al., 2007); wolves and caribou (Hervieux et al., 2014). The interactions are natural but present a need for accurate information on natural predation levels and impacts to prey (Hervieux et al., 2014) to avoid unjustified persecution of the predator (Granquist et al., 2018), and for effective management of all species concerned (Marshall et al., 2016).

A key goal of investigating predator prey interactions involves determining prey inter- and intra-specific diversity, dietary proportions, and abundances or biomass consumed by the predator (reviewed by Pompanon et al., 2012). Developments in eDNA extraction and metabarcoding techniques are demonstrating reliability for this level of environmental monitoring (Thomsen & Willerslev 2015) by: (i) identifying prey at high taxonomic resolution and when missed by other methods (Bowen & Iverson, 2013; Stat et al. 2019); (ii) estimating dietary proportions, reconstructing biomass and abundances of prey consumed through relative genetic importance (Deagle et al., 2019); (iii) identifying species’ intraspecific genetic diversity within environmental samples for wildlife forensics and population estimation (Sigsgaard et al., 2016; Seersholm et al., 2018; Tsuji et al., 2020).

One wildlife conflict in southeastern Australia involves the recovery of long-nosed fur seals (*Arctocephalus forsteri*) and their potential to threaten populations of little penguin (*Eudyptula minor*). The fur seals were decimated by fur trade through the 1800’s and culling into the late 1900’s due to perceived competition for resources with fishermen (Shaughnessy et al., 1999). Long-nosed fur seals are the only mainland Australian seal species with increasing population trends, reported at 97,200 in the state of South Australia (2013–14 census; Shaughnessy et al., 2015) where an estimated 83% of their recorded pup production occurs. While the original population size is unknown, harvesting records suggest that the current population represents a small fraction of that prior to European colonisation and exploitation (Ling, 2014).

Little penguins are a popular tourist attraction and valued species to communities across southern Australia (Tisdell & Wilson, 2012), and estimated at 470,000 individuals (BirdLife International, 2021). Yet, 60% of sites have unknown population trends, 29% of colonies are declining, most persist on offshore islands in southern Australia and are difficult to census (BirdLife International, 2021). Major contributors to decline include: (i) changes in land-use and predators introduced by European settlers (Dann, 1991; Kirkwood et al., 2014), (ii) susceptibility to hyperthermia during more frequent terrestrial heat waves (Lauren Tworkowski, La Trobe University, unpublished data), and (iii) large-scale changes to food webs caused by climate change and competition with fisheries (Ropert-Coudert et al., 2019). Little penguins and other seabirds were identified in juvenile, sub-adult, and adult male long-nosed fur seals diets, at two locations in southern Australia and at relatively low frequencies (Page et al. 2005; Hardy et al. 2017; Goldsworthy et al. 2019). However, the number of penguins consumed, and the impact are unknown, particularly for ‘unquantifiable remains’, such as feathers and when prey are not consumed whole. Page et al. (2005) proposed a single scat containing feathers was equivalent to a single bird consumed; however, mass-balanced trophodynamic modelling in the Great Australian Bight marine ecosystem suggested that this likely overestimated predation (Goldsworthy et al. 2013).

Both species are federally protected and garner significant cultural and conservation value (*Environment Protection and Biodiversity Conservation Act*, 1975 & 1999), albeit listed as ‘Least Concern’ by the IUCN Redlist (IUCN 2020). The recovery of Australian seal species continues to conflict with some communities and fisheries (Shaughnessy et al., 2003; Goldsworthy & Page, 2007; Cummings et al., 2019) due to their potential predation impacts to prey species. While it is realistic to expect high levels of predation to affect prey population size or behaviour (Visser et al., 2008), persistent campaigning to cull the long-nosed fur seal population in South Australia are growing despite an absence of quantitative information (Goldsworthy et al. 2019).

To assess predation incidence and potential impact on little penguin and seabirds across southeastern Australia, we apply two surveillance techniques – morphometric (hard-part) and DNA metabarcoding (genetic) assays – to long-nosed fur seal scats. Due to known biological and methodological differences in dietary information (Casper et al., 2007; Tollit et al., 2009), and differences in the quantities of DNA obtained from hard-parts compared to soft tissues (McDonald & Griffith, 2011), we consider these techniques complementary. Specifically, (i) we compare overall seabird and little penguin detection rates across groups of samples, rather than sample-by-sample; (ii) we investigate the diversity and relative importance of seabirds consumed by long-nosed fur seals; and (iii) we explore a minimum estimate of penguin abundance consumed by analyzing mitochondrial haplotype diversity among little penguin DNA obtained.

## Methods

### Sample collection

Individual predator scat samples (n = 99) were collected across multiple time points from four long-nosed fur seal breeding colonies in Bass Strait and NSW, southeastern Australia (Fig. 1 & 2). Pup abundances are illustrated as a conventional proxy for relative seal population (Fig. 1; Appendix S1.1). Most samples were collected from the two larger colonies, Barunguba and Cape Bridgewater, in spring (September) 2016 and summer (January) 2017, with additional samples included from spring 2015 and summer 2016 at Cape Bridgewater. Samples from Gabo Island were collected from summer 2017. One sample was opportunistically collected from Deen Maar Island and included in assays. Sample sizes resulted from balancing adequate replication per site with availability of fresh samples.

Whole and fresh (< 48 h old) scats were sampled to minimise bias from differential DNA degradation or partial loss of material. Whole scats were thoroughly mixed with individual disposable spatulas at point of collection and a 2 mL subsample was taken from each field-homogenized scat for DNA-based analyses of prey remains. The remaining whole scats were collected for analyses of morphological prey remains, using individual, zip-lock bags. Samples were stored within hours of collection between -10˚ and -20˚C in portable freezers (WAECO) for up to 7 d in the field and transferred to -20˚C freezer facilities.

### Identification of seabird morphological and genetic remains

All prey items were identified from hard-parts using methods described by Page et al. (2005). Birds were identified using feathers and other remains such as feet, flippers, and heads (Fig. 2, Appendix S1.2).

Prey DNA extractions used 250 mg of faecal subsamples and MoBio PowerSoil® DNA Isolation Kits ([www.mobio.com](http://www.mobio.com)) with modifications to the manufacturer’s instructions made in response to extraction optimisation (Appendix S1.3). DNA was eluted in 10 mM Tris buffer, MoBio PowerSoil® C6 solution, ([www.mobio.com](http://www.mobio.com)) and stored at -20˚C. Nuclear DNA for positive controls was extracted from muscle tissue (25 mg) of a domestic chicken (*Gallus gallus domesticus*) and a little penguin using with Bioline Isolate II Genomic DNA Kits ([www.bioline.com/us/](https://www.bioline.com/us/)) as per manufacturer instructions.

In total, 99 faecal DNA sample extracts at two DNA concentrations (neat and 1:10 dilutions), as well as extraction blanks (n = 5), PCR blanks (n = 4), and positive controls (n = 2) were screened in duplicates by diagnostic endpoint PCR (dPCR) using the Bird12sa/h assay (Cooper, 1994) (Table S1 & S2, Appendix S1). Duplicate dPCR products were run on 1.5% agarose gels to determine the presence/absence of amplified target bird DNA in each duplicate. A total of 32 samples showed target amplicons in both or one duplicate, but not in the extraction and PCR controls. We sequenced each of the 32 samples that tested positive for birds, and two extraction blanks and one positive control (n = 35 samples for sequencing). A single-step fusion tagging PCR procedure was used to attach and assign unique MID (Multiplex IDentifier) tag combinations, next generation sequencing (NGS) adaptors and the Bird12sa/h assay. The sequencing workflow – single-step fusion PCR (Appendix S1), library build, sequencing (150 bp paired-end Illumina Miseq: v2 Nano 150 bp) and demultiplexing – was performed by Ramaciotti Centre for Genomics, University of New South Wales.

Bioinformatics and sequence quality filtering procedures are described in reproducible detail in Appendix S1.3. We used Geneious R8.1.5 (Kearse et al., 2012) to merge the paired-end sequences (2x ~150 bp fragments, with overlap of 70 bp) and retain only those with exact flanking sequences – MID tags, primers, and adapters. Primers, adapters, and tags were removed to leave the complete target sequences in each sample. These were quality filtered and clustered into molecular operational taxonomic units (OTUs) using *UPARSE* algorithm and performed in *USEARCH* (Edgar, 2010; Edgar & Flyvbjerg, 2015). Low abundance sequences (below a threshold of 1% of the total abundance of all unique sequences) were removed to reduce the occurrence of sequencing error and chimeras, and sequences were then clustered using a 97% similarity criterion (similar to Berry et al., 2017). Thus 7370 unique seabird DNA sequences, representing a total of 64,700 disaggregated bird sequences, were parsed to standard sequence filtering and OTU clustering pipeline (with cluster size threshold value of 73), resulting in 47,478 filtered sequences across 99 samples, and these were clustered to 5 OTUs.

Consensus sequences for each OTU were queried against the National Center for Biotechnology Information’s (NCBI) GenBank nucleotide database using the algorithm BLASTn (Basic Local Alignment Search Tool) (Benson et al., 2005). The resulting ‘blasted’ sequences were assigned to taxa, following criteria and taxonomic reference databases outlined in Hardy et al. (2017) and Appendix S1.3 (Table S3). These criteria maximised confidence in making a taxonomic identification and minimised risk of false positives.

### Haplotype polymorphism analysis

We sought to identify a minimum number of individual little penguin by exploring mtDNA haplotypes from 12S rRNA sequences obtained. While dependent on sequence fidelity, such approaches have been used to explore intraspecific diversity (similar to Seersholm et al., 2018). We imported the quality-filtered file containing 47,478 seabird DNA sequences, produced just prior to OTU clustering, we disaggregated and matched these sequences in relation to sample identifier and formed these into clusters of unique sequences in Geneious. From the six samples that contained abundant penguin DNA (Table S4), we selected the nine most abundant unique sequences. Each of these represents a sequence abundance of greater than 7.5% of the total sequence abundance of the sample (Table S5). This process was used to exclude beyond reasonable doubt, any further sequences that could be attributed to sequencing error.

We produced a minimum spanning haplotype network using the software *PopART* (Leigh & Bryant, 2015) from an alignment of these nine penguin sequences to visualise relationships between haplotypes consumed, and between samples from the different locations sampled. All samples containing little penguin DNA (n = 10) were then searched for the presence of identified haplotypes. We estimated the number of penguins likely consumed based on the number of haplotypes within each sample. Additionally, as samples were collected across multiple days from each location and sampling time, we treated each sample as from distinct predators or predation events.

### Statistical analyses

To account for different sampling times and locations, samples were assigned one of seven unique grouping factors that combined location and time (e.g., Barunguba, January 2017). The single scat sample collected from Deen Maar Island was not included in statistical tests, but results were reported for future comparisons. The overall detection rates of seabirds and specifically little penguins using different dietary analysis techniques (hard-part vs. DNA; Table S6, Appendix S2) were examined using two generalised linear models (GLMs), constructed in the *stats* package in R version 4.0.3 (R Core Team, 2020). A binomial error distribution for presence-absence data was used and additive term included to account for long-nosed fur seal group (location and time). Model fit was assessed using deviance explained and variable significance (p < 0.05).

## Results

Overall, the detection rates of seabirds were statistically similar for each method (Fig. 3, Table S3). However, DNA metabarcoding offered additional information: (i) absolute and relative abundance information for amounts of DNA recovered (Fig. S1 & S2, Table S4), (ii) improved sensitivity in detecting multiple prey taxa within a single scat (Fig. 4), and (iii) exploration of little penguin 12S rRNA genetic diversity enabled the estimation a minimum number of predated penguins to the be estimated (Fig. 5, Table S5).

### Comparing diagnostic hard-part and genetic assays

Seabirds were detected in 29.3% (n = 29) of samples using diagnostic hard-parts, and 21.2% (n = 21) of samples using DNA metabarcoding (Fig. 3a). Most of these detections were little penguins, detected in 25.3% (n = 25) of samples with hard-parts and 10.1% (n = 10) of samples with DNA (Fig. 3b). Of these positive detections obtained after quality control and filtering, the majority of DNA sequences for all seabird taxa were identified in 9 out of 21 samples, and for little penguins in 6 out of 10 samples, relative to the total DNA obtained for each taxon (Figs 3 & S1, Table S4), and providing a lower conservative estimate for predation incidence. The same seabird taxa were detected by simultaneously in half the positive samples (n = 10), and half of these (n = 5) contained both little penguin hard-parts and DNA (Fig. 3). Five samples contained DNA and hard-parts that did not belong to the same seabird taxon (Fig. 4), and the remaining positive samples from each method represent a detection made by one method alone (n = 6 for DNA, and n = 13 for hard-parts). The combined proportion of samples that were positive for seabirds was 40% (n = 40), and 30% for little penguins (n = 30).

Mean detection rates were statistically similar for both methods for seabirds and penguins (Table S6). There was greater variability in detection rates across locations using hard-parts compared to DNA (Fig. S3). Minor, albeit statistically significant, differences were observed across sampling groups for seabird detection rates (Fig. S3a, Table S3), but not for penguins (Fig. S3b) (GLM binomial seabird detection ~ location: p-value = 0.017; Table S3). Detection variability was high across sampling locations, times and methods used (Fig. S3a). Little penguins were detected by hard-parts from Gabo Island samples, however only a few sequences of little penguin DNA were detected at Gabo Island and Deen Maar Island, and these sequences did not pass DNA quality filtering.

### Seabird diversity in long-nosed fur seal diets

DNA-based metabarcoding was more sensitive in detecting taxonomic mixtures in scat samples compared to hard-part analysis (Fig. 4), with 2 distinct seabird taxa detected in 5 samples and a single seabird taxon in the remaining 16 samples. No samples contained more than one identified bird taxon using diagnostic hard-parts (Fig. 4a). Little penguins were the main seabird prey species detected using both analyses (Fig. 4), and this was reflected in all data from DNA – frequency of occurrence (Fig. 4), total abundance of sequences (Fig. S1), and relative sequence abundance (Fig. S2).

Morphological analysis revealed two additional taxa: a shearwater family group (Procellaridae spp.) (n = 2 samples), and Australasian gannet (*Morus serrator*) (n = 1) (Fig. 4). DNA metabarcoding detected abundant DNA from two distinct shearwater taxa, also at family-level, taxa in 5% (n = 5) and 9.1% (n = 9) of samples, respectively (Tables S3 & S4, Appendix S2). Black-browed albatross (*Thalassarche melanophris*) and greater crested tern (*Sterna bergii*) occurred in one sample apiece (Fig. 4, Tables S3 & S4). Parallel use of both DNA metabarcoding and hard-part analysis revealed a greater diversity of taxa than either method alone.

### Towards quantifying little penguin consumption

From the six scats containing abundant penguin DNA, a total of five mtDNA haplotypes were identified in samples from Cape Bridgewater (haplotypes 1–2 and 4–5) and Barunguba (haplotypes 1–3) (Fig. 5a). Only haplotype 3 was unique to Barunguba, the remaining five haplotypes were detected at both sites (Figs. 5b). Haplotype 1 was identified three different single sources of DNA from penguins from southeastern Australia (see reference material in Appendix S1.4). Across all ten penguin positive scat samples, six contained a single haplotype, whilst the remaining four contained between 2–4 haplotypes (Fig. 5b). Taking two distinct genetic haplotypes present within a sample to represent at least two distinct individual birds consumed, we estimate at least 16 individual penguins were consumed across the 99 scat samples, from two sampling locations and multiple seasons.

## Discussion

Validating and applying modern surveillance tools, such as metabarcoding, to complex conservation scenarios can better inform decision making, also providing transferable . We leveraged metabarcoding alongside traditional diet analysis methods, to investigate a wildlife conflict in southeastern Australia. We provide an updated and improved predation prevalence range for seabirds overall (9–29%) and little penguins (6–25%) in long-nosed fur seal diet. We confirm that little penguins remain the most frequently consumed seabird by long-nosed fur seals in comparison to other avian taxa. Here, DNA metabarcoding also offered key advantages over morphological analysis – quantitative information on: (i) absolute and relative abundances of taxa recovered using DNA, (ii) detection of multiple prey taxa within a single scat sample, and (iii) estimates of at least 16 penguins by exploring the spatial and temporal distribution of haplotypes.

Previous studies using either assay techniques have identified little penguin remains at relatively low frequencies in relation to seals’ total diets (5.9% in Page et al. 2005, <2% in Hardy et al. 2017, ~13% in Goldsworthy et al. 2019). The lower range of estimates observed here (9% of samples for seabirds, 6% for penguins) and based on samples containing large quantities of prey DNA corroborate these previously reported predation rates for the region. However, the upper range of estimates observed in this study (10% and 25% of samples, respectively for DNA and hard-parts) and recent crashes in little penguin colonies (R. R. McIntosh, pers. comm.) signal a need for broader and increased monitoring of predation mortality. Predatory behaviours could be transmitted to other predator populations, particularly in response to food web disruption under ocean warming and changes in prey availability and this could have cascading effects on penguin populations. Analysis of the predator’s total diet is also warranted to gauge the relative importance of different prey items, besides seabirds.

While both metabarcoding and morphological methods provided statistically similar results, we emphasize that these represent complementary but quasi-independent assays of predation. Different detection rates reported between these methods in this study are also common across a broad range of predator and prey taxa (Tollit et al., 2009; Zarzoso-Lacoste et al., 2013). Many methodological and biological factors differentially affect the recovery of diagnostic genetic and morphological materials. In another pinniped species, prey soft tissues had a gut passage rate of 48 hours and up to 7 days for hard-parts (Tollit et al., 2009). Soft tissues may contain greater DNA quantities and concentrations, particularly mitochondrial (Mumma et al., 2016; Granquist et al., 2018), and could be preferentially amplified compared to DNA available from hard-parts, (i.e., feather, fur, or bone). DNA from these tissues is of poor quality and requires a different process for extraction (McDonald & Griffith, 2011; Rothe & Nagy, 2016). Typically, studies report higher detection rates from genetic assays compared to morphological analyses (Egeter et al., 2015; Mumma et al., 2016), but the lower detection rates here are consistent with other pinniped studies (Tollit et al., 2009) and with over-representation of feathers across fur seal scats (Goldsworthy et al., 2019), explaining the higher detection rate from these hard-parts.

Distinct variation in penguin metabarcodes obtained, beyond that attributed to sequencing error, led us to explore how many individuals might be within the data (similar to Seersholm et al., 2018 and Tsuji et al., 2020). While not the ideal locus to explore intra-species diversity, we nonetheless identified 5 haplotypes consumed, across time and space this suggested at least 16 penguins were consumed. This is very likely an underestimate of predation, firstly because we used the highly conserved gene for 12S ribosomal RNA (Banks et al., 2002), selected for proven reliability in detecting seabirds (Hardy et al. 2017). Targeting alternative markers (variable barcodes or microsatellites) could reveal greater genetic diversity, however we did not succeed with a COI barcode (Appendix S1.2). To avoid interpreting error (PCR or sequencing) as separate individuals we followed stringent sequence quality and abundance filtering to exclude the possibility of false positives beyond sequencing error, and we likely excluded several low DNA-abundance, true positives (Deagle et al., 2013).

Most scats with penguin DNA contained a single haplotype, however four scats contained up to 4 distinct genetic haplotypes, suggesting that long-nosed fur seals can consume multiple penguins in a single foraging trip or within 48h of sampling. This result provides a significantly more reliable method of estimating predation incidence and impact than previous assumptions that each scat containing feathers corresponded to a single bird (Page et al., 2005, Mumma et al. 2016). If little penguin predation becomes an important individual foraging strategy even for some long-nosed fur seals, this could have serious negative impacts for isolated penguin populations. DNA-based methods are transferable across systems and offer vast potential for technological and methodological improvements (Tsuji et al., 2020) over traditional morphological diet assays.

Finally, our results demonstrate a need for research and development of techniques at the nexus of population genetics and environmental sampling – including screening for predator genetic diversity to identify individuals in a population contributing to predation of a sensitive or valuable species (Wegge et al., 2012), developing species-specific probes using older and cheaper technology (Fox et al., 2012), cross-validation of eDNA data with quantitative PCR (Murray et al. 2011), or development of penguin-specific DNA-to-tissue-based correction factors could provide consumed biomass information (Thomas et al., 2014). Additionally, predator impacts need to be considered and managed within up-to-date cumulative impact assessments for threats. We have delivered an important step towards this for little penguins in south-eastern Australia. Similar to other wildlife conflict situations, endemic predation is natural and often habitat degradation, environmental change and invasive species are more significant sources of impact to susceptible species (Hervieux et al., 2014; Marshall et al., 2016; Ropert-Coudert et al., 2019).

## Supporting Information

PINP\_Bird\_supplement.docx.

## Data Availability

Datasets and code used for figures and analyses will be placed in public online repository upon acceptance of this manuscript for publication.

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## Figure Legends

**Figure 1.** a) Long-nosed fur seal scat collection sites (n = total number of samples), including pup abundances for sampling locations (McIntosh et al., 2014) as an index of seal population relative importance of sites. Sampled sites were: Cape Bridgewater (38.3013° S, 141.4062° E) and nearby Deen Maar Island (formerly Lady Julia Percy Island, 38.4161° S, 142.0038° E) from western Bass Strait, Victoria; Gabo Island in eastern Bass Strait, Victoria (37.5649° S, 149.9133° E); and Barunguba (formerly known as Montague Island 36.2510° S, 150.2270° E) at the northeastern breeding range in New South Wales (NSW). Species distributions shown for b) long-nosed fur seals and c) little penguins (data from ALA, 2019).

**Figure 2.** a) A long-nosed fur seal, *Arctocephalus forsteri*, from Barunguba, NSW; b) the little penguin, *Eudyptula minor*, often burrowing near fur seal colonies; c) and d) seabird remains are conspicuous at long-nosed fur seal haul-outs and colonies, among scats and regurgitates.

**Figure 3.** Detections of a) seabird and b) little penguin diagnostic hard-parts (‘hp’) and DNA (‘dna’), as a percentage of all long-nosed fur seal samples (n = 99). We report all genetic sequences obtained from standard sequence quality control and filtering ‘DNA (all)’, and for samples that contained large sequence quantities of sequences ‘DNA (abundant)’ (> 90% of filtered sequences); and the number of samples that contained both the morphological and genetic remains of the same seabird (‘same taxon’).

**Figure 4.** The diversity of seabirds identifiedin long-nosed fur seal samples: a) using hard-part analyses (n = 29) and b) using DNA-based methods (n = 21). GI = Gabo Island.

**Figure 5.** Little penguin genetic diversity (for ~230 bp 12S rRNA gene) a) presented as a minimum spanning network of five distinct haplotypes, and b) number of haplotypes contained within each penguin-positive sample, including haplotype sequence abundances within samples.

## Figures

**Map

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**Figure 1.**

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**Figure 2.**



**Figure 3.**



**Figure 4.**



**Figure 5.**