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Recovering whole mitogenome sequences from Eurasian otter (*Lutra lutra*) spraint samples: a metagenomic approach

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Non-invasive genetic monitoring of endangered species and populations is a crucial component of conservation biology, especially for elusive species such as the Eurasian otter (*Lutra lutra*). Leveraging techniques from the fields of metagenomics and ancient DNA, here we present a laboratory and bioinformatic pipeline for recovering whole mitochondrial genomes (mitogenomes) through shotgun metagenomic sequencing of Eurasian otter spraint (faecal) samples. Using this approach, we successfully assembled whole mitogenomes from 20 out of 27 analysed spraint samples, collected along the river Usk in south Wales (UK). The three recovered haplotypes in these samples were identical to sequences previously documented in high-quality DNA from muscle tissue samples of otters from Wales, validating our approach. We recovered higher mitochondrial depth in spraint samples collected in spring than winter and in samples collected within less than 24 hours. However, our limited sample size prevented us from determining factors which influenced mitochondrial depth within a multivariate

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framework. In addition to assembling otter mitogenomes and characterizing fungal and bacterial diversity, we also obtained mitogenomes of known prey species of otters, highlighting the value of metagenomic sequencing from non-invasively collected faecal samples.

1. Introduction

Genetic monitoring can provide critical information for the conservation management of endangered and protected species, yielding insights into various demographic factors such as population size [1], sex identification [2], levels of inbreeding [3], occurrence and severity of past bottlenecks [4,5], as well as allowing the tracking of individuals and genetic clusters within populations [6]. Furthermore, conservation genetics can help to identify evolutionarily distinct and unique species, subspecies or populations of a species [7], as well as exploring health indicators such as dietary status, parasitic load [8] and gut microbiome diversity [9].

Non-invasive genetic sampling enables researchers to obtain genetic information from a population without disturbing or harming individuals through handling and trapping [10]. Furthermore, non-invasive approaches are particularly useful when studying elusive species and/or those that reside in habitats that are difficult to access. For example, the presence of elusive predatory species, including snow leopards (*Panthera uncia*), has been confirmed in remote locations using environmental DNA (eDNA) sampled from footprints [11]. Another elusive mammal studied extensively using non-invasive genetic methods for over two decades is the Eurasian otter (*Lutra lutra*) [4,6,12–17]. While hair samples can be used to genetically monitor this species [14], most studies use spraint (faeces) for analysis, because they are easy to sample. This is because otters regularly deposit spraint onto conspicuous features, such as boulders and tree stumps next to waterbodies [18]. DNA analysis from spraint is therefore a commonly used approach in non-invasive studies of Eurasian otter [4,19].

Extracting information needed from non-invasive genetic samples first requires choosing appropriate genetic and/or genomic markers for genotyping (e.g. microsatellites, barcodes, nuclear and plastid genomes [20]), depending on the research question in hand. Various types of genetic and genomic markers can provide different information. For example, the entire mitochondrial genome (mitogenome) can be used to obtain demographic [21] and phylogeographic insights [22,23]. Since the first mitogenome of the Eurasian otter was published in 2009 [24], various studies have used mitogenome sequences to identify genetically distinct subspecies and populations, including those that are conflicting with current taxonomic classification [23,25]. Harnessing the increased taxonomic and population genetic resolution offered by mitogenomes compared with short sequence alignments [23] is of particular importance for identifying populations of otter that are of conservation concern.

Whole mitogenomes for Eurasian otters have previously been obtained using polymerase chain reaction (PCR)-based approaches, based on DNA extracted from tissue or blood [24–27]. Also, a multiplex PCR method was recently applied to characterizing the whole mitogenome of a spraint sample [27]. Furthermore, Kim and Jo [28] and du Plessis *et al.* [29] applied low-coverage whole-genome shotgun sequencing to reconstruct the mitogenome of Eurasian otters from muscle and from two historical specimens, respectively.

Using DNA extracted from tissue relies either on invasive sampling or on acquiring samples from dead individuals. While the latter can yield high-quality DNA, ad hoc collection of deceased specimens can limit sample sizes, and corpses are not always found in locations of research interest. Non-invasive genetic sampling using more readily available and abundant spraint samples therefore provides a promising alternative. However, DNA in faecal samples is known to degrade over time [30], driven by exposure to fluctuating temperatures and humidity [16]. Because epithelial cells of the host found in faeces contain high quantities of mitochondria [31], many copies of the mitogenome should be present, relative to the less abundant copies of the nuclear genome. In degraded and low-quality samples, this contrast in copy number between mitochondrial and nuclear loci can be the difference between being able to accurately characterize variation and not, as evidenced by many ancient DNA studies not having sufficient sequencing coverage to characterize nuclear variation, while obtaining (near-) complete mitochondrial genomes from the same specimens [32].

Here we investigated whether reconstruction of full mitogenome sequences from Eurasian otter spraint samples is feasible, using a short-read shotgun metagenomic sequencing approach applied to

samples collected from a river catchment in south Wales (UK). Furthermore, we aimed to investigate the impact of sequencing effort and the number of microbial reads on mitochondrial depth. Finally, we contextualized the obtained spraint mitogenome data by comparative phylogenetic analysis, including previously published Eurasian otter mitogenomes from the United Kingdom.

2. Material and methods

2.1. Sample collection

Spraint samples were collected from three sites along the river Usk (Afon Wysg), south Wales, UK (figure 1), between the Usk Reservoir and the village of Llanellen. Sampling was conducted for five consecutive days in winter (27 February 2023 to 06 March 2023) and spring (26 March 2023 to 04 April 2023), allowing us to categorize spraint as 'fresh' (deposited since the previous day, i.e. <24 hours old), versus 'older/unknown age' spraint. See electronic supplementary material, section 1, for further details.

2.2. DNA extractions and sequencing

QIAamp Fast Stool DNA Mini Kits (QIAGEN, Hilden, Germany) were used to extract total DNA from spraint samples, largely following the manufacturer's protocol, 'isolation of DNA from stool for pathogen detection'. This protocol involved a cell lysis stage at 95°C, to improve lysis and thus DNA recovery from microbial cells. An additional cell disruption step was also included, whereby 300 mg of 0.1 mm glass beads and 100 mg of 0.5 mm glass beads (Thistle Scientific Ltd, UK) were added to 0.18–0.2 g of spraint sample, alongside the Qiagen InhibitEX Buffer at the start of the lysis phase. Then, a bead beater (FastPrep-24™ Classic Bead Beating Grinder and Lysis System, MP Biomedicals™, California) was used to homogenize the samples for 3 min at 5 m s^{-1}. See electronic supplementary material, section 2, for a comprehensive description of deviations from the manufacturer's protocol. A negative (no sample) control per extraction batch was included to monitor for contamination.

DNA concentrations (ng μl^{-1}) of samples and negative controls were determined via fluorometric quantification, using Qubit 1X dsDNA HS Assay Kits and a Qubit 4 Fluorometer (Thermo Fisher Scientific, Germany), following manufacturer's specifications. All negative control concentrations were below levels of detection, thus deemed free of contamination and not sequenced. To achieve the minimum DNA concentration stipulated by Novogene (10 ng μl^{-1} in minimum volume of 20 µl), some samples were re-extracted, pooled and concentrated using an Eppendorf 5301 Vacufuge Concentrator System set to 60°C. All sample extractions, including three that did not meet this minimum concentration (O_0404_LL, O_0328_BR_A and O_0326_BR_B), were sent for library preparation and shotgun metagenomic sequencing (150 bp paired end sequencing) on an Illumina NovaSeq 6000 at Novogene (Cambridge, UK). A library pool was made for winter samples, and another for spring samples, which were sequenced separately.

2.3. Bioinformatic analyses

2.3.1. Bacterial and fungal identification

To assess the impact of microbial variation in spraint on mitochondrial sequencing depth, bacterial and fungal reads were taxonomically identified and normalized to parts per million (ppm) (figure 2). Raw sequence quality was visualized before and after trimming, using FastQC (v. 0.11.9) [34]. Trimmomatic (v. 0.39) [35] was used to remove adapter and low-quality sequences to minimize data loss and maximize quality, using the following parameters, as recommended on the software's GitHub page (https://github.com/usadellab/Trimmomatic). The removal of adapters used two seed mismatches, a palindrome clip threshold of 30, and a simple clip threshold of 10. Any bases with a quality score of less than three at both the start and ends of reads were also removed. A sliding window trim was carried out, using a window size of four bases and a minimum average quality of 25 per window. Following trimming, reads more than 36 base pairs in length were kept, and unpaired reads were discarded.

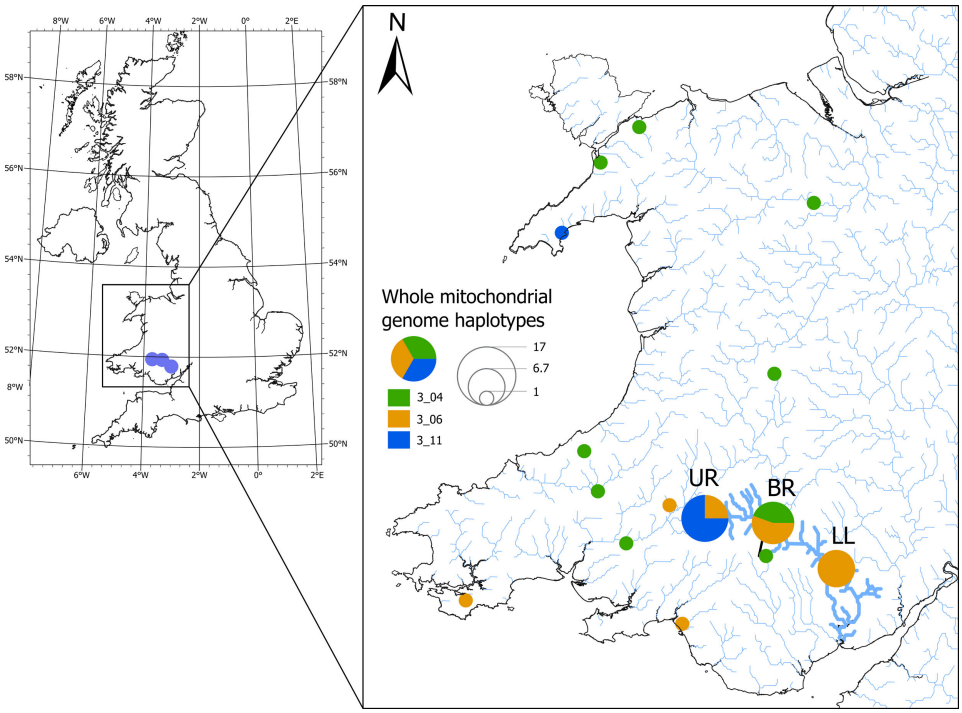

Figure 1. Sample locations of spraints and whole mitogenome haplotypes of Eurasian otters (*Lutra lutra*) in Britain (left) and Wales (right). Three spraint sampling locations along the river Usk, south Wales, are indicated as pie charts, from west to east: Usk Reservoir (UR, $n = 11$), Brynich Lock (BR, $n = 9$) and Llanellen (LL, $n = 7$). Other rivers in Wales are shown as thin blue lines, and the river Usk is in bold. Single-colour dots represent locations of previously described whole mitogenome haplotypes from Wales (3_04, 3_06 and 3_11; colour-coded) [33].

Using the Nextflow management system (v. 21.20) [36] and Singularity (v. 3.8.7) containers [37], the 'main_AMR++.nf' workflow and '—kraken' pipeline of AMR++ (v. 3.0) [38] were executed, to taxonomically identify fungi and bacteria. Taxonomic identification of trimmed reads was carried out using Kraken2 (v. 2.1.1) [39] with the PlusPFP Kraken2 database [40], which contained the Standard, RefSeq, Protozoa, Fungi and Plant Kraken databases. Relative abundances of taxa were visualized using Krona 2.8 [41].

2.3.2. Normalization

The number of reads per sample, and per bacterial and fungal phylum was calculated. The relative abundance of each taxon was calculated and normalized manually in Microsoft Excel using the ppm approach [42], where the number of read hits per taxon was divided by the per million paired reads per sample. This method was chosen to avoid bias from sequencing depth variation among samples [43,44].

$$\frac{\sum_{i=1}^{n} N_{i\ read\ hits}}{D/10^6}$$

N = number of read hits per taxon
D = total number of paired reads per sample.

2.3.3. Whole mitogenome assembly

To evaluate the presence, quantity and quality of otter DNA, a pipeline originally developed for analysis of ancient DNA was implemented to identify otter reads for mitogenome assembly. Raw reads were filtered, trimmed and mapped to the Eurasian otter reference genome, mLutLut1.2 [45], using the PALEOMIX v. 1.3.7 pipeline [46]. Adapter sequences, low quality and ambiguous bases, and short reads were filtered out using AdapterRemoval [47], and overlapping read pairs were merged. After merging, reads less than 25 bp long were trimmed, along with ambiguous bases and bases with a

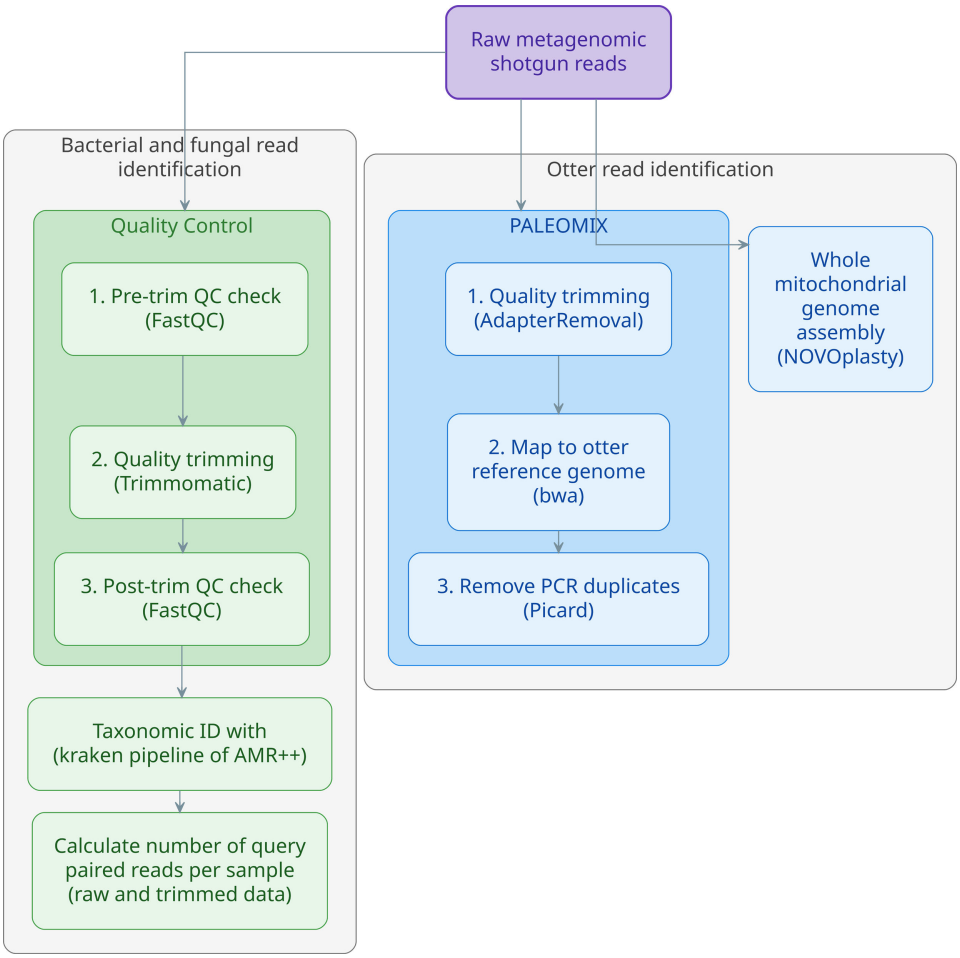

Figure 2. Flow chart of bioinformatic analyses conducted. Details and citations of software/packages are provided in the main text.

Phred score of less than 2 at the start or end of a read. A one-third mismatch rate was allowed when detecting adapter sequences (mm: 3, minlength: 25, collapse: yes, trimns: yes, trimqualities: yes). Reads were mapped against the reference genome using bwa backtrack v. 0.7.17 [48,49], with a minimum mapping quality of 30, and PCR duplicates were filtered using Picard v. 2.22.2 MarkDuplicates (https://broadinstitute.github.io/picard/). The Kraken2 database, used in bacterial and fungal identification described above, identified a significant proportion of human (*Homo sapiens*) reads in some samples; however, this is the only vertebrate included in this database. To further investigate this signal, the PALEOMIX pipeline was also conducted for the human reference genome (GRCh38.p14; accession GCF_000001405.40). Contamination from other vertebrates was not explored in this study, beyond BLAST analysis of all contigs assembled by NOVOPlasty (see below).

Whole mitogenome sequences were assembled using NOVOPlasty v. 4.3.1 [50] and subsequently submitted to BLAST v. 2.12.0 (see electronic supplementary material, section 3). The otter reference genome mitochondrial scaffold was used as a seed (GenBank: LR822067.1), and recommended settings were used (estimated sequence length 12 000–22 000 bp, k-mer size of 33, insert size calculated and quality scores not used). These analyses were repeated with the human reference genome mitochondrial scaffold (National Center for Biotechnology Information (NCBI): GCF_000001405.40) as the seed.

2.4. Factors influencing mitochondrial depth

Based on mapping the metagenomic reads for each sample against the otter mitochondrial scaffold LR822067.1, further investigations into which factors influenced depth were conducted using R v. 4.2.1 [51] within RStudio [52] and package tidyverse [53]. To minimize multiple testing, we assessed collinearity between the 43 microbial phyla, using the corrplot R package [54]. Both visualization (electronic supplementary material, section 4, figure S1) and a Pearson's correlation test showed that

total read counts of all bacterial and fungal phyla were collinear (r = 0.638, t = 4.15, d.f. = 25, $p \leq$ 0.01). Therefore, the total bacterial count data were used as a proxy to represent both bacterial and fungal counts in the following analyses. The association between mitochondrial depth and sequencing effort (total number of reads generated per sample), season, spraint age (less than 24 hours, greater than 24 hours or unknown), total bacterial read counts, total human read counts and their two-way interactions was explored using generalized linear models (GLMs). Model residuals were checked to evaluate model fit, and models were minimized using stepwise deletion, based on a change of Akaike information criterion (AIC) greater than 2, using the 'drop1' function.

2.5. Mitochondrial variation

We investigated whole mitogenome variation of Eurasian otters in Britain using published data (47 modern mitogenomes [23,33,55] and two historical sequences [29]), combined with newly generated mitogenome data from the present study (n = 20). For the latter, we only included those assembled to a circular sequence when using the Eurasian otter reference mitogenome as a seed. This resulted in a total of 69 whole mitogenomes included in subsequent phylogenetic analyses.

Sequences were aligned in Geneious Prime v. 2024.0.3 (https://www.geneious.com) with MAFFT v. 7.81, using the '—auto' flag [55,56]. In Geneious, the tandem repeat region within the control region was removed from position 15 937–16 225 (relative to the reference mitochondrial sequence, LR822067) [23], resulting in a final alignment length of 16 251 bp. PopArt v. 1.7 [57] was used to produce a 95% credibility limit Templeton, Crandall & Sing (TCS) network of all sequences [58], using the PopArt implementation of TCS in which ambiguous sites (International Union of Pure and Applied Chemistry (IUPAC) codes) and indels are treated as missing data. Mitochondrial diversity summary statistics (number of haplotypes, number of unique haplotypes, segregating sites, haplotype diversity and nucleotide diversity) were calculated using the R packages pegas [59] and ape [60], based on the entire sequence alignment.

3. Results

3.1. Samples

A total of 27 otter spraint samples were collected, of which 10 were less than 24 hours old, four greater than 24 hours old and 13 of unknown age. Samples of unknown age were not recorded the day before sample collection but appeared to be old deposits (e.g. grey, dry) and so were unlikely to be less than 24 hours old. Per extraction, DNA yield ranged from 56.6 to 1728.0 ng (mean: 649.0 ng, s.d. 468.3). Following the pooling of some repeated extractions and attempts to increase concentration by Vacufuge centrifugation, all samples except three had concentrations above the minimum requirements stipulated by Novogene UK (exceptions: samples O_0404_LL, 8.5 ng μl^{-1}; O_0328_BR_A, 4.4 ng μl^{-1}; and O_0326_BR_B, 5.9 ng μl^{-1}). The total number of read pairs and sample data sizes ranged from 1 091 102 reads and 0.17 Gb (sample O_0329_UR, unknown age) to 69 047 510 reads and 9.9 Gb (sample O_0404_LL, unknown age). For further metadata, please see electronic supplementary material, table S4. Across the 27 spraint samples, we detected 40 bacterial phyla, with the top three most abundant bacterial phyla being Proteobacteria (41.6%), Actinobacteria (31.5%) and Firmicutes (22.2%); the remaining 4.7% of phyla belonged to the other 37 bacterial phyla that were also identified. Three fungal phyla were also identified: Ascomycota (91.4%), Basidiomycota (7.9%) and Microsporidia (0.8%).

3.1.1. Otter DNA evaluation

The PALEOMIX analyses showed that the number of reads mapping to the Eurasian otter reference genome ranged from 1563 to 9 566 148. As such, the proportion of Eurasian otter reads (or endogenous DNA content) ranged from 0.01 to 14.5% of the total reads (see electronic supplementary material, table S4). PCR duplicates (resulting from library preparation/amplification) ranged from 16 to 44% of reads across samples (see electronic supplementary material, table S4), and after the removal of these duplicates, whole-genome (nuclear and mitochondrial) sequencing depth ranged from 0.00009 to 0.5×, mitogenome depth from 0.5 to 166.9×, and mean mitogenome depth was 44.4× (s.e.m. 8.3×).

Mitochondrial depth was particularly high (greater than 65×) in 7 of the 27 samples and was less than 5× for only four samples. The number of reads mapping to the human reference genome ranged from 1233 to 33 708, reflecting a range in proportion of human reads from 0.002 to 0.211% of total reads. The number of reads mapping to the Eurasian otter reference genome was greater than the number mapping to the human reference genome in all samples, except for O_0328_BR_A (23 638 reads mapped to otter and 33 708 to human) (see electronic supplementary material, table S4, for further details).

3.1.2. Whole mitogenome assembly

When using the Eurasian otter reference mitogenome as a seed, the 20 samples with the highest mitochondrial coverage (13.5–166.9×) were assembled to circular sequences by NOVOPlasty. Seven of these samples were less than 24 hours old, 12 were of unknown age and one was greater than 24 hours old. Contigs were generated for the next six highest coverage samples, and for only one sample (O_0329_UR; with unknown age and a mitochondrial depth of 0.5×), neither circular molecules nor scaffolds were obtained by NOVOplasty. Three of the six samples that generated contigs were fresh (less than 24 hours old), two were greater than 24 hours old, and one was of unknown age.

When using the human reference as a seed, 14 samples were assembled to a circular molecule that was identified with BLAST as Eurasian otter (electronic supplementary material, tables S4 and S5). Additionally, we obtained two circular mitogenomes of fish species (*Cottus perifretum* common bullhead from sample O_0228_LL_A and *Gasterosteus aculeatus* three-spined stickleback from sample O_0404_LL; electronic supplementary material, table S5). For the remaining samples, contigs of varying lengths were assembled, matching to Eurasian otter, common bullhead and common frog (*Rana temporaria*) (electronic supplementary material, table S4). The fresh sample O_0328_BR_A, for which the number of reads mapped to the human reference genome outnumbered those mapped to the Eurasian otter reference genome (33 708 human reads versus 23 638 otter reads), NOVOplasty assembled a circular otter mitogenome, regardless of whether the human or otter mitogenome seed was used (electronic supplementary material, table S5). The lowest depth sample (O_0329_UR) failed when run in NOVOplasty using both otter and human as seed.

3.2. Predictors of mitochondrial depth

Mean mitochondrial sequencing depth was lower in winter (11.9×, s.e.m.: 3.2) than in spring (58.1×, s.e.m.: 10.2). Also, depth was higher in spraint less than 24 hours old (41.6×, s.e.m.: 15.3) than in samples greater than 24 hours old (7.4×, s.e.m.: 3.1). Despite using various data transformations, error families and link functions, all tested GLMs violated assumptions. In particular, high leverage points were a consistent issue, and we considered the removal of such outlier data as inappropriate due to the small sample size of spraint samples analysed (see electronic supplementary material, section 4.2 for further information). As such, we cannot determine conclusively which factors underlie the variation in mitochondrial sequencing depth among samples.

3.3. Whole mitogenome variation in British otters

The alignment of 69 sequences of 16 251 bp comprised 20 unique haplotypes that were defined by 154 segregating sites. Among the 20 newly generated mitogenome sequences from the Usk river basin, we identified three haplotypes (12 segregating sites), all of which had previously been found in Eurasian otter samples from Wales, all belonging to mitochondrial Lineage 3 (figure 3; lineage terminology *sensu* du Plessis *et al*. [23,33]. Genetic diversity statistics (e.g. haplotype and nucleotide diversity) of the newly analysed Usk river basin spraint samples were similar to those previously reported from Wales (electronic supplementary material, section 5).

Combining the newly obtained data with previously published results showed similar geographic haplotype distributions (figure 1) known for two of the haplotypes (haplotype 3_06 being restricted to south Wales and haplotype 3_04 found throughout Wales), while haplotype 3_11 (previously only identified in one individual in north Wales) based on our results is also present in the Usk catchment.

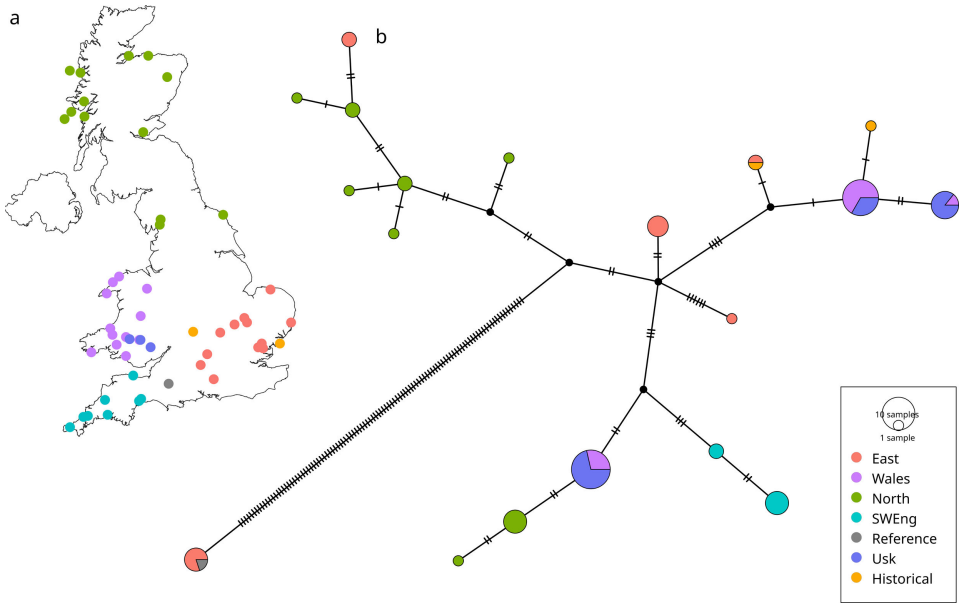

Figure 3. Distribution and phylogenetic relationships of Eurasian otter (*Lutra lutra*) mitogenome sequences in Britain. (a) Sampling locations within Britain, with newly generated mitogenomes ($n = 20$) from the river Usk shown in dark blue and previously published mitogenome data ($n = 49$) colour-coded by source population (east, Wales, north, southwest England) [23,33]. The reference mitogenome by Mead *et al.* [45] (grey) and historical mitogenomes [29] (yellow) are also shown. (b) TCS haplotype network of mitogenome sequences in otters from Britain, with circle size corresponding to haplotype frequency. Dashes on branches denote mutational steps.

4. Discussion

Here, we demonstrate that Eurasian otter whole mitogenome sequences can be reconstructed using shotgun metagenomic sequencing of DNA from field-collected spraint samples. To the best of our knowledge, this has not been shown previously. For elusive species such as the Eurasian otter, this mitogenomic approach provides a relatively simple tool for non-invasive genetic monitoring, yielding higher resolution among matrilines than shorter barcode sequences such as the control region or *Cytochrome B* [23,33]. All three mitogenome haplotypes identified from spraint samples in our study have previously been described in Welsh otters using tissue samples, validating this novel methodology.

4.1. Eurasian otter mitogenome reconstruction: a new approach

It has previously been established that Eurasian otter mitogenomes can be assembled using low-coverage shotgun sequencing data from tissue samples [28]. Here, we show that this can also be achieved using non-invasive spraint samples. While this is a novel application for the Eurasian otter, the construction of mitogenomes from metagenomic shotgun sequencing derived from faecal samples has been successful in other mammal species [61,62].

Detecting the presence of elusive species using faecal-derived mitogenomes has been applied in otter conservation. For example, PCR-based reconstruction of mitogenomes showed, for the first time, sympatric presence of Eurasian otter and Asian short-clawed otter (*Aonyx cinereus*) in the Nakai-Nam Theun National Park (Lao People's Democratic Republic), which camera trapping had failed to show [63]. Therefore, non-invasive genetic sampling represents a highly useful tool for non-invasive monitoring of elusive species such as otters.

Another field where mitogenome sequencing has proven useful is taxonomic investigation and classification of mammals [64], including otter (*Lutra*) species [23,25]. For Eurasian otters, mitogenome data have uncovered deeply divergent mitochondrial lineages co-occurring in the UK [33], which were previously undetected from analyses of short mitochondrial barcodes [65]. We expect that using non-invasive spraint samples for mitogenome analysis will represent an important tool for genetic monitoring of otters. This approach will be of particular use in South America, Asia, North Africa and the Middle East, where otters are difficult to survey [23,33,63].

The generally non-recombining mitochondrial genome in animals is inherited as one locus and does not (necessarily) represent patterns across the nuclear genome. Indeed, widespread discordance between mitochondrial and nuclear markers has been described for many animal taxa [66], which highlights the importance of also analysing nuclear loci in phylogenetic, phylogeographic and taxonomic studies [67]. While we in the present study only obtained nuclear otter genome sequencing depth of less than 0.54× per spraint sample, increased sequencing effort would enable population genetic analysis of nuclear variation. Resulting multilocus genotypes would provide insights, e.g. into population structure, relatedness and temporal changes in effective population size, and allow estimation of the number of otters present in a study area—as demonstrated by Balestrieri *et al*. with microsatellite data [2]. Identification of unique individuals would be necessary to remove any duplicates in our dataset (i.e. spraint samples that derive from the same individual). This would subsequently enable meaningful comparisons of haplotype and nucleotide diversity with Welsh mitogenome data from du Plessis *et al.* [33].

Beyond the reconstructed Eurasian otter mitogenomes, our spraint metagenomic sequencing also yielded the mitogenomes of the prey fish species, *Cottus perifretum* and *Gasterosteus aculeatus*. Similarly, metagenomics has been used to reconstruct copepod prey mitogenomes from faecal samples of predatory pipefish [68]. Our approach, therefore, offers insights into not only population genetics of otters but also dietary information. We further note that our approach can be tied in with metagenomics-based characterization of host microbiomes and monitoring of antimicrobial resistance from faecal samples [9,69]. Given the breadth of insight to be gathered from metagenomic sequencing of spraint samples, especially from species of conservation interest, we concur with Ang *et al.* [70] who described faecal samples as an 'underappreciated source of information'.

4.2. Mitochondrial variation

Previous studies of mitogenomic variation in Welsh otters, based on analysis of tissue samples, found three haplotypes, all of which belong to Lineage 3, the dominant mitogenome lineage found in Britain and Europe [23,33]. Here, we detected the same three haplotypes from spraint samples collected along the river Usk. Our faecal-derived metagenomic sequence data, therefore, provides congruent results, in the context of mitogenome data derived from tissue samples.

When combining our mitogenome results with those of previous studies, we show that haplotype 3_04 occurs across Wales, whereas 3_06 has thus far only been found in south Wales [23,33]. From previous work, haplotype 3_11 had only been observed once, in an adult male individual (mLutLut51) from northwest Wales (figure 1; electronic supplementary material S1 of du Plessis *et al.* [33]). We here show that the same haplotype also occurs in south Wales. Based on the limited mitogenome sample size available to date, this suggests absence of strong matrilineal population genetic structuring among Welsh otters, consistent with previous microsatellite and mitochondrial DNA control region data [65,71].

Our findings add to existing evidence that the geographic distribution of the strongly divergent Lineage 1 in the southeast of England, putatively derived from anthropogenic introductions, at some point in the past decades, of Eurasian otters from Asia [23,29,33]. 'Non-European origin' mitochondrial DNA haplotypes in European otter samples have previously been suggested to have derived from captive stocks, in which European and Asian individuals had been mixed [72].

4.3. Factors impacting mitogenome reconstruction

A drawback of non-invasive genetic analysis includes that the DNA in the collected samples (such as faeces, hair or eDNA [10]) is generally found in low copy numbers and degraded due to various abiotic conditions (e.g. temperature and humidity [16]) and digestive enzymatic degradation in the case of faecal samples [73]. This typically leads to low quality and quantities of DNA and subsequently elevated error rates in genotyping [16,74]. The application of shotgun metagenomic sequencing, however, can partly alleviate this issue and has revolutionized the study of highly fragmented ancient DNA [20]. We therefore advocate that, similarly, shotgun sequencing of spraint samples allows the generation of robust data, by removing the reliance on intact, long DNA, while harnessing the power of high sequencing coverage from high-throughput sequencing approaches.

Our study provides a valuable proof of concept, but the sample size was too limited to enable a detailed analysis of the factors influencing mitochondrial sequencing depth. This also prevented the

exploration of nonlinear relationships using a generalized additive model, which would have used more degrees of freedom [75]. Furthermore, the sample collection was designed for a different study [76], and so did not gather abiotic information, such as temperature and humidity conditions at each local spraint site. In future studies, such metadata could be used to explore abiotic influences on resulting mitochondrial sequencing depth.

Based on our analyses, sample freshness (i.e. spraints being collected within less than 24 hours) did not guarantee successful mitogenome reconstruction. We found that full mitogenomes were reconstructed from old as well as fresh spraint, while mitogenomes from three fresh spraint samples in this study were only reconstructed to contig level, rather than obtaining full circular mitogenomes. Similarly, a recent study reconstructed mitogenomes of volcano rabbits (*Romerolagus diazi*) from only two of four fresh faecal samples [62]. The applicability of our method also to spraint samples that are not fresh (i.e. greater than 24 hours) is promising, reducing the need for repeated site visits sometimes used for otter genetic monitoring to allow collection of fresh spraints [16]. This will be particularly useful where surveys are time-limited or cover large areas. We suggest that while the collection of fresh spraints should be prioritized to mitigate against DNA degradation, older spraint samples can also yield full mitogenomes.

One fresh sample (O_0328_BR_A) yielded more human than otter sequencing reads, although this did not prevent successful otter mitogenome reconstruction. Contamination within the laboratory was considered unlikely because the DNA concentrations of the negative extraction controls were below the limit of detection (less than 0.1 ng μl^{-1}). This sample was predominantly comprised of anal jelly, rather than faecal matter, and was found on a rock close to the water's surface. It is possible that human eDNA found in the river water may have contaminated the sample, assuming that the sample was splashed prior to collection. As in our present study, human contamination in metagenomic sequencing data from langur (*Presbytis* spp.) faeces did not prevent target species mitogenome reconstruction [70].

Genetic analyses can be expensive [10] and therefore challenging to fit into conservation project budgets. Metagenomic sequencing of each sample in the current study cost circa £208 per sample from Novogene UK, which included DNA quality control, library preparation, sequencing, metagenome analysis, VAT and cloud delivery costs. The per-sample cost of a metagenomic-based approach is higher than when using long-amplicon PCR approaches for mitogenome reconstruction. In addition, high concentrations of input DNA are required to create a DNA library for metagenomic sequencing, which can be difficult to achieve when working with non-invasive samples. Despite these drawbacks, we note the high mitochondrial depth (greater than 65×) of some samples, and the successful mitogenome reconstruction from 20 out of 27 samples. Lower sequencing depth, and hence lower costs, would probably be sufficient to reconstruct otter mitogenomes in future investigations.

Surveys requiring direct observation of individuals in the field are not required for non-invasive approaches, which is particularly beneficial when working with elusive, nocturnal and widely dispersed species, such as otters [77]. Ferreira *et al.* [78] saved money and time in the field and identified more individuals when using non-invasive sampling of faeces of elusive Cabrera voles (*Microtus cabrerae*), instead of traditional trapping methods. These benefits make non-invasive sampling a cost-effective approach. Furthermore, metagenomics provides far more data than targeted PCR approaches, including dietary, resistome and microbiome information [8,9]. Therefore, metagenomics in combination with non-invasive sampling could be cost-effective in the long term, by enabling a plethora of questions to be addressed using one dataset.

5. Conclusions

Here, we show that mitogenome reconstruction is possible using metagenomic analysis of spraint samples from Eurasian otters. This shows that tissue-derived DNA is not critical for mitogenome research, thus removing the need for invasive sampling or collection of dead specimens. Our shotgun sequencing approach does not require long strands of intact DNA, enabling analysis of non-fresh spraint samples which exhibit increased DNA degradation. Although fresh samples are preferable, the utility of older specimens is encouraging for future research and monitoring projects.

Overall, these benefits could make future mitogenomic research into this charismatic mammal easier and more accessible. Finally, not only is mitogenome reconstruction available with this method, but a variety of other research questions can be answered using metagenomics, including dietary and microbiome research.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. All newly generated raw reads have been deposited on SRA (BioProject PRJNA1301358, BioSamples SAMN50440896–SAMN50440922; SRA SRR34857688–SRR34857714). Previously published haplotype data were taken from NCBI Nucleotide Database (OR633269-86 and BK064833-5). Bioinformatic code used to conduct the analyses is available on GitHub (https://github.com/sduplessis1/EurasianOtter_Spraint_mtDNA) and found in the electronic supplementary material [79].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. S.J.d.P.: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing—original draft, writing—review and editing; J.E.S.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, validation, visualization, writing—original draft, writing—review and editing; M.G.: funding acquisition, resources, writing—review and editing; P.K.: data curation, formal analysis, methodology, resources, supervision, writing—review and editing; E.A.C.: methodology, project administration, supervision, validation, writing—review and editing; F.H.: conceptualization, funding acquisition, project administration, resources, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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