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go_batch: A snakemake pipeline to assemble mitochondrial and ribosomal sequences from genome skims.

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Abstract

Low coverage “genome-skims” are often used to assemble organelle genomes and ribosomal gene sequences for cost effective phylogenetic and barcoding studies. Natural history collections hold invaluable biological information, yet degraded DNA often hinders PCR based analysis. However, with improvements to molecular techniques and sequencing technology, it is possible to use ancient DNA methods to generate libraries, and sequence the short fragments to generate genome skims from museum collections.

Here we introduce "*go_batch*", a bioinformatic pipeline written in snakemake designed to unlock the genomic potential of historical museum specimens using genome skimming. Specifically, *go_batch* allows the batch assembly and annotation of mitochondrial genomes and nuclear ribosomal genes from low-coverage skims. The utility of the pipeline is demonstrated by analysing a novel genome skimming dataset from historical sollariellid gastropod samples.

We demonstrate that *go_batch* was able to recover previously unattainable mitochondrial genomes and ribosomal genes from sollariellid gastropods. In addition, phylogenetic analysis of these gene sequences helped resolve complex taxonomic relationships, with manual validation and taxonomic expertise.

The generation of bioinformatic pipelines that can process sequence data from the vast repository of specimens held in natural history museum collections are likely to make significant contributions to our understanding of evolution and species discovery, ultimately aiding conservation efforts in the face of a changing planet.

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Introduction

Natural history collections are home to more than one billion expertly verified specimens worldwide (Bartolozzi et al., 2023) as well as large numbers of unsorted and unidentified bulk samples, and as such represent a vast repository of biological data that remains largely untapped. Challenges associated with such material include poor preservation, the use of unknown preservatives and age of material. Advances in novel laboratory techniques (Ruane & Austin, 2017; Straube et al., 2021) and next generation sequencing (NGS) technology overcomes many of these obstacles and make it possible to obtain DNA sequences from historical specimens, unlocking the potential for wide-ranging genomic analyses. Using natural history collections provides the opportunity to sample species that are rarely collected or even extinct and from areas of the world that are poorly sampled. It also avoids the need for fieldwork which can be costly, time consuming and in some cases, dangerous and may involve complicated regulatory issues.

Genome skimming has gained increasing popularity as an approach for barcoding specimens from historical museum collections. The term “genome skimming” refers to the generation of low coverage NGS data and was first coined by Straub et al (2012). Although genome skimming does not generate data with sufficient coverage to assemble entire genome sequences, there are sufficient reads to assemble sequences that are present in the genome in multiple copies and are therefore still well represented in the sequence data. Common targets for genome skimming studies include organelle genomes (a typical cell has many organelles but one nucleus) and nuclear ribosomal genes (there are 100s of rRNA nuclear genes, typically arranged in arrays). For many years organelle genes (mitochondria: *cox1*, chloroplasts: *matK* and *rbcL*) have been used as barcodes in DNA based taxonomy given their high copy number and availability of “universal” primers that work on a wide range of species, but increasingly whole organelle genomes are increasingly the focus of barcoding studies, or even the entire genome skim dataset as a ”DNA-mark” (Bohmann et al., 2020).

When working with historical specimens in particular, genome skimming offers many advantages over polymerase chain reaction (PCR) amplification and sequencing. Optimally, high yields of high molecular weight genomic DNA are required for PCR, but degraded and low yield DNA are also suitable for short read NGS (such as Illumina). The wet lab work is relatively straightforward, only requiring DNA extraction and library methods optimised for degraded DNA. Genome skimming also has additional benefits over targeted PCR since multiple loci can be recovered at the same time without development and optimisation of PCR primers. With advances in bioinformatic tools, it is likely that low coverage genome skimming datasets will have even greater utility in the future. For example, recent kmer based approaches have been developed for genome skims to investigate phylogenetic relationships (Sarmashghi et al., 2017) and genome properties (Sarmashghi et al., 2021). Finally, genome skimming is increasingly cost effective as the cost of NGS sequencing continues to decrease. In the

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light of these advantages, genome skimming is seen as a hugely scalable process that is suitable for batch recovery of barcode genes from museum collections.

However, few bioinformatic pipelines are available for assembly of organelle and nuclear ribosomal sequences from genome skimming data. Notable exceptions include MitoZ (Meng et al., 2019) and NOVOWrap (Wu et al., 2021) for the assembly and annotation of mitochondrial genomes. In addition, plastaumatic (W. Chen et al., 2022) is available for chloroplast assembly and annotation and PhyloHerb (Cai et al., 2022) can be used for the assembly of chloroplast and nuclear ribosomal repeats without annotation. These tools were not designed with historical and/or degraded samples in mind and do not account for issues such as contamination and the assembly of non-target sequences. In addition, these tools do not implement phylogenetic analysis of the annotated genes identified. Other targeted assembly approaches are available including Orthoskim (Pouchon et al., 2022), but this is not available as part of a pipeline that can be scaled across many samples.

This study introduces *go_batch*, a pipeline written in snakemake for batch assembly and annotation of mitochondrial genomes and nuclear ribosomal genes, and phylogenetic analysis from genome skimming data. The pipeline wraps 12 published bioinformatic tools as well as custom python and R scripts and is designed to cope with poor quality data from historical collections, permitting large scale genome skimming studies from museum specimens. This pipeline takes raw NGS reads from single or multiple libraries and produces (1) assembled sequences, (2) summary of assembled sequences to check for contamination, (3) annotated genes in BED and fasta format (4) alignments and (5) phylogenetic analysis of annotated genes.

Building on previous pipelines, our approach can be: (1) run in on a single machine or in parallel on a High Performance Computing cluster, (2) utilised to recover both organelle and nuclear ribosomal sequences, (3) uses GetOrganelle which an independent review found to be the best performing assembly tool (Freudenthal et al., 2020; Jin et al., 2020), (4) performs basic assembly checking for contamination and non-target sequences commonly found in historical samples and (5) generates phylogenetic gene trees based on from annotated genes.

To demonstrate the utility of *go_batch*, we use the pipeline to analyse a novel genome skimming dataset for the gastropod family Solariellidae (hereafter solariellid gastropods). This group was selected as it represents many of the challenges associated with genome skimming museum collections. Solariellids are small marine snails found predominantly in deep-water. Many species are rare and as a family they are poorly represented in museum collections worldwide, with few live-collected specimens: many species are known only from a single, dry and often damaged shell. Although solariellid gastropods have been the focus of previous phylogenetic studies (Sumner-Rooney et al., 2016; Williams et al., 2013, 2022), these studies have relied on partial sequence from only four genes, which have not fully resolved relationships among genera. As such, our understanding of solariellid evolution would greatly benefit from increased gene sampling, but there are no

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published reference genomes for the group and limited genomic data available on public databases. Where 'good' universal primers exist, attempts to include key taxa in previous studies has not been possible as PCRs have failed, likely due to degraded fragment size. Given their rarity, small size and frequently poor preservation, solariellids are an excellent test case for the utility of genome skim data and pipelines designed for historical specimens.

Material and methods

Solariellid sample selection and sequencing

A total of 25 samples were selected, with representatives of 18 genera, selecting samples to encompass the diversity of the Solariellid family, including several species with dubious generic assignments (Table 1). Samples differ in several ways that likely affected DNA quality and yield (Supplementary Table 1), for example, time since collection (1967-2015) and preservation method (dry shell with dehydrated body tissues or live-collected snail preserved in 70- 99% ethanol). In addition, some shells were cracked, allowing the rapid penetration of ethanol for snails that can seal their bodies inside their shells by closing their operculum. Samples also differ in time kept in storage (initially at 4º C and then at -20º C) since DNA was extracted (2010–2020; Supplementary Table 1).

DNA was isolated using Qiagen DNeasy blood and tissue kit, and quantified using a Qubit fluorimeter and HS assay kit. A Tapestation 2200 was also used to assess DNA integrity prior to library preparation. Polymerase Chain Reaction (PCR) amplification and Sanger sequencing of mitochondrial (*cox1*, 16S and 12S) and ribosomal genes (28S) were attempted for each sample to compare with our genome skimming approach. Illumina Libraries were prepared using Illumina libraries were made using a SparQ DNA Frag and Library Prep kit (QuantaBio) and sparQ PureMag Beads (QuantaBio), with Sparq Adaptor Barcode sets A and B (QuantaBio), with bespoke modifications (See Supporting Information Methods). Libraries were normalised and pooled equally before being sent to Novogene (Cambridge, UK) for sequencing. The single indexed libraries were sequenced on an Illumina Novaseq on an S4 300 cycle flowcell using 150bp paired reads.

Additional sequence data for '*Solariella*' *varicosa* was provided by Andrea Waeschenbach (Natural History Museum London, UK). Raw sequence data for two outgroups from the family Turbinidae were also analysed, including: *Turbo cornutus* (Kim et al., 2022; SRR15496837) and *Lunella* aff. *cinerea* (Williams et al., 2014). These outgroup sequences provide the possibility of comparing published assembled and manually curated organelle genomes with the results from our pipeline using the same raw sequence data.

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Table 1 – Sample details for 25 solariellid gastropods and two outgroup species used in this study with museum registration numbers or NCBI registration for sequence data (*Turbo cornutus* only), ocean of origin, detailed collection location, and depth. AMS: Australian Museum; MNHN: Muséum national d'Histoire naturelle; SMNH: Swedish Museum of Natural History; MNSA: KwaZulu-Natal Museum; NMNZ: Museum of New Zealand Te Papa Tongarewa; NHMUK: Natural History Museum, London. Inverted commas around generic names indicates uncertainty about generic assignment based on this or previous studies. Previously published data for *Turbo cornutus* (Kim et al. ,2022) and *Lunella aff. cinerea* (Williams et al., 2014) were also included in this study.

Species	Specimen voucher	Ocean	Latitude	Longitude	Depth (m)
Archiminolia oleacea	AMS C.133269	Indo-West Pacific	-24.375	153.285	192-229
Arxellia herosae	MNHN-IM-2009-28739	Indo-West Pacific	-24.717	168.167	298-324
Bathymophila gravida	NMNZ M.299691	Indo-West Pacific	-36.146	178.202	712-924
'Bathymophila' sp. 18	MNHN-IM-2009-23080	Indo-West Pacific	-22.317	171.333	925
Bathymophila-Like sp. 12	MNHN-IM-2009-28741	Indo-West Pacific	-19.667	-178.167	314-377
Chonospeira nuda	SMNH 127100	North East Pacific	36.367	-122.417	999
Clade D sp. d	MNHN-IM-2013-59648	Indo-West Pacific	22.050	119.067	1306-1756
Elaphriella wareni	MNHN-IM-2013-45837	Indo-West Pacific	-8.617	151.783	705-817
Ilanga whitechurchi	NMSA W9631	South West Indian Ocean	-33.167	28.033	90
Lamellitrochus sp. 6	MNHN-IM-2013-60491	Caribbean	16.350	-60.900	111-162
'Lamellitrochus' carinatus	MNHN-IM-2009-31169	Caribbean	16.360	-61.579	29
Microgaza rotella	MNHN-IM-2013-8023	Caribbean	16.400	-61.550	130
Phragmomphalina tenuiseptum	NMNZ M299700	Indo-West Pacific	-31.867	172.433	780-790
Solariella amabilis	NHMUK 20180166	North Atlantic	62.191	5.567	150-200
Solariella sp. 7	MNHN-IM-2019-12000	Indo-West Pacific	-24.800	168.150	250-270
'Solariella' carvalhoi	MNHN-IM-2013-61297	Caribbean	15.800	-61.467	379-428
'Solariella' obscura	NHMUK 20230529	North Atlantic	69.803	30.693	04-Dec
'Solariella' varicosa	NHMUK 20120235	North Atlantic	70.067	29.200	10-174
Spectamen bellulum	NHMUK 20110452	Indo-West Pacific	-26.943	153.404	31
'Spectamen' franciscanum	NMSA V1091	South West Indian Ocean	-34.783	23.983	171
Suavotrochus lubricus	MNHN-IM-2013-61096	Caribbean	16.033	-61.233	266-388
'Suavotrochus' sp. 2	MNHN-IM-2013-61502	Caribbean	15.783	-61.200	550-562
'Zetela' alphonsi	SMNH 10387	South East Pacific	-36.361	-73.725	865
Zetela kopua	NMNZ M.131532	Indo-West Pacific	-45.403	173.980	1386
Zetela textilis	NMNZ M.035478	Indo-West Pacific	-42.637	176.283	256-311
OUTGROUPS					
Lunella aff. *cinerea*	NHMUK 20100448	Indo-West Pacific	-12.554	130.876	NA
Turbo cornutus	SRR15496837	Indo-West Pacific	33.454	126.949	NA

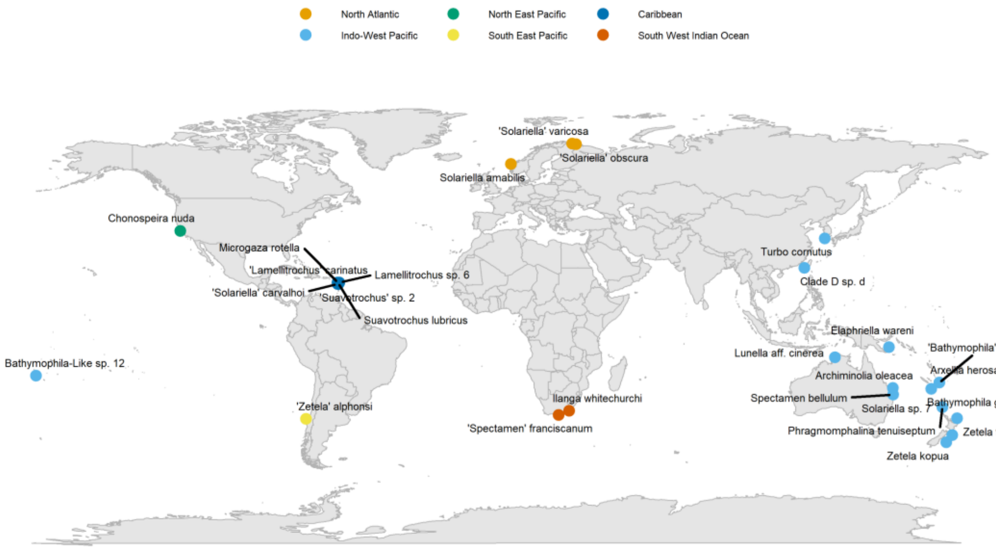

Figure 1 – Map showing collection localities for solariellid gastropods samples used in this study.

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Pipeline description

As input, the pipeline requires two main inputs: (1) a config.yml file and a (2) samples.csv file. The config file outlines the main parameters including the target sequence type (animal_mt, embplant_cp or anonym for, mitochondrial, chloroplast and ribosomal sequences respectively, as defined by GetOrganelle), paths to reference databases (blast database, NCBI taxdump, MITOS) and number of threads to use. The samples.csv file is a list of the samples including in the analysis with paths to forward and reverse reads, and paths to the gene and seed databases required by GetOrganelle. The pipeline accepts NGS data from short read platforms (e.g., Illumina) in paired fastq format.

The pipeline starts by processing the data from each sample, using fastp (S. Chen et al., 2018) to detect and remove adapter sequences with quality filtering disabled, as recommended for GetOrganelle. GetOrganelle (Jin et al., 2020) is then used to assemble the target sequence of interest. If the target sequence is an organelle genome (animal_mt or embplant_pt), GetOrganelle is implemented with the following parameters: `--reduce-reads-for-coverage inf --max-reads inf -R 20`. If the target sequence is another gene e.g. ribosomal, the following parameters are used following the authors suggestions: `-F anonym --reduce-reads-for-coverage inf --max-reads inf -R 10 --max-extending-len 100 -P 0`. Sequences assembled by GetOrganelle are typically named based on the output of SPAdes (Prjibelski et al., 2020), which can produce long sequence names. Therefore, sequences are renamed to <sample_name>_contig<n> if there are multiple contigs or <sample_name>_circular if a single circular sequence is found. Note that GetOrganelle can produce more than one assembled sequence where there are different possible paths e.g. mitochondrial genomes contain inverted repeats. However, the pipeline simply selects the first assembled sequence for downstream analyses as the main outputs are the annotated gene sequences and the correct orientation of repeat regions is not necessary. Basic assembly statistics are summarised using SeqKit (Shen et al., 2016). Next, the assembly quality is evaluated using a blastn search (Camacho et al., 2009) against a database specified in the config.yaml file and mapping input reads to the assembled sequence using minamp2 (Li, 2018). This information is summarised using blobtools (Laetsch et al., 2017) and the likely taxonomy of the assembled sequence is define using the taxrule "bestsumorder". Following the assembly quality check, assembled sequences are annotated using MITOS2 (Bernt et al., 2013), or barrnap (https://github.com/tseemann/barrnap) for organelle or ribosomal sequences respectively. Following assembly and annotation, a plot is created to visualise the location of annotated genes, coverage and proportion of mismatches in mapped reads.

Once the sequences are assembled and annotated, the checkpoint function of snakemake is used to recover all protein coding genes assembled across samples. For each protein coding gene recovered, mafft (Katoh & Standley, 2013) is used to align sequences with the following parameters: `--maxiterate 1000 --`

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`globalpair -adjustdirection`. The alignments are trimmed using either Gblocks (Castresana, 2000) or Clipkit (Steenwyk et al., 2020) as specified in the specified in the config.yaml file. Phylogenetic analysis is then implemented in with IQ-TREE2 (Minh et al., 2020) and consensus trees are plotted in R using the ggtree package (R Core team, 2020; Yu et al., 2017).

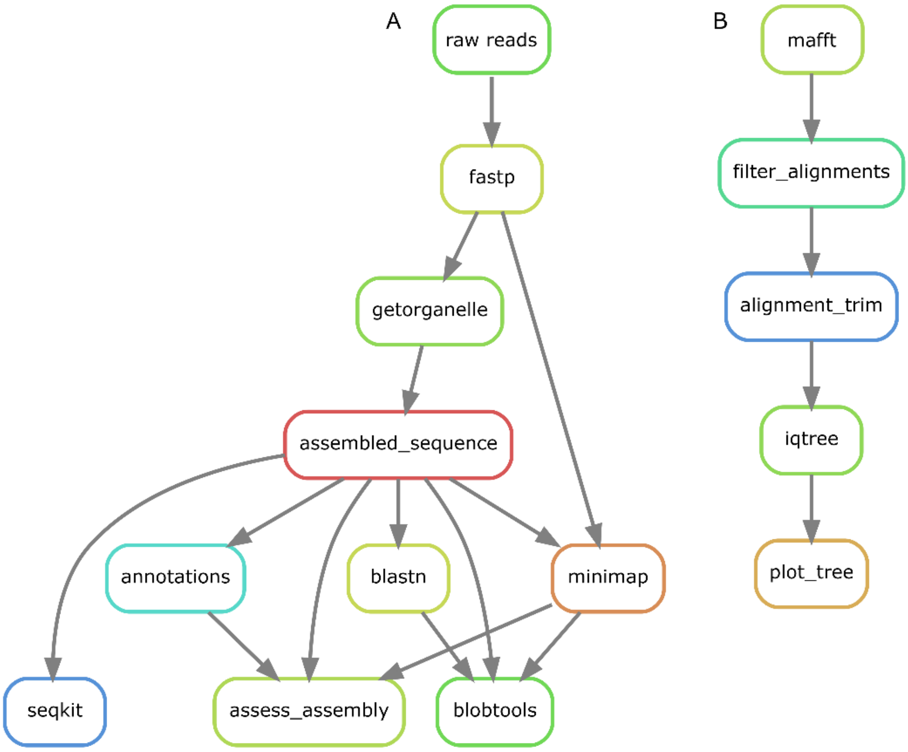

Figure 2 – Schematic diagram of pipeline workflow. Workflow A is applied to all samples provided in the samples.csv. Workflow B is applied to all annotated gene sequences found across assembled sequences.

The pipeline output was manually checked to identify possible contamination in assembled sequences for a given sample or individual genes. Specifically, the blobtools output and was checked for sequences with unusual blast hits and the gene trees were reviewed by taxonomic experts to identify incongruent relationships. Manually checked alignments were reanalysed with IQ-TREE if the alignment was edited and combined into a partitioned alignment using a custom python script. Phylogenetic relationships were then inferred using two methods including astral which uses individual gene trees as input and (Zhang et al., 2018) and a partitioned gene analysis using IQ-TREE.

Results

Amplification of four genes (28S, COI, 12S and 16S) was attempted soon after DNA was first extracted, and the results are listed in Supplementary Table 1. In some cases, PCRs were successful, but clean sequence could not be obtained, usually because of low yield and noisy background. Often, only 12S, the smallest PCR fragment could be amplified and sequenced, suggesting that DNA was degraded. The DNA Integrity Number

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(DIN) is automatically assigned by the instrument following an algorithm based on the signal distribution across the size range. A DIN of 10 indicates highly intact DNA fragments, whilst a DIN of 1 indicates a highly degraded DNA sample (Supplementary Table 1). DNA quality ranged from not detectable for the poorest samples to 6.5 for the best.

Approximately 130Mb of raw sequence data was generated for all samples, with an average of 31M raw reads per sample. Overall, *go_batch* successfully recovered mitochondrial genome sequences from 25/28 samples of which one sample had a circular mitochondrial genome (Figure 3), and ribosomal gene sequences from 25/28 samples.

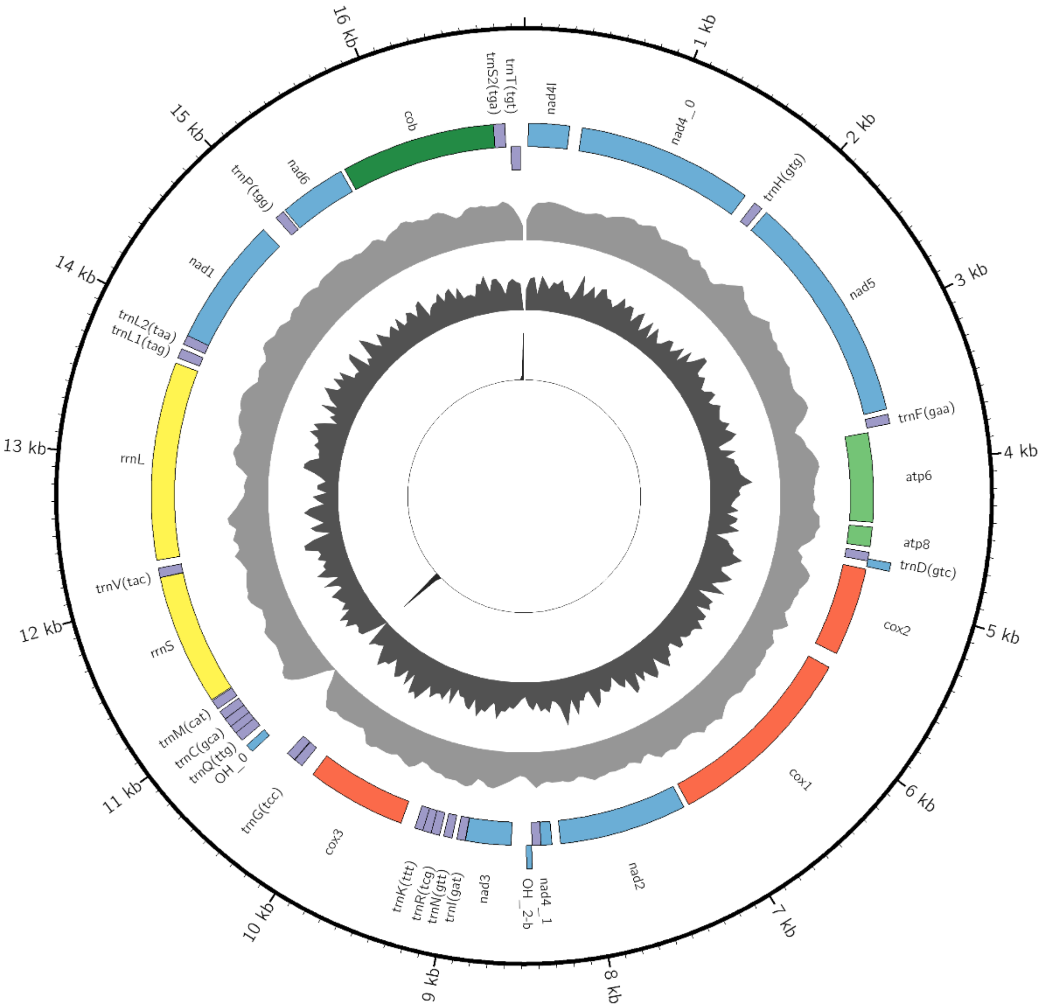

Figure 3 - Assembled circular sequence for *Zetela kopua* with the following attributes from outside to inside: sequence position, annotation names, annotations on the + strand, annotations on the - strand, coverage, GC content (max=y) and repeat content (max=z).

Phylogenetic analysis of the partitioned alignment in IQ-TREE (Figure 4) resolved relationships between the sollaridellid samples included in this study. However, some of the support values in basal branches are poorly supported (<75).

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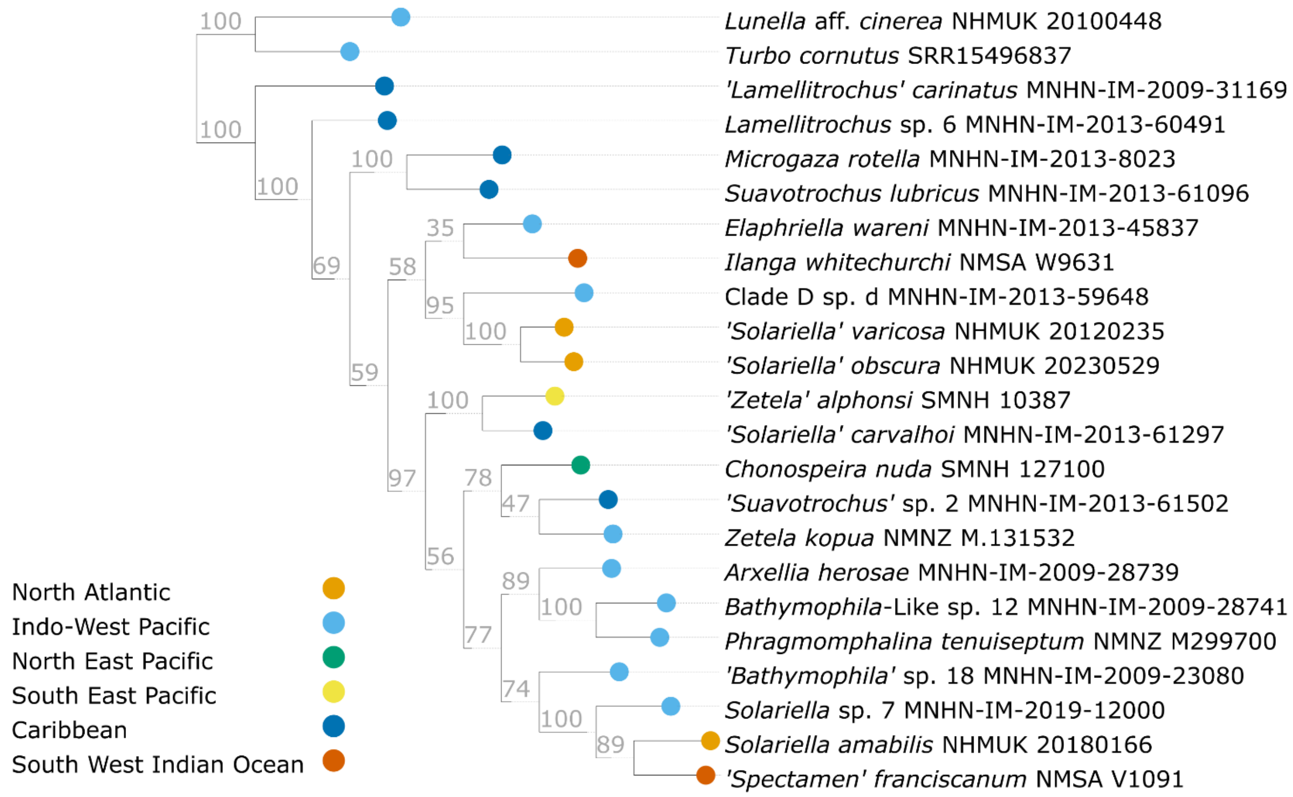
100
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56
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77
74
100
89
Lunella aff. *cinerea* NHMUK 20100448
Turbo cornutus SRR15496837
'*Lamellitrochus*' *carinatus* MNHN-IM-2009-31169
Lamellitrochus sp. 6 MNHN-IM-2013-60491
Microgaza rotella MNHN-IM-2013-8023
Suavotrochus lubricus MNHN-IM-2013-61096
Elaphriella wareni MNHN-IM-2013-45837
Ilanga whitechurchi NMSA W9631
Clade D sp. d MNHN-IM-2013-59648
'*Solariella*' *varicosa* NHMUK 20120235
'*Solariella*' *obscura* NHMUK 20230529
'*Zetela*' *alphonsi* SMNH 10387
'*Solariella*' *carvalhoi* MNHN-IM-2013-61297
Chonospeira nuda SMNH 127100
'*Suavotrochus*' sp. 2 MNHN-IM-2013-61502
Zetela kopua NMNZ M.131532
Arxellia herosae MNHN-IM-2009-28739
Bathymophila-Like sp. 12 MNHN-IM-2009-28741
Phragmomphalina tenuiseptum NMNZ M299700
'*Bathymophila*' sp. 18 MNHN-IM-2009-23080
Solariella sp. 7 MNHN-IM-2019-12000
Solariella amabilis NHMUK 20180166
'*Spectamen*' *franciscanum* NMSA V1091
North Atlantic
Indo-West Pacific
North East Pacific
South East Pacific
Caribbean
South West Indian Ocean

Figure 4 - Partitioned maximum likelihood tree of 17 mitochondrial protein-coding genes, two mitochondrial ribosomal genes and one nuclear ribosomal gene (28S) generated using IQ-TREE and visualised using ete3. The tree is rooted on the outgroup taxa and values on branches are bootstrap values.

Discussion

This study demonstrates the utility of *go_batch*, a snakemake pipeline for the assembly and annotation of organelle and ribosomal genes from genome skimming datasets, using a novel dataset from solariellid gastropods. Some of the mitochondrial genomes and ribosomal genes generated are the first sequenced for the family Solariellidae. Complete or partial mitochondrial genomes were obtained for 25 out of 28 specimens, including specimens with DNA extracted more than ten years ago, from dehydrated tissue samples and specimens preserved with uncracked shells in 70% ethanol, samples collected more than 50 years ago, and with highly degraded DNA. Better results were obtained for dehydrated tissues from dry shells (2/3 successful) than snails preserved in low percentage (70–80%) ethanol with uncracked shells (10/12).

The *go_batch* pipeline is designed to cope with poor quality data from historical collections, permitting large scale genome skimming studies from museum specimens. Given the current biodiversity crisis and lack of taxonomic expertise, it has become more important than ever to document biodiversity before it is lost. By sequencing natural history collections at scale using bioinformatic tools such as *go_batch*, researchers can increase the rate of phylogenetic and barcoding studies, and ultimately species discovery. Although *go_batch* simplifies the bioinformatic analyses significantly, our study highlighted that it was important to manually

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check the assembled sequences for contamination or poorly annotated sequences. In addition, taxonomic expertise was necessary to identify incongruent phylogenetic relationships.

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Supporting Information

Tables

Supplementary Table 1 - Summary of sample quality for extracted DNA and details of factors affecting DNA including the year of sample collection, preservative (ethanol or dry shell), if shell was cracked to allow penetration of ethanol, year DNA was extracted, DNA Integrity Number (DIN) and the amplification success for four partial gene sequences (nuclear 28S rRNA, and mitochondrial genes: COI, 16S rRNA and 12S rRNA). PCR success is summarised as genbank number if PCR were successful and published, "SEQ" if the PCR was successful and sequenced but unpublished, or "PCR only" if the gene could be amplified but not sequenced.

Specimen	Year collected	Preservative	Shell cracked?	Year DNA extracted	DIN	PCR 28S	PCR COI	PCR 16S	PCR 12S
Archinimolia oleacea	1977	70% ethanol	N	2011	none	–	–	–	–
Arxellia herosae	2001	dry	–	2011	1.8	–	–	–	HF585844
Bathymophila gravida	2001	80% ethanol	N	2010	1	PCR only	PCR only	–	–
‘Bathymophila' sp. 18	2011	95% ethanol	N	2013	6.2	LT575957	–	LT575910	LT575928
Bathymophila-Like sp. 12	1999	dry	–	2010	1	–	–	–	HF585775
Chonospeira nuda	2009	95% ethanol	N	2013	1.9	–	SEQ	SEQ	SEQ
Clade D sp. d	2015	95% ethanol	Y	2020	1.3	–	SEQ	–	–
Elaphriella wareni	2014	95% ethanol	Y	2019	2.8	SEQ	–	–	SEQ
Ilanga whitechurchi	2013	99%?ethanol	N	2014	none	–	–	OK393755	–
Lamellitrochus sp. 6	2015	95% ethanol	Y	2020	5.8	OK393809	OK392062	OK393760	OK393784
‘Lamellitrochus' carinatus	2012	95% ethanol	Y	2013	5.7	SEQ	SEQ	SEQ	SEQ
Microgaza rotella	2012	95% ethanol	Y	2013	6.1	LT575964	LT575902	LT575920	LT575947
Phragmomphalina tenuiseptum	1988	80% ethanol	N	2010	1.6	PCR only	PCR only	–	PCR only
Solariella amabilis	1970	70% ethanol	N	2011	1	–	–	–	HF585871
Solariella sp. 7	1992	dry	–	2011	none	–	–	–	HF585874
‘Solariella' carvalhoi	2015	95% ethanol	Y	2020	6.5	OK393814	OK392068	OK393764	OK393789
‘Solariella' obscura	1967	70% ethanol	N	2011	1	–	–	–	PCR only
'Solariella' varicosa	1967	70% ethanol	N	2011	–	–	–	–	HF585720
Spectamen bellulum	2005	99% ethanol	N	2010	3.3	SEQ	–	PCR only	HE800677
‘Spectamen’ franciscanum	1995	75% ethanol	N	2010	1	–	PCR only	–	PCR only
Suavotrochus lubricus	2015	95% ethanol	Y	2020	3.7	SEQ	SEQ	SEQ	SEQ
‘Suavotrochus’ sp. 2	2015	95% ethanol	Y	2020	5.7	SEQ	SEQ	SEQ	SEQ
‘Zetela’ alphonsi	2006	70% ethanol	N	2010	none	PCR only	–	–	PCR only
Zetela kopua	1979	80% ethanol	N	2010	1.8	PCR only	PCR only	–	–
Zetela textilis	1974	80% ethanol	N	2010	none	–	PCR only	–	–

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Methods

The least degraded samples had a DIN of 3-10 and sufficient DNA was available to add 10 ng to each reaction. A fragmentation time of 16 mins was found to be sufficient to create libraries of 150-215 bp, the adaptor was diluted 1 in 5, and the library was amplified with 10 PCR cycles.

Degraded samples (DIN<3) were treated individually with trial and error at each step of library preparation. Libraries were prepared a few at a time with adjustments made to subsequent library preps based on QC results. Many of these samples were also of low concentration, meaning it was not possible to add the recommended 10ng DNA per reaction. To avoid further damage to the DNA, it was not concentrated; the maximum available volume of dilute sample was used, and the protocol adjusted for low input, as detailed in the user protocol provided with the kit. After library preparation all libraries were analysed with a Tapestation 2200 D1000 kit (Agilent).

A fragmentation time of 4 minutes was initially trialled for a subset of particularly poor samples (DIN<2), but following a comparison of 4 and 10 mins, there was little discernible effect on library quality. For ease of processing, all subsequent libraries were made with 10 min fragmentation time.

Many libraries from degraded samples showed high concentrations of adaptor-dimer and so were cleaned using SparQ PureMag beads (QuantaBio) at 1.8x. A repeat PCR was performed on 2 of the libraries which showed extremely high quantities of adaptor-dimer, low library concentration and possible bubble product.