Steps and Protocols for eDNA Analysis in Estuaries

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Introduction

Range-expanding species are defined as those that extend outside their historic geographic range without human intervention (Jeschke et al., 2013; Wallingford et al., 2020). When these species initiate novel interactions with existing species that adversely affect the native community, such as predation or competition, range-expanding species are also considered invasive. Examples of invasive range-expanding species include the squid *Dosidicus gigas*, the crab *Callinectes bocourti*, and the insect *Coelopa pilipes* (Sorte et al., 2010).

Monitoring the position of range-expanding species is critical for learning the pace and direction of their movements into new environments. Many different techniques can be used to survey species' abundance and distributions in the field (Coleman et al., 2023). Traditional techniques include observation, mark-recapture-release, and quadrant surveys; for aquatic species, gill nets, electrofishing, and fyke nets have also been employed (Coleman et al., 2023; Spear et al., 2020; Piggott et al., 2020). However, a new method that has developed over the last decade is environmental DNA (eDNA) surveying (Lenzi et al., 2023; Wu et al., 2023). The eDNA detection methods are based on the retrieval of minute quantities of DNA from an environment (soil, water, or air) that is left by organisms through processes such as shedding or excretion (Nagarajan et al., 2022). Because fragments of informative DNA can be amplified from the sample, eDNA can be used to detect common, elusive, or rare species in an ecosystem (Rishan et al., 2023). By employing different amplification primers, eDNA can be tailored for single-species detection (for example, tracking a single range-expanding species) or for surveying entire communities (referred to as metabarcoding; Nagarajan et al., 2022). In this project, I focused on learning eDNA methodology, focusing on three key steps: field sampling

for eDNA in an estuary, extracting and amplifying DNA in the laboratory, and analyzing sequences amplified from eDNA using bioinformatics tools.

Aratus pisonii, commonly known as the mangrove tree crab, was the primary focus of my project because of its recent detection in salt marshes on Sapelo Island, Georgia, within the domain of the Georgia Coastal Ecosystems LTER project (Figure 1). *A. pisonii* is native to tropical areas such as the Caribbean Islands, Trinidad and Tobago, Northern Brazil, and Florida (Beever et al., 1979; Von Hagen, 1977), and is a member of the Sesarmidae family. Unexpectedly, this species was identified as a range-expanding species approximately 10 years ago and has now migrated northward into Georgia marshes ahead of its native habitat, the mangrove forest (Riley et al., 2014). It is possible that a warming climate enabled this expansion, and therefore that *A. pisonii* is just one of many species whose ranges will change in the next decades. Such shifts in species ranges are problematic for habitat management, conservation, and restoration efforts. Mangrove crabs, including *A. pisonii*, bioturbate and consume litter, and influence nutrient recycling (Algoni, 2002; Buranelli & Mantelatto, 2019).

In order to become familiar with different aspects of the eDNA approach I first collected water samples from two locations on Sapelo Island, one of which was a site where *A. pisonii* has been observed (Long Tabby Creek). Next, I carried out DNA extraction and amplification on these samples using a primer set that broadly targets invertebrates. Finally, I applied bioinformatics analysis techniques to an existing sequence dataset of eDNA amplicons obtained from a California estuary.

Field Sampling

Coastal water samples for environmental DNA (eDNA) extraction were collected from Long Tabby Creek (from the Department of Natural Resources (DNR) dock) and in South End Creek (from the University of Georgia Marine Institute (UGAMI) boat lift) on Sapelo Island, GA, USA on June 11 and 12, 2023 (Figure 2). Water was transported to the lab and pre-filtered through a 160 µm mesh into acid-washed polycarbonate containers. Samples were filtered the following day using a peristaltic pump with a 142 mm filtration apparatus for large volume filtration, and a vacuum pump with 47 mm and 25 mm glass filter holders for small volume filtration. Water samples were filtered with two different filter types and various pore sizes, either polyethersulfone membrane filters (Supor) with 0.8, 0.45, and 0.2-micron pore sizes, or glass fiber filters (Whatman) with 0.7 (GF/F), 1.2 (GF/C), or 1.6 (GF/A) micron pore sizes. Filters were stored at -20°C until transported in an ice chest to the University of Georgia, Marine Science Department in Athens, GA, USA, and then stored in a -20° freezer until ready for processing. In addition to estuarine water sampling, Jonah Rigdon (Research Technician II) hand-collected four *A. pisonii* from Sapelo Island, GA, USA in October 2022 for the positive control tissue extraction.

In this component of my project, I had my first experience in estuarine water sampling. I became familiar with the water filtration protocol for eDNA sampling, including the use of peristaltic pumps and filter holders, the selection of appropriate filters (diameter and pore size), and the identification and packing of materials needed for sampling. I also improved some soft skills such as problem-solving (the field site had limited supplies on hand compared to a lab on campus and I had to work around the lack of access to certain equipment types in the field),

collaboration with other graduate students and staff (Jeremy Schreier, trained me, and Jonah Rigdon helped with crab collection), and public speaking (sharing my experiences) with undergraduates participating in the UGAMI summer course. I also identified tips and tricks that would have been helpful and that I will keep in mind for future field work, such as recording GPS coordinates for sample and specimen collection, designing and pre-printing data collection sheets, refreshing my memory for tying different knots, and taking pictures of each filter sample.

DNA Extraction and Amplification

Filter optimization – To acquire eDNA from the sample filters, I used the DNEasy Blood and Tissue Kit (Qiagen). First, I tested the two types of filter material with the extraction kit. The Whatman filters were ruled out because they disintegrated during the extraction protocol, which has been shown to decrease the efficiency and reliability of extractions (Chevrinais et al., 2023). Among the Supor filters, I chose to use the 47 mm Super-200 samples because the Supor-450 and Supor-800 were used only with the 142 mm filtration apparatus, and this size filter was determined to be more time-consuming and complex. For example, I would need to carry out independent extractions for sections of a single filter because of the extraction kit capacity, or to calculate the volume of estuarine water passed through just a section of the filter relative to the full size. Finally, because eDNA is of unknown size and differs across species (Jo et al., 2019; Moushomi et al., 2019), I chose to use the smallest (0.2 micron) pore size filters to better capture eDNA. I therefore focused on 47 mm Supor-200 filters with a 0.2-micron pore size.

eDNA extraction – eDNA filters were thawed on ice and cut into smaller pieces with sterilized scissors into a centrifuge tube. Extraction was carried out with the DNEasy Blood and Tissue Kit following the manufacturer's instructions with minor modifications based on Chevrinais et al., 2023 and Danzinger & Frederick., 2022. The procedure generally involved treating a filter with proteinase K to degrade proteins and RNase A to degrade RNA. Following an incubation at 56℃ for a minimum of 2 h, AL buffer was added to the filter mixture in a centrifuge tube. An additional incubation of 10 min was added to the protocol (Chevrinais et al., 2023). Next, DNA was precipitated with the addition of 100% ethanol. Once the mechanical and chemical lysis steps were completed, the liquid was separated from the filter and transferred into a DNeasy column to bind, wash, and elute extracted DNA.

Tissue sample DNA extraction – DNA was also extracted from tissue samples of *A. pisonii* to test whether two published PCR primer sets that broadly target invertebrates were able to amplify DNA from *A. pisonii*. Primer pair COL6b_F and COH6_R (Buranelli & Mantelatto., 2019) amplifies a 658-bp fragment of the mtDNA COI gene (Schubart & Huber, 2006) and was chosen because it was shown previously to amplify *A. pisonii* DNA (Buranelli & Mantelatto, 2019). Primer pair LCO1490_F and HCO2198_R amplifies a 710-bp fragment of the mtDNA COI gene (Folmer et al., 1994) and was chosen because it is commonly used for invertebrate studies.

DNA from *A. pisonii* gill and muscle tissue was also isolated using the Qiagen DNeasy Blood and Tissue Kit. The procedure was similar to the eDNA extraction except that the modifications to the manufacturer's instructions described above were not incorporated. I used different amounts of tissue wet weight (25, 20, 15, & 10 mg) and a negative control (0 mg) in the

extractions to determine the optimal tissue weight for DNA yield. A Bio101 FastPrep FP120 Cell Disruptor was used before the incubation step to break up tissue and release DNA. I also tested different durations for the 56ºC incubation (3 h or overnight). The best results were achieved with 10 mg tissue material, with both 3 h and overnight incubation times yielding equivalent DNA. After extraction, DNA concentration was measured using a Nanodrop spectrophotometer (Model: ND-1000). All samples were diluted (1:10) in AE buffer to decrease the concentration of potential PCR inhibitors from the raw extraction mix and then stored at -20ºC until further processing.

PCR Amplification – Amplification of *A. pisonii* DNA was carried out with Schubart and Huber (2006) and Folmer et al. (1994) primer pairs and the Phusion High-Fidelity PCR Master Mix (Thermo Scientific). The manufacturer's recommendations were followed for reaction ingredients and amounts (water, forward and reverse primer, and DNA template). An Eppendorf Mastercycler X50a was used for PCR amplification with initial denaturation at 98℃ for 30 s, 30 cycles, denaturation at 98℃ for 10 s, annealing at temperatures ranging from 49-58℃ for 30 s, extension at 72℃ for 30 s/kb, and final extension at 72℃ for 10 min, hold at 4℃. The range of annealing temperatures was used to identify optimal conditions for primer binding to DNA.

Amplification of eDNA was carried out with a different invertebrate primer pair that was an updated version of the Schubart and Huber (2006) and Folmer et al. (1994) primers. This mlCOIintF and jgHCO2198 primer pair (Leray et al., 2013) amplifies a shorter fragment (313 bp) and is more amenable to Illumina sequencing. To prepare for Illumina sequencing, overhangs were added to the forward and reverse primers: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-Forward-Specific-Primer-Sequence 3'

and 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-Reverse-Specific-Primer-Sequence 3' (https://dna.uga.edu/microbiome-service/). eDNA was similarly amplified with Phusion High-Fidelity PCR Master Mix. Initial results indicated no amplification, and given more time, my next step would have been to optimize PCR conditions as described above.

Gel electrophoresis and extraction – A. pisonii amplified DNA was visualized and photographed in a 1% agarose gel using the UVP Epi Chem II Darkroom. Bands indicating the location of amplification products were cut out of gels by hand and DNA was excised using a Monarch DNA Gel Extraction Kit according to the manufacturer's instructions. The *A. pisoni* recovered amplicons were sequenced by Sanger Sequencing at Genewiz, Inc. Sequences were blasted against the NCBI database to determine whether they matched the target species.

In my training on DNA extraction and amplification for eDNA analysis, I learned that PCR amplification is highly sensitive to a variety of conditions. Trial and error approaches are necessary to identify the appropriate conditions for each primer and DNA type, and published PCR conditions for a primer set will very likely need to be modified. Other technical procedures I learned included how to distinguish DNA absorbance (260 nm) from that of reagents and excess material peaks (230 nm) (Figure 3), make and run gels (Figure 4), extract DNA from gels, and operate a thermal cycler.

Bioinformatics Analysis

The final component of my project was to learn analysis techniques for eDNA amplicons. To do this, I obtained an unanalyzed eDNA amplicon dataset from Bodega Bay Estuary, CA,

USA from an ongoing project in Dr. Holly Bik's lab in the Department of Marine Sciences at UGA (Patricia Holt-Torres, Tiago J. Pereira, Mirayana Marcelino-Barros, Gabriel Serrano, Jay Stachowicz, and H. Bik, unpublished data).

A common bioinformatics tool for eDNA sequence analysis is the QIIME2 platform (Estaki et al., 2020). This tool contains reference datasets for many of the gene regions that are commonly targeted in eDNA studies, including the cytochrome oxidase I (COI) gene region that was the focus of the Sapelo Island study, and the 18S rRNA V1-V2 gene region that was targeted in the Bodega Bay study. The Bodega Bay dataset has a total of 168 samples and includes three localities: Campbell Cove (CC), West Park (WP), and Mason's Marina (MM) and two sample types (seagrass or bare sediment). The three sites can be categorized in terms of their disturbance regimes (disturbed or pristine), and salinity and tidal gradients (High, Mid, or Low). Samples were sequenced with Illumina MiSeq 2x300, producing paired-end reads of 300 nucleotides in length.

My amplicon dataset analysis began after the demultiplexing, and quality control steps were carried out by the Bik lab using dada2 software in QIIME2. I was given a feature table 'artifact' and the corresponding metadata file, which I then integrated to create a visualization file that made the data viewable in QIIME2. From there, I explored available analyses that can be performed on amplicon data, including constructing a phylogenetic tree and investigating alpha and beta diversity analysis. I also learned to filter the metadata file to exclude certain variables in the data (e.g. blank, negative control, and mock community samples) to ask specific questions about environmental factors that may have correlated with the amplicon data (Figure 5).

Conclusion

As an undergraduate, I became interested in learning about eDNA because of my interest in conservation biology and my goal to learn and apply techniques for restoring and maintaining healthy habitats. This internship project allowed me to become familiar with an emerging method for the analysis of the composition of a community and track how it changes over time. I have learned new techniques and gained more confidence and experience in field, laboratory, and computational skills. The addition of these skills and experiences aligns with my goal of training in conservation methodologies. My motivations to contribute to the field of conservation biology include the importance of water as a resource and habitat for animals and for the ecosystems they play a part in. This internship has increased my interest in conservation biology, and I will continue to pursue a career in this field.

Figure 1. *Aratus pisonii*, range-expanding mangrove crab (Photo courtesy of Jacob Shalack).

(https://gcelter.marsci.uga.edu/public/app/species_details.asp?id=Aratus%20pisonii).

Figure 2. A-C. Water sampling for eDNA at Long Tabby Creek (DNR dock) on Sapelo Island, GA, USA, June 11, 2023. D. Vacuum filtration set up (front) and peristaltic filtration set up (back) in a UGAMI laboratory.

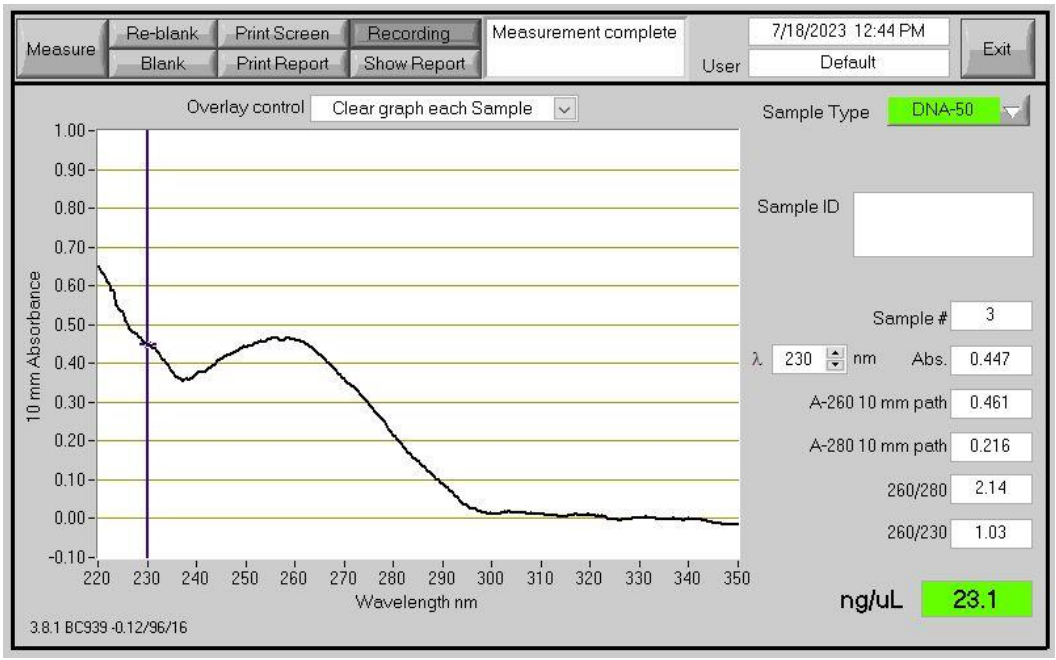

Figure 3. Visualization of extracted DNA, represented by the peak near 260 nm, measured on a Nanodrop spectrophotometer (Model: ND-1000).

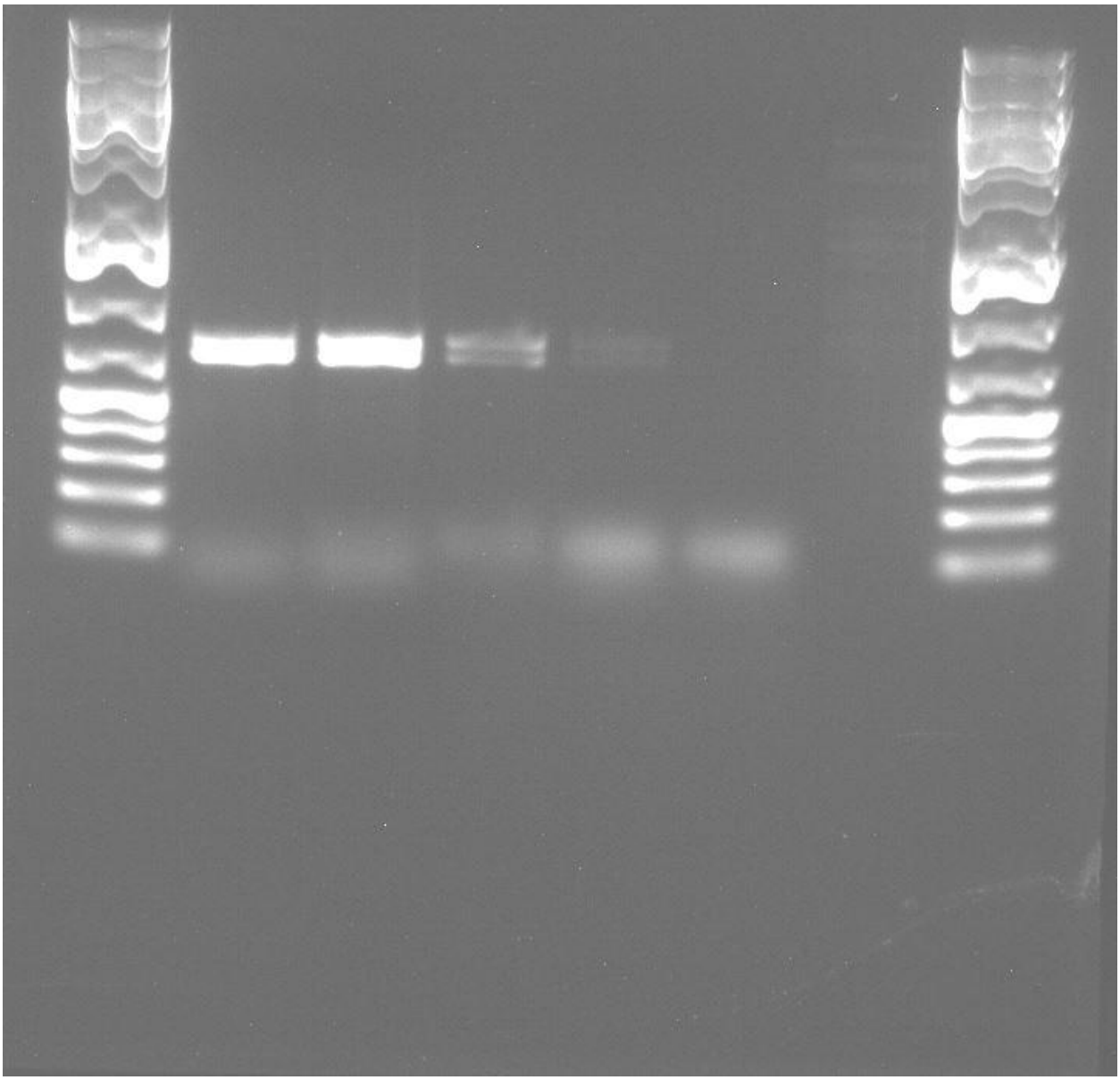

Figure 4. Example of PCR amplification of the *A. pisonii* cytochrome oxidase subunit I gene (COI) on Dec. 22, 2022, using primer pair LCO1490_F and HCO2198_R (Folmer et al., 1994).

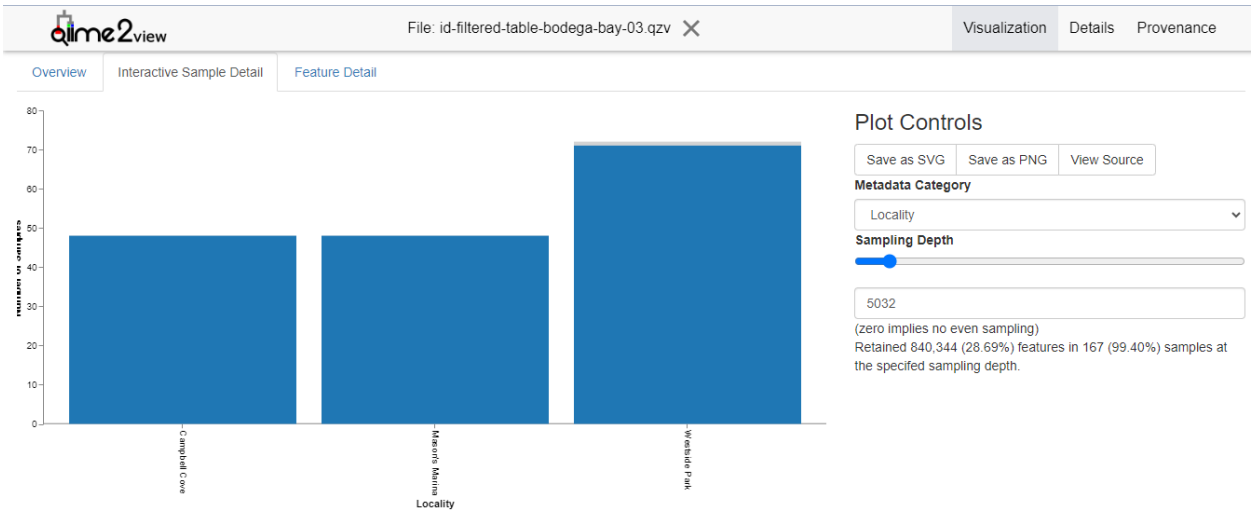

Figure 5. Screenshot of Bodega Bay amplicon data analyzed in Qiime2. This Interactive Sample Detail tab enables the selection of samples based on metadata, in this example by categorizing based on sampling localities.

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