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Article

Optimizing an eDNA assay and field deployment to detect decapod species in O‘ahu streams

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SUMMARY

Environmental DNA (eDNA) survey methods have been used to detect both cryptic and invasive species. Native Hawaiian decapod species (‘opae) are often relatively hard to observe manually due to low density, resulting in the need for the development of more sensitive monitoring and surveillance tools. Native ‘opae have been historically hard to detect, and eDNA methods may allow researchers to detect them when manual surveys cannot. The purpose of our study was to determine if eDNA could either complement or validate stream species presence determined by traditional biomonitoring methods. We hypothesized that an eDNA method would allow for successful detection of decapods, which are ten-legged crustaceans. Using this assay, we were able to successfully detect decapods that aligned with manual monitoring surveys and detected species that were not manually observed. Our research indicates that eDNA can complement manual monitoring techniques and discover cryptic species in the environment.

INTRODUCTION

‘Opae, or native decapod crustaceans, are culturally and biologically important to Hawai‘i (1). These native crustaceans reside in streams and estuaries throughout Hawaii (1). On O‘ahu specifically, there are two native crustacean species, *Macrobrachium grandimanus* (‘opae oeha’a) and *Atyoida bisulcata* (‘opae kala ‘ole) (1). Historical Hawaiian newspaper articles indicate that these native decapod crustaceans were not only used as a food source, but also as bioindicators of stream health (1). However, declines in populations of *M. grandimanus* and *A. bisulcata* have been occurring on O’ahu, largely due to urbanization—specifically the channelization of freshwater streams—and the introduction of invasive species (1). In the past, these species would migrate with the moon phases, but habitat disruptions are suspected to have altered migration patterns, potentially contributing to the decline in their populations (1).

Monitoring the current *M. grandimanus* and *A. bisulcata* populations through manual surveys has been difficult thus far. Traditional manual observations are costly, time-consuming, and require taxonomic expertise, which is a physically demanding and resource-intensive field of study (2). Additionally, the detection of native populations is often difficult due to their low population densities, resulting in the need for more sensitive monitoring and surveillance tools (3). *A. bisulcata* has never been observed while *M. grandimanus* has been minimally observed based on data from Pa‘ēpa‘ē o Waikolu, a program that manually monitors biodiversity on O‘ahu (4). *M. grandimanus* prefer low-flow waterways and are rarely observed in channelized streams in urban Honolulu. *A. bisulcata* has not been observed in these streams, which brings into question the stability of native species populations (1).

Environmental DNA, or eDNA, refers to fragments of DNA that are present in a sampled environmental substrate (5). All species, whether on land or in the water, can leave trace amounts of their DNA in the environment through skin cells, scales, feces, or urine (6). Researchers can use samples from the environment to detect the presence of species based on their DNA (7). Metabarcoding is a method that identifies species from environmental DNA through high-throughput sequencing of a DNA marker (8). eDNA metabarcoding surveys have been used to successfully detect invasive species or rare, native, or endemic species, especially those that are at risk of extinction due to the introduction of non-native species (9, 10). Furthermore, eDNA surveys were previously conducted in the Arctic to assess large-scale marine invertebrate diversity and found a substantial amount of hidden diversity (11).

Although using eDNA to detect the presence/absence of rare species has been well researched, eDNA surveys have not been extensively used to detect decapod crustacean species in Hawaii (6). The use of eDNA surveys, in addition to the traditional manual surveys, may be helpful in determining the absence or presence of native decapod crustaceans. eDNA surveys have the potential to complement or validate the observations recorded during the traditional biomonitoring survey methods. In addition, this approach provides numerous advantages due to its time efficiency and non-intrusive nature (12). Therefore, we sought to test whether eDNA can be used to detect unseen diversity within decapod crustaceans within Hawaiian streams and whether eDNA surveys can effectively supplement manual stream surveys by detecting what cannot be seen.

The purpose of our research was to determine the feasibility of using eDNA as an additional survey tool for the detection of rare native Hawaiian decapods and invasive decapods to support traditional survey techniques while developing a robust protocol for widespread use. We hypothesized that water collection, DNA extraction, PCR conditions, and standardization of sequencing eDNA protocols would allow the successful detection of decapods in environmental samples where these decapods have been manually surveyed. We found that eDNA was able to detect decapod species that

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Stream Sampling Locations	Kaimuki High School		Mānoa Marketplace		Waihi		Waiakeakua	
Monitoring Type	Manual	eDNA	Manual	eDNA	Manual	eDNA	Manual	eDNA
A. bisulcata								
M. grandimanus								
M. lar								
N. denticulatasinensis								
P. clarkii								
Unclassified Species	N/A		N/A		N/A		N/A	

Table 1: Manual and eDNA detection of invasive species and native species correlation. Green represents a species being detected and orange represents that species not detected. N/A = not applicable.

aligned with traditional manual monitoring methods and even a rarer invasive species that manual monitoring did not, although no native species were detected with either method at most sites.

RESULTS

We took eDNA water samples across four stream sites on the same day as the manual monitoring survey **(Table 1)**. We then compared both sets of data to determine species detection **(Table 1).** Through sampling streams using an eDNA approach, we detected the following invasive decapod crustaceans: *Procambarus clarkii* (swamp crayfish), *Neocaradina denticulata sinensis* (cherry shrimp), *Machrobachium lar* (tahitian prawn) using a local database containing 752 DNA sequences, retrieved from GenBank, spanning 35 unique sequences of decapod crustaceans found in Hawaii streams (13).

Our sequencing results showed that while the native decapod crustaceans were not detected using eDNA, various invasive decapods were present in all four streams. *M. lar* (91%) and *N. denticulata sinensis* (8%) were detected at the Kaimukī School study site with 1% unclassified reads **(Table 1)**. We successfully detected *P. clarkii* (2%), *M. lar* (97%), and *N. denticulata sinensis* (1%) with 0.5% unclassified reads using an eDNA approach in the Mānoa Marketplace sample **(Table 1).** *P. clarkii* (13%), *Macrobrachium lar* (74%) and *N. denticulata sinensis* (13%) were detected in Waihī stream **(Table 1)**. *N. denticulata sinensis* (11%) and *P. clarkii* (88%) were detected in Waiakeakua Stream **(Table 1)**. Most importantly, in all of the streams we surveyed, the invasive decapods found in the manual survey were also discovered in the eDNA survey **(Table 1).** Furthermore, our eDNA survey was able to detect species *M. lar* that was not collected during the manual survey, indicating that eDNA be used to detect unseen diversity within decapod crustaceans within Hawaiian streams. We found additional invasive decapod diversity using eDNA in Mānoa Marketplace, Kaimukī, Waihī, and Waiakeakua stream sites **(Table 1).**

Our data suggests that eDNA surveys may effectively supplement manual stream surveys by detecting what cannot be seen. We tested sample collection methods and PCR annealing temperatures prior to experimentation for robust decapod detection. Centrifugation of the water sample provided consistent and detectable amounts of DNA from the DNA extraction. We used touchdown PCR to amplify the 16s rRNA gene. Samples were collected the same day as the manual survey program and processed immediately after collection. We conducted DNA extraction and PCR amplifications in separate locations to avoid contamination.

DISCUSSION

Our study aimed to determine if eDNA could either complement or validate stream species presence determined by traditional biomonitoring methods. Our results supported the hypothesis that an eDNA method would allow for successful detection of decapods as we successfully detected decapods across all four streams. Decapods commonly detected by manual survey methods were confirmed by the eDNA sequencing results. While we were not able to detect native ‘opae using eDNA, we detected various invasive decapods. The eDNA approach consistently identified species manually observed across all sites, and revealed additional decapod diversity across Mānoa Marketplace, Kaimuki High School, and Waihī sites. The relative abundance of the decapods is not quantitative and suggests that species with a higher relative abundance may have a larger population within the streams, shed more DNA, or be close to the spot where the sample was taken (6).

We were able to detect *N. denticulata sinensis* and *M. lar* at the Kaimukī High School site where manual observation did not detect these species. We detected *N. denticulata sinensis* and *M. lar* in Mānoa Marketplace and *M. lar* in Waihī**.** We were not able to detect native ‘opae species using eDNA, which highlights either species absence or lack of ability to detect ‘opae through eDNA techniques. One study focused on the detection of abundant decapod species, but did not detect rare or hard-to-detect species (14). Steps of eDNA monitoring such as DNA extraction, PCR, and sequencing are not without biases, which may impact relative abundance or even detection of a species (15). If there is not enough DNA of the target species present in a sample, results will show that the target species was not detected (15). Our stream eDNA assay indicates that it could be possible that native ‘opae cannot be feasibly detected using eDNA due to a low presence of DNA, as there was low abundance detected manually.

One previous study noted that different sampling locations within a single site were needed for a comprehensive understanding of the species present in a study site (9). Furthermore, some shrimp species undertake seasonal migrations. To account for this, one study detecting *Palaemon paucidens*

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sampled shores during spring-summer and deep waters during the autumn-winter seasons, in accordance with the seasonal migration patterns of the species (14). Similarly, for further work in sampling ‘opae, migration seasons could be studied within the context of urbanization. There is much research work and field work to be done in order to detect native ‘opae using an eDNA approach, but continuous research suggests this method can detect native ‘opae oeha’a with reasonable accuracy.

It is important to mention that native species *(M. grandimanus*) have been regularly found in the Kaimukī study site, but eDNA methods did not detect the endemic species. Yet, tahitian prawn (*M. lar*) were detected, which has almost never been found in Mānoa or Palolo. It is possible that *M. grandimanus* was misclassified *as M. lar* as they are both part of the same genus due to the degradation of the eDNA (15). However, misclassification is highly unlikely as both species have sequences available in the Genbank database and are relatively equally represented in the local database. It is far more likely that *M. grandimanus* was absent or minimally present at the time of sampling.

Some future actions to take within the research and sampling could be studying migration and molting seasons. Urbanization and channelization may have impacted native shrimp migration abilities and times, affecting their detection. If due to species absence, this study suggests that researchers and conservationists should take the next steps to better understand barriers to migration, as the native species is not able to successfully travel upstream. If the barriers to migration are more well understood, local governments can put more effort into reducing and removing infrastructure that is detrimental to the ecosystems on O‘ahu.

With further field deployments in other streams, the pairing of eDNA and manual surveys would provide a more complete picture regarding whether the eDNA approach can be used as an effective supplemental technique in Hawaiian streams. If able to detect native decapod crustaceans using an eDNA approach, the understanding of biodiversity regarding native species on O‘ahu will improve, broadening the implications of this research study. Specific research on endemic species of ‘opae, such as studying migration patterns or mating seasons, can lead to a greater understanding of the biodiversity on O‘ahu. These future studies could also help conservationists and the general public better implement conservation methods to help support native stream species.

There were some limitations to our study, as the samples served as a snapshot in time and not as a representation of all decapod presence within the stream. The quality of water could have also affected filtration methods, meaning that certain site samples could have extracted cleaner DNA than other samples with more sediment/waste, affecting detection results. Furthermore, only one sample was taken per site. Thus, future longitudinal studies with multiple samples taken at different time points and better control of water quality are warranted.

The findings of our study contribute to the field of eDNA, due to its novelty in regard to the combination of location, system, and technique. This method of surveying decapod crustaceans in Hawaii using eDNA has not been explored before and was achieved through optimizing DNA extractions for decapod crustacean detection in Hawaiian freshwater streams and optimizing PCR conditions using MiDeca for freshwater application (16). This study also serves as a preliminary comparison between a manual survey and an eDNA survey in Hawaiian freshwater streams that can be expanded onto future research projects.

In conclusion, based on prior studies detecting decapods using eDNA, it can be reasonably inferred that using an eDNA approach as a tool for biomonitoring is feasible, but further research on the ability to detect endemic species needs to be conducted. Furthermore, the invasive species of decapods detected from this research study suggests invasive species takeover and the potential of using the optimized primers and workflow to further detect decapod DNA presence or absence in stream water. Our research highlights the implications of using eDNA as a supplementary biomonitoring tool. The demonstrated success in detecting decapods can be applied towards other biomonitoring efforts worldwide to confirm and add information to manual monitoring databases.

MATERIALS AND METHODS

Study area and water collection

Water samples were taken from study sites from Mānoa and Palolo streams, specifically Waihī, Waiakeakua, Kaimukī, and Mānoa Marketplace sites. These sample sites were specifically selected because native decapod crustaceans have been recently observed in these locations (11). Four hundred mL of water were collected from the streams specified above. Bottles and caps were submerged and rinsed in the water at the sample location three times prior to sample collection. Samples were transported back to the lab on ice. Upon arrival, samples were centrifuged and the pellet following the centrifugation was kept.

DNA extraction

Either the NEB Monarch Genomic DNA Purification Kit (cat. #T3010) or the Qiagen DNeasy Blood and Tissue Kit (cat. #69504 and #69506) was used to extract genomic DNA and eDNA. We found no differences in the quality of cleaned DNA, and two kits were used as the NEB Monarch Genomic

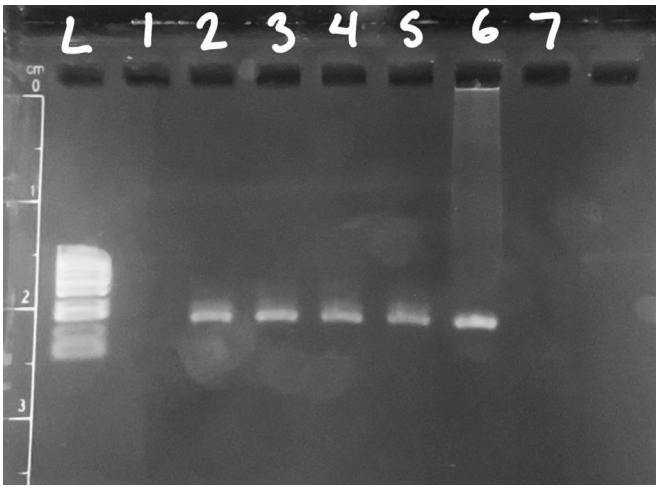

Figure 1: Example gel from Waihi and Waiakeakua study sites in Mānoa stream. L=HyperLadderTM 100bp, 1=negative control, 2=Waihi replicate 1, 3=Waihi replicate 2, 4=Waiakeakua replicate 1, 5=Waiakeakua replicate 2, 6=positive control, 7=negative control.

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DNA Purification Kit was more cost-friendly. gDNA was extracted from a small piece of tissue using one of the kits above to be included as part of a custom database. The eDNA was extracted from the pellet of centrifuged stream water. Extractions were performed following manufacturer protocols with the following modifications when using the Qiagen DNeasy Blood and Tissue Kit: tissue was lysed overnight in 100 µl of Buffer AL at 95 degrees C and DNA was eluted step-wise with 50uL then 35uL of Buffer AE into two separate tubes.

MiDeca primers

The MiDeca primers are decapod-specific primers used for eDNA metabarcoding using tissue-derived DNA extracts (18). These 16S primers were successfully able to classify 250 species, belonging to 186 genera and 65 families across the suborder Dendrobranchiata and 10 of the 11 infraorders of the suborder Pleocyemata (16). The sequence of the MiDeca-forward primer (MiDeca-F) was 5´-GGA CGA TAA GAC CCT ATA AA-3´, and the sequence of the MiDeca-reverse primer (MiDeca-R) was 5´-ACG CTG TTA TCC CTA AAG T-3´ (18).

Standard PCR protocols (0.5 uM of each primer per 25uL reaction) were followed using Hotstart MyTaq (Bioline cat. #490005-938). Touchdown PCR was used to improve amplification for difficult targets. For touchdown PCR, annealing temperature was decreased by 2°C every 2 cycles for 10 cycles, followed by 25 cycles at the lowest annealing temperature at 47°C. 1 uL of ladder and 2 uL of PCR product were loaded onto a 15 mL 2.0% agarose gel stained with 1 uL gel red. Successful PCR was confirmed through gel electrophoresis on a 2.0% agarose gel **(Figure 1)**.

Post-PCR cleaning, quantification, end prep, barcode ligation, and sequencing

The PCR product was purified following standard protocol using Beckman Coulter AMPure XP Beads (cat. #A63880) and quantified using the Invitrogen™ Qubit dsDNA High Sensitivity Assay (cat # Q32854). The PCR product was prepared for sequencing using the Native Barcoding (Cat# NBD-104) and Ligation Sequencing (Cat# LSK-109) kits (Oxford Nanopore Technologies, UK). Sequencing was conducted on the Oxford Nanopore Technology MinION platform using the Flongle Flow Cell. Run length was set to 72 hours, and minimum length cutoff was set to 20 bp. Base-calling was conducted using Guppy v5.0.14 using the "dna_r9.4.1_450bps_hac.cfg" model (Oxford Nanopore Technologies, UK).

Data Processing and Analysis

A custom database of decapod species historically observed at sampling sites was created. Whole mitochondrial genomes were the first choice to include in the database; however, in cases where full mitochondrial genomes were not available, full-length 16S sequences were used (github.com/ehill-iolani/decona-gui). All sequences within the custom database were retrieved from GenBank. The bioinformatic analysis was conducted using the "Decona" pipeline in combination

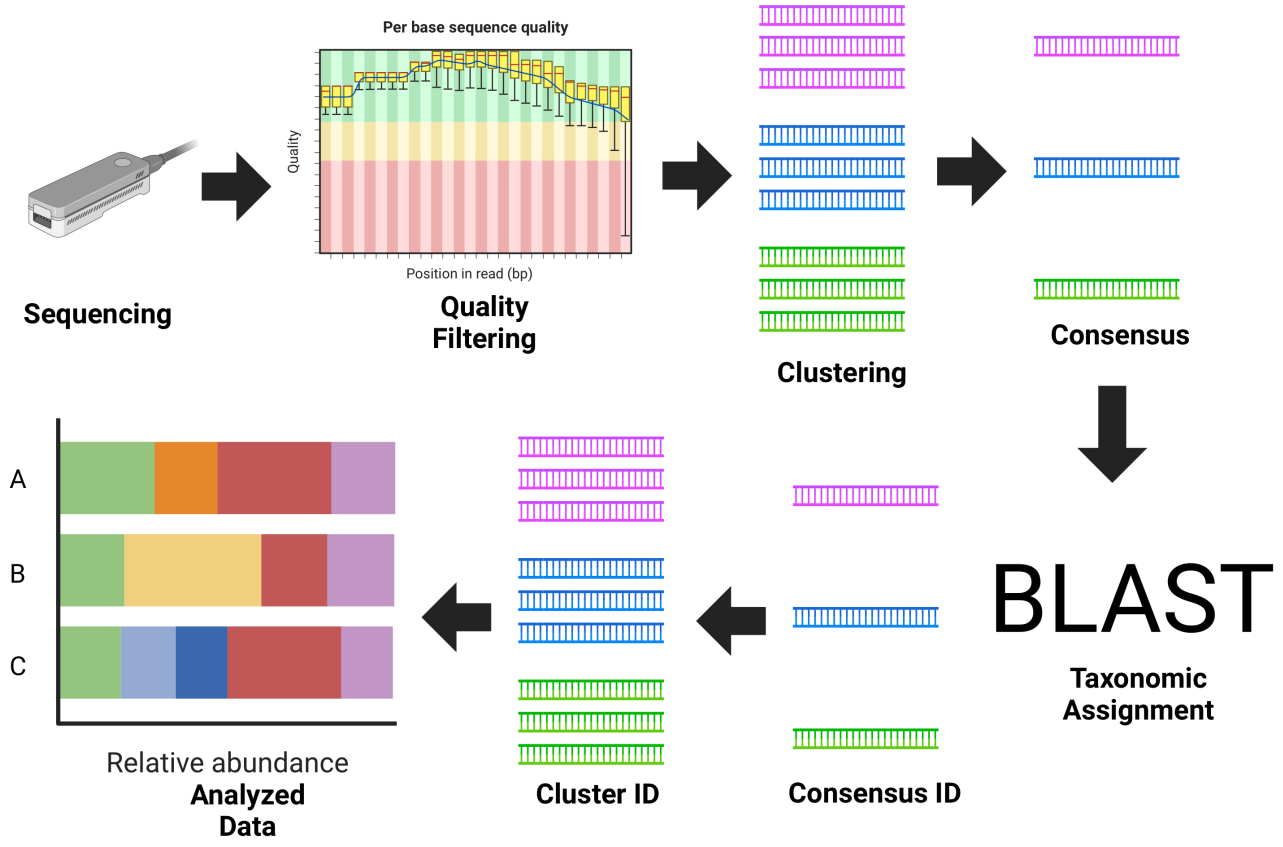

Figure 2: Example gel from Waihi and Waiakeakua study sites in Mānoa stream. L=HyperLadderTM 100bp, 1=negative control, 2=Waihi replicate 1, 3=Waihi replicate 2, 4=Waiakeakua replicate 1, 5=Waiakeakua replicate 2, 6=positive control, 7=negative control.

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with NCBI- BLAST (Basic Local Alignment Search Tool) (17). Briefly, amplicon reads were filtered by quality and length with Nanofilt v2.7.1 by using a Q score > 10 with a size between 170 and 300 bases as thresholds (18). These thresholds ensured that poor-quality reads or off-target sequences would not be clustered. After filtering, reads occurring at >5x frequency with >95% sequence identity based on a k-mer length of 5 were clustered using CD-HIT (19). Clustered reads were aligned using Minimap2 v2.17-r941 (19). Draft consensus sequences were then generated using Racon v1.4.21 (20) and further polished using Medaka v1.1.2 (21). Workflow of data processing and analysis was then visualized using R **(Figure 2)**.

Consensus sequences were searched with BLAST against a local database generated specifically for decapod crustaceans present on O'ahu using species-specific sequences that are publicly available through GenBank. DNA extraction of different decapod crustaceans (Procambarus clarkii, Neocaridina denticulata sinensis, Macrobrachium lar) found in these streams have been completed to help build the sequence database for eDNA analysis in the future. Any consensus sequence that returned a >95% sequence identity from the local BLAST was recorded as an identified species. Consensus sequences returning with <95% sequence identity were marked as "unclassified". The BLAST and the output were processed using a custom R script to extract the size of the read clusters used to generate the consensus sequence for each unique hit (github.com/ehill-iolani/decona_plus). The number of reads per species for each sampling site was then used to determine species presence/absence **(Table 1)**.

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