Amer. Malac. Bull. **38**(2): 129–135 (2021)

Historical DNA from museum shell collections: evaluating the suitability of dried micromollusks for molecular systematics

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Abstract: Natural history collections are an invaluable resource that can inform systematic studies and biodiversity discovery, and also contribute to understanding changes in species abundance and distributions over time. The decline in abundance and diversity of Pacific Island land snails has been a major conservation concern for more than six decades, but only the largest and most colorful snails are protected under the US Endangered Species act, and few are listed as critically endangered on the IUCN Red List. Like most invertebrates, the conservation status of many Hawaiian land snails still need assessment. Molecular data are highly informative for revising species limits and understanding evolutionary patterns and processes, but with as much as 70% of Hawaiian land snails already extinct, few fresh samples are available from which to extract DNA. To overcome the lack of material suitable for DNA barcoding, we test whether short DNA fragments of 225 to 355 bp can be sequenced from museum snail shells containing dried tissues collected more than 50 years ago. Short DNA sequences (225 bp) were obtained from 66.7% of lots, while longer DNA sequences (355 bp) were successfully sequenced from 24.2% of lots. Snail specimens stored in natural history cabinets for more than 100 years were successfully sequenced, supporting the inclusion of these materials for modern biodiversity studies. Molecular data from this study represents a small proportion of Hawaiian microsnail species housed among the millions of specimens in the Bishop Museum in Hawaii and other natural history collections. Additional resources and focused efforts are needed to scale this approach to incorporate many more of the hundreds of snail species in need of assessment in Hawaii. More broadly, there are large representative collections of endangered Pacific Island non-marine snails in many natural history museums that may be suitable for molecular work, either with DNA barcoding or other genomic approaches.

Key words: biodiversity, degraded DNA, invertebrates, micromolluscs, museum

In the current era of rapid biodiversity loss, a fuller understanding of species diversity and biogeography are critical to inform conservation actions (Ceballos *et al.* 2015, McNeely 2002). Invertebrates comprise over 95% of animal species and are integral to functioning ecosystems, but our knowledge of invertebrate diversity and the conservation status of those species is woefully inadequate: only 8.5–10% of mollusk species have been assessed for the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Cowie *et al.* 2017). This reflects both the general scarcity of abundance and distribution data for invertebrates, as well as the paucity of funding currently directed to sustaining this work. Committees and working groups have been attempting to assess the conservation status of these invertebrates, but efforts to protect biodiversity continue to be hampered by inadequate knowledge of species diversity and inaccurate taxonomy (Dubois 2003, Dubois 2011). Known as the Linnean shortfall (Hortal *et al.* 2015), the insufficient knowledge of Earth's biodiversity is unevenly distributed taxonomically with estimates indicating that approximately 3% of land mammals and 33% of amphibian species are undescribed compared to 80% of arthropod species (Giam *et al.* 2012, Stork 2018). Global anthropogenic impacts on native ecosystems are increasing, and striking declines in insects, spiders and other invertebrates continue to make science headlines highlighting the urgency for increased conservation efforts to mitigate further declines in diversity (Eisenhauer *et al.* 2019, Hubbard *et al.* 2014, Rix *et al.* 2017, Seibold *et al.* 2019).

Natural history collections play a critical role in studies to understand species diversity and distributions (Meineke *et al.* 2018). These resources are an incredible trove of data on biodiversity from expeditions to remote localities and are indispensable in documenting and studying rare, threatened, and extinct species. However, historical museum specimens of gastropods and many invertebrates have been difficult to incorporate into molecular systematic studies with standard DNA sequencing approaches. The majority of snails in museum collections are dry shells (Bouchet and Strong 2010, Sierwald *et al.* 2018), and mollusks preserved in wet collections (pre-1990s) were often fixed in formalin solutions that fragment the DNA and cross-link it to proteins, but also modify the nucleotides (Do and Dobrovic 2015, Williams *et al.* 1999). Even if specimens have been directly fixed in molecular grade ethanol, high molecular weight DNA degrades over

time and leads to a decline in the ability to amplify standard loci from samples, even in samples preserved 10 to 20 years ago (Miller *et al.* 2013).

Fortunately, over the last decade, researchers have improved methods to obtain DNA sequences from museum specimens. To date, most historical specimens sequenced from museum collections have been arthropods or vertebrates, for which dried tissues suitable for DNA analysis are readily available (Bi *et al.* 2013, Rowe *et al.* 2011, Staats *et al.* 2013, Tin *et al.* 2014), although some older ethanol-preserved museum specimens have been sequenced as well (Jaksch *et al.* 2016, Derkarabetian *et al.* 2019, Wood *et al.* 2018). Gastropod DNA has been sequenced from dried tissues of a few terrestrial and marine taxa via Sanger sequencing (Korábek *et al.* 2015, Moretzsohn 2001) and genomic approaches (Abdelkrim *et al.* 2018). There has also been success in sequencing DNA from gastropod and bivalve shells (Andree and López 2013, Geist *et al.* 2008, Villanea *et al.* 2016). Sequencing DNA from shells has the added advantage that the shells can be treated chemically prior to DNA extraction to remove fungi and microorganisms (Hawk and Geller 2018). These results show great potential for sequencing degraded DNA from mollusks, however, shell size is probably a contributing factor in the success rate, with large species with thick shells yielding comparatively high amounts of DNA (Geist *et al.* 2008). In comparison, the majority of Pacific Island land snails are micromollusks (Pilsbry and Cooke 1914–1916, Cooke and Kondo 1960) with very thin shells less than 5 mm in length. Sequencing DNA from shells is less likely to be feasible for these terrestrial microsnails due to the thinness of their shells (Martin *et al.* 2021).

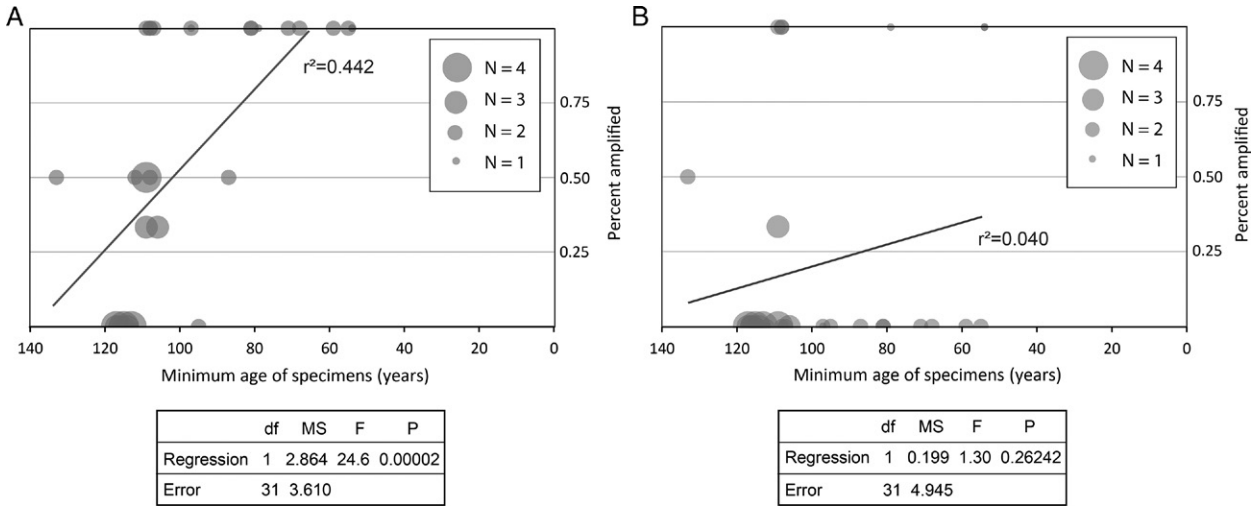
Few Pacific Island land snails have been assessed for their conservation status despite being among the most threatened animal groups globally (Régnier *et al.* 2009, Régnier *et al.* 2015, Chiba and Cowie 2016). The decline in snail abundance and diversity is particularly pronounced in Hawaii, where more than half of the species are presumed to have gone extinct in the last century, and the remaining species are critically endangered (Solem 1990, Yeung and Hayes 2018). DNA sequences have incredible potential for guiding systematic revisions of taxa that have few diagnostic shell features, many of which lack good descriptions of reproductive anatomy that is typically used to delineate species. Mitochondrial sequences like COI are among the loci most frequently sequenced in systematic studies, as they are relatively easy to amplify and are useful for species delimitation of many taxa (DeSalle and Goldstein 2019). Since 2010, extensive surveys across the Hawaiian Islands have searched for remnant populations of native land snails to obtain animals for captive rearing and systematic studies needed for conservation. Unfortunately, many species are no longer found where they were formerly abundant, and the only specimens for study are from the historical collections at museums. Dried tissues remain in many microsnail shells, but the degree of fragmentation of DNA in specimens collected approximately 50 to 120 years ago is unknown.

Dried tissues in mollusk shells are likely to include not only mollusk DNA, but exogenous DNA from microorganisms or fungi, which will be extracted with mollusk DNA. However, these shells are often some of the only samples available for systematic studies of species that are now rare or extinct. As part of a systematic study of Hawaiian land snails we examined the efficacy of obtaining DNA fragments from dried microsnails via DNA extraction and amplification with custom short-fragment primers. Mitochondrial DNA fragments of two different sizes are amplified and we assess how the age of the specimens influences success of DNA sequencing.

MATERIALS AND METHODS

The Bernice Pauahi Bishop Museum (BPBM) malacology collection houses the most extensive collection of Pacific Island land snails, focused heavily on Hawaiian Island species. Shells of *Pacificella* spp. and *Lamellidea* spp. (Achatinellidae: Pacificellinae) in the BPBM dry collection were selected as a case study and examined for dried tissues, which can be visualized through the thin, semi-transparent shells. The specimens chosen for analysis represent a diversity of Pacificellinae species acquired over decades of collecting from the late 1800's to 1967. A "lot" is defined here as a group of specimens of the same taxon (i.e., species) collected at the same time from the same locality. Seventy-five specimens were chosen from 33 lots, with one to three specimens sampled per lot (depending on the number of specimens), but four specimens selected from four of the lots of biogeographic or taxonomic interest. Collection dates were unavailable for most specimens in this study, therefore, the date specimens were added to the museum ledger was used as the minimum age of specimen. All specimens used in this study were recorded in the ledger prior to 1967, of which 5 lots were entered into the ledger after the death of the collector. The minimum age for these 5 lots were therefore based on the year of death for the collectors: A. Garrett (1887), C.M.F. Ancey (1906), and D.D. Baldwin (1912).

Total DNA was extracted using the E.Z.N.A. Mollusk DNA Kit (Omega Bio-Tek) from the shell containing dried tissues, and because most specimens are thin-shelled and less than 3 mm in shell height, the entire specimen was generally dissolved in the process. Prior to extraction, each shell was rinsed with sterile deionized water. Specimens were incubated for three days in in 350 µl lysis buffer containing 200 µg proteinase-K, and 100 µg of proteinase-K were added each day because extended incubation times have been found to increase DNA yield (Silva *et al.* 2019). Genomic DNA was



	df	MS	F	P
Regression	1	2.864	24.6	0.00002
Error	31	3.610		

	df	MS	F	P
Regression	1	0.199	1.30	0.26242
Error	31	4.945		

Figure 1. Regression analyses of the success sequencing short fragments of COI from Pacificellinae species. The size of the circles is proportional to the number of specimens extracted from each lot (between 1 and 4 specimens). A. 225 bp fragments. B. 355 bp fragments.

eluted with 30 µl of elution buffer, which was reapplied to the column membrane for a second elution to increase yield. A negative control without tissue was included with each extraction set to detect any contamination by exogeneous DNA.

Mitochondrial cytochrome *c* oxidase subunit I (COI) sequences from recently collected *Lamellidea* Pilsbry, 1910 and *Pacificella* Odhner, 1922 specimens were used to design custom PCR primers to amplify 225 base pair (bp) and 355 bp fragments of COI from museum specimens. Both combinations of primers were tested on high-molecular weight DNA from recently collected specimens to ensure the primers successfully amplified the targeted regions. Amplified products visualized on a 2% agarose gel stained with ethidium bromide. Single product amplicons were sent to Eurofins Scientific (Louisville, KY, USA) for PCR clean-up, cycle sequencing and electrophoresis on an ABI 3730XL. The DNA chromatograms were checked visually in Geneious v. 9.1.8 (Kearse *et al*. 2012) and consensus sequences were produced from forward and reverse sequences. Sequences for each locus were aligned using MUSCLE, as implemented in Geneious and searched against the GenBank database using BLAST to check for non-mollusk sequences. Sequencing was considered successful if the sequence was obtained in at least one direction and clustered with other Pacificellinae sequences in our phylogenetic analyses. All sequences from this study have been submitted to the Barcode of Life Database (BOLD).

The success in amplifying and sequencing DNA is reported as a proportion of the specimens extracted and the proportion of lots extracted. Sequencing success was plotted in Microsoft Excel (Office 365) against the minimum age of the specimens. The number of specimens from which DNA was extracted, amplified and sequenced is summarized for three-time frames representing specimen minimum age: 50–80 years old (7 lots), 81–110 years old (14 lots), and 110+ years old (12 lots). An analysis of variance (ANOVA) for a simple linear regression was used to examine success of amplification and sequencing based on specimen age using the software Minitab® 19 (www.minitab.com).

RESULTS

All DNA sequences obtained with the custom primers were confirmed as mollusk DNA with a BLAST search against GenBank and clustered with Pacificellinae sequences in our phylogenetic analyses. A few samples amplified initially but failed to sequence and could not be successfully re-amplified. Short 225 bp DNA sequences were obtained from 46.7% of specimens (35/75), with at least one specimen sequenced from 66.7% (22/33) of the lots. Fewer sequences were obtained for the larger, 355 bp, DNA fragments: 14.7% of specimens (11/75) from 24.2% (8/33) of the lots. Amplification and sequencing success of the 225 bp fragment was correlated with specimen age ($r^2 = 0.442$, $p < 0.0001$, Fig. 1A), and samples 50–80 years old were 100% successful (11/11 specimens, 7/7 lots). Samples 81–110 years old were successfully amplified 67.7% (21/31) of the time and sequenced 61.3% (19/31) of the time, and at least one sequence was obtained for 92.9% of lots (13/14). With the oldest specimens, 110–140 years old, only 6.1% of samples amplified and sequenced successfully (2/33), with a sequence obtained from 16.7% of lots (2/12).

Fragments of 355 bp were successfully sequenced from a smaller number of specimens. Samples 50–80 years old were successfully amplified and sequenced from only 3 of 11 (27.3%) specimens, representing 43% (3/7) of extracted lots.

Samples 81–110 years old were successful in 22.6% (7/31) of specimens, or 29% (4/14) of lots. Only 3% (1/33) of specimens 110–140 years old amplified and sequenced successfully, which was 8% (1/12) of lots. However, the age of the specimens was not a significant predictor ($r^2 = 0.040$, $p = 0.262$) of amplification success for the 355 bp fragment (Fig. 1B).

DISCUSSION

Over time, DNA in collected specimens degrades into smaller fragments. As a result, DNA in museum specimens is often less than 500 bp in length (Cooper 1994), shorter than the regions often used for DNA barcoding (Hebert *et al.* 2003) and is present in lower concentrations (McGaughran 2020). Extraction, amplification and sequencing of DNA from dried tissues in this study were successful for nearly half (46.7%) of all specimens attempted for the short 225 bp DNA fragment, and 14.7% of the time for the larger 355 bp fragment. The amplification success of the larger DNA fragment is slightly lower than the 20% success for a 313 bp fragment from dried cowry tissue (Moretzsohn 2001). This difference in amplification success might be attributed to the 42 bp larger fragment used in this study, differences in extraction protocols in the removal of PCR inhibitors, or the fact that cowries are larger snails that may have more tissues remaining in their shells than microsnails.

Few studies have investigated DNA quality in museum collections of mollusks, and only one included a variety of medium to large sized ethanol-preserved mollusks. Jaksch *et al.* (2016) found that a 400 bp fragment of mitochondrial DNA (16S) could be amplified from 36% of specimens collected before 1900 and 40.5% of samples overall, with high variability in PCR success. Additional studies are needed to optimize DNA extraction and amplification approaches for historical gastropod samples, but the present study demonstrates the feasibility of obtaining DNA sequences from tissues in dry collections of micromollusks.

The almost complete lack of amplification from specimens more than 110 years old was unexpected given the success with samples between 50 to 100 years old. A decline in amplification success would be expected with the fragmenting of DNA over time, but the abrupt change in amplification success with samples catalogued a few years later could indicate that other factors have contributed to DNA degradation in those samples. Naturally, the absence of precise collection dates makes it difficult to ascertain the true relationship between specimen age and DNA fragmentation, but the observed pattern is still notable. Notes in the Bishop Museum ledger indicate that some of the lots now over 110 years old were previously treated with ether and alcohol to kill fungus. Fungus might be expected in shells if the snail had already died prior to collection but remained in moist leaf litter. If samples partially rotted before being dried, DNA would be more highly degraded in these samples.

The sudden decline in amplification success at 110 years age is reflected in the significant relationship between age of the specimen and amplification of 225 bp fragments ($r^2 = 0.442$, $p < 0.0001$, Fig. 1A). However, there was no significant relationship between age and amplification success of 355 bp fragments, at least within the time frame investigated. If specimens between 20 and 50 years were available for this study, we might expect that a greater proportion of those samples would amplify and that a stronger relationship between age of specimens and amplification success could emerge. Unfortunately, there was a decline in field collecting of mollusks including Pacific Island land snails in the 1960s, around the same time that declines in collection effort were noted in some other US Natural History Museums (Pearce and Arnold 2016).

The oldest specimens extracted for this study were collected by A. Garrett during expeditions to the Society Islands in 1857 and 1860–1863. He also lived in Huahine (an island within the Polynesian Society Islands) from 1870–1887 (Thomas 1979). Despite the considerable age of these specimens, a 355 bp DNA fragment was successfully amplified and sequenced for 1 of 2 of the extracted specimens, and there was faint amplification of the second specimen. That both of these samples showed some amplification is unusual in our dataset; none of the other specimens over 115 years had any visible amplification, while the second Garrett specimen might be sequenced with further troubleshooting (e.g., concentrating PCR product from multiple PCRs). It is unclear why DNA in tissue within Garrett's collected specimens is better preserved than the other samples of comparable age.

In this study, any remaining body tissues of micromollusks were dissolved along with their thin shells, and it is possible that the DNA sequenced was from either the shell or the body tissues within the shells. Although the thinness of the shells suggests that there would be little DNA present in micromollusk shells for amplification (Martin *et al.* 2021), this remains to be tested using micromollusk shells that have been cleaned of all remaining body tissues. However, the inability to distinguish the source of the DNA in this study does not detract from the findings that museum specimens are a valuable source of DNA for studying biodiversity.

The short-fragment primers developed for this study were also tested on tissue samples of Pacificellinae specimens collected 10 years ago and sacrificed by drowning prior to fixation/preservation in ethanol. A sample of 18 snails from different collection sites produced no DNA amplification for either a 225 bp or 125 bp fragment of COI, indicating substantial DNA degradation. This is consistent with the findings by Schander and Hagnell (2003) who demonstrated that drowning snails before preservation degrades DNA

and even as little as 30 minutes results in noticeable degradation compared to preserving the snail directly in alcohol. An alternative method of preparing snails for preservation is the *Niku-Nuki* method, which instantly kills the snails so they can be quickly preserved in ethanol for molecular and morphological analyses (Fukuda *et al*. 2008). This also has the advantage of denaturing DNAases and killing fungi and bacteria that can cause additional degradation when a snail dies from drowning.

The amplification of 225 bp to 355 bp DNA fragments in this study indicates that dried mollusk specimens are suitable for molecular systematic studies, including genomic approaches using high throughput sequencing. The PCR-based approaches for sequencing short fragments of DNA from degraded samples are most practical for sequencing small numbers of specimens, as it does not entail the initial cost-investment of producing DNA libraries or anchored-hybrid enrichment probes. Studies with greater numbers of specimens can take advantage of multiplexing PCR products to reduce sequencing costs with high throughput approaches (Cruaud *et al*. 2017). The limitations of this approach are that short DNA sequences obtained using a PCR-based approach contain few informative sites for phylogenetic analysis, and that using this method to sequence multiple loci is laborious. Nonetheless, short sequences obtained via PCR can be useful when incorporated into a larger dataset. As DNA sequencing technology and genomic techniques continue to advance, genomic approaches to obtaining DNA from museum specimens will increasingly be utilized (Nachman 2013) to obtain larger amounts of data at lower cost, facilitating biodiversity studies across broader timescales.

It is promising that DNA sequences can be obtained from dried microsnails in museum collections, as the Pacific Island land snails are facing a conservation crisis (Chiba and Cowie 2016), and the taxonomic and conservation status of many microsnails remains uncertain. These results obtained here indicate that dried microsnails in the Bishop Museum collection have the potential to yield DNA fragments of 225 bp or larger and can provide data needed to begin assessing their historical and contemporary diversity. Genetic data alone cannot alleviate the taxonomic impediment (Dubois 2011) but may provide a powerful tool to assist in delineating species and evaluating current species distributions. In the face of the biodiversity crisis, shell collections should be re-examined as a potential source of genetic data, particularly for rare and threatened species.

ACKNOWLEDGMENTS

We thank numerous volunteers, interns, staff and affiliated researchers working in the Malacology Collection and Pacific Center for Molecular Biodiversity at Bishop Museum, especially collections managers Jaynee R. Kim and Kelli DeLeon, as well as the broader malacology conservation community in Hawaii for their tireless dedication to improving our understanding and conservation of the highly imperiled land snail fauna. Recently collected snails used as part of this study were approved under DLNR scientific permits for native invertebrate research (FHM11-264, FHM12-294, FHM13-324, FHM15-357, FHM16-401, FHM17-426, I1053, I1271). Funding for Hawaiian land snail research and this project was provided by NSF (DBI-1902328, DEB-1656231, DBI-1561774, to NWY, KAH; DEB-1120906 to KAH). This is Contribution No. 2001-002 to the Bishop Museum Hawaii Biological Survey.

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Submitted: 01 February 2021; **Accepted:** 03 May 2021; **Published:** 16 December 2021