Reviving Galápagos snails: Ancient DNA extraction and amplification from threatened Galápagos endemic land snail species

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ABSTRACT

Snail shells represent an abundant source of information about the organisms that build them, which is particularly vital and relevant for species that are locally or globally extinct. The long-term persistence of snail shells in the environment or in collections allows for the inference of morphological, ecological, and in some cases genetic information on multiple temporal scales. Access to genetic information from snail shells can thus be a valuable asset, yet previous protocols for extraction of DNA from empty shells have met with extremely low success rates, particularly from shells weathered from long term exposure to environmental conditions. Here processed 35 snail shells collected from the Galápagos islands, some from species that have not been observed alive in the past 50 years. We amplified and sequenced short fragments [≤ 259 base pairs (bps)] of mitochondrial DNA from 18 specimens. Our results indicate that the implementation of an ancient DNA (aDNA) extraction protocol and thoughtful primer design to target short DNA fragments can result in a high success rate of recovering mitochondrial DNA data from such specimens, providing support for the use of such methodologies to supplement phylogenies of living land mollusks.

The Galápagos islands are well known for the inspirational role they played in the development of the theory of evolution by natural selection (Darwin, 1859). Importantly, being among the best-preserved and protected oceanic archipelagos in the world, the Galápagos islands continue to serve as a natural laboratory to address key questions in evolutionary biology. With 69 described species, Galápagos endemic land snails of the genus *Naesiotus* (Bulimulidae) represent the most species-rich adaptive radiation of these islands. The Galápagos *Naesiotus* species have diversified in morphology and ecology, presumably adapting to a wide range of ecological conditions. Species are found on all major islands, and have successfully colonized all vegetation zones except for the littoral zone (Parent & Crespi, 2006). The last 40 years have been marked by dramatic declines in most Galápagos snail populations, making it very difficult, if not impossible in some cases, to find live individuals in the field. Given the population declines, museum collections represent an invaluable source of information for these species, as preserved specimens can be used to collect morphological, ecological (based on sampling locations), and genetic data. However, extraction and amplification of DNA from preserved tissue has remained challenging mainly because well-preserved animal soft tissue, material suitable for extraction of genetic material is rarely available. Nonetheless, “empty” mollusk shells have been preserved in great numbers in museum collections and are still recoverable directly from the field. While the extraction of genetic material from mollusk shells is not a novel concept, previous studies have been met with limited success, particularly when attempting extraction from weathered and/or subfossil shells (Andree & Lopez, 2013; Caldeira et al., 2004; Geist, Wunderlich & Kuehn, 2008).

RESULTS

Table 1. Amplification success for the two extraction methods used in this study. Amplification was attempted up to four times for each sample. **a)** Refers to the first 28 shells extracted, 14 for each method, **b)** “Direct comparison” refers to the 7 additional snail shells which were each split in half and tested using both methods. **c)** “All specimens” lists results for the total 35 shells used in this study.

		Phenol-Chloroform		Fast method		Both Methods	
a)	**First set of specimens (28)**	**Samples (n)**	**Percentage (%)**	**Samples (n)**	**Percentage (%)**	**Samples (n)**	**Percentage (%)**
	Total samples	14	100	14	100	28	100
	Successful amplification (any primer set)	8	57	7	50	14	50
	Successful amplification (all primer sets)	1	7	2	14	3	11
	Inhibited samples	5	36	11	79	16	57
	Long amplicon (244bp)	2	14	3	21	6	21
	Medium amplicon (189bp)	5	36	4	29	9	32
	Short amplicon (157bp)	5	36	4	29	9	32
b)	**Direct comparison specimens (7)**	**Samples (n)**	**Percentage (%)**	**Samples (n)**	**Percentage (%)**	**Samples (n)**	**Percentage (%)**
	Total samples	7	100	7	100	7	100
	Successful amplification (any primer set)	3	43	3	43	3	43
	Successful amplification (all primer sets)	2	29	2	29	2	29
	Inhibited samples	1	14	7	100	7	100
	Long amplicon (244bp)	2	29	1	14	2	29
	Medium amplicon (189bp)	2	29	2	29	2	29
	Short amplicon (157bp)	3	43	3	43	3	43
c)	**All specimens (35)**	**Samples (n)**	**Percentage (%)**	**Samples (n)**	**Percentage (%)**	**Samples (n)**	**Percentage (%)**
	Total samples	21	100	21	100	35	100
	Successful amplification (any primer set)	10	48	10	48	17	49
	Successful amplification (all primer sets)	3	14	4	19	5	14
	Inhibited samples	6	29	18	86	24	69
	Long amplicon (244bp)	4	19	4	19	8	23
	Medium amplicon (189bp)	7	33	6	29	11	31
	Short amplicon (157bp)	8	38	7	33	12	34

DISCUSSION

Following the low success rates of snail shell DNA recovery in previous literature, we propose that the successful analysis of such DNA can be markedly improved by following ancient DNA (aDNA) methodologies, as we believe DNA of sufficient quality for PCR amplification from snail shells can indeed remain intact for long periods of time. In this study, we performed DNA extractions from 35 land snail shells collected from the Galápagos islands. Some of these shells correspond to species that have not been observed in the last 50 years, and may have become extinct in the interim. We describe and compare two simple protocols for the extraction of DNA from weathered snail shells, and amplify and sequence a 472 bp portion of COI of the mitochondrial genome in three shorter, overlapping fragments. Sequences from these specimens were placed in a phylogenetic tree illustrating the evolutionary relationships among extant Galápagos land snails of the same genus. The contrast between the extremely low success rates of snail shell DNA recovery in previous literature, and the high success rate employing aDNA protocols is highly suggestive that snail shell DNA should be treated as aDNA, regardless of the age or state of the sample. Based on the differential amplification success of the different-length fragments, we suggest that efforts for amplification of aDNA extracted from shells be directed to primer sets targeting small amplicons (for example, in our study ≤259 bps), which is common in aDNA protocols. The high inhibition rate associated with the samples studied here is indicative of snail shells containing PCR inhibitors which are co-extracted with DNA. The Kemp et al 2007 method was found to be better suited to remove co-extracted PCR inhibitors. It should be noted, however, that inhibitors were successfully removed from all samples after one or two additional rounds of silica extractions, regardless of which extraction method was employed.

Figure 1. Examples of extant species of the genus *Naesiotus* (Bulimulidae), endemic to the Galápagos islands, and snail shells collected in the field before extraction. Discoloration from exposure to the elements was characteristic of the specimens used in this study. Photographs courtesy of Christine E. Parent.

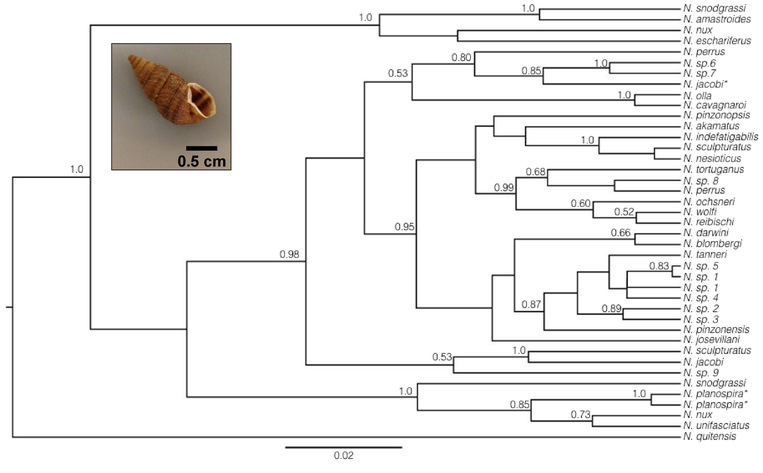

Figure 2. Maximum clade credibility tree showing phylogenetic placement of three threatened snails sequenced in this study (marked with asterisks) with extant species from the Galápagos Islands. Clade support (as probability value) is indicated above the nodes with greater than 0.5 probability support.

METHODS

Snail shells were collected during fieldwork conducted between 1965-1966 and 2000-2014 on the islands of Santa Cruz, Isabela, Floreana, Gardner (near Floreana), Pinzon, and Rabida. All shells were collected from the ground, stored in plastic vials at room temperature until they were used in our study. Twenty-eight snail shells were divided into two sets of 14, each set used to evaluate one of the two extraction methods described below. An additional seven shells were mechanically broken down with a hammer, and the pulverized product divided into approximate halves by volume. This was done to directly evaluate two extraction methods on the same samples. The two extraction methods used in this study are described in detail in:

Phenol-Chloroform:
Kemp et al. 2007 Genetic analysis of early holocene skeletal remains from Alaska and its implications for the settlement of the Americas. American Journal of Physical Anthropology, 132: 605-621.

Fast method
Cui et al. 2013 Ancient DNA Analysis of Mid-Holocene Individuals from the Northwest Coast of North America Reveals Different Evolutionary Paths for Mitogenomes. PLoS ONE, 8: e66948.

To test for the presence of co-extracted inhibitors in the DNA extracts, every sample was tested through PCR against an internal positive control (IPC). The IPC constituted of 1.5 µL of DNA extracted from ancient goose remains which was added to every PCR reaction, as described below, and amplified using goose specific primers instead of snail primers, as described in detail in Kemp et al. (2014, for a schematic illustration, see Fig. 1). To test the efficiency of PCR for targets of different sizes we targeted fragments spanning 244, 189, and 157 bp in length (referred to the amplicons they produce as long, medium, and short fragments).

To amplify a portion of the COI region, we performed 15 µL PCRs following Kemp and colleagues (2007). Negative controls (PCR reactions to which no DNA template was added) accompanied every set of PCRs to monitor the presence of contaminating DNA. This was done in addition to the extraction negative controls, which also accompanied every set of PCRs. We used samples of DNA extracted from soft tissues of Naesiotus specimens as PCR positive controls, which we kept and added to the PCRs in a separate laboratory, just prior to initiating the reactions. We used NCBI’s BLAST tool on all amplicons to confirm taxonomic identity of the sequences. Because we expect our specimens to represent threatened or extinct species that have never been sequenced before, we considered sequences whose closest match was in the genus *Naesiotus* to confirm the aDNA is endogenous to the shell material.

We constructed a phylogenetic tree to determine the evolutionary relationship of the shell specimens in this study relative to other Galapagos Naesiotus species. In addition to the sequences we generated here, we used the partial mtDNA COI sequences of 37 Galapagos Naesiotus species and the same partial mtDNA COI sequence from N. quitensis, a species occurring on mainland Ecuador, as an outgroup (Parent and Crespi 2006).

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