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Reviving Galápagos snails: ancient DNA extraction and amplification from shells of probably extinct Galápagos endemic land snails

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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. Access to genetic information from snail shells can be valuable, 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 we present two simple protocols for the extraction and amplification of DNA from empty land snail shells from specimens of Galápagos endemic snails, including presumably extinct species. We processed 35 shells of the genus *Naesiotus* (Bulimulidae) from the Galápagos islands, some from species that have not been observed alive in the past 50 years. We amplified and sequenced short fragments ($\leq$244 bp) of mitochondrial DNA (mtDNA) from 18 specimens. Our results indicate that the implementation of an ancient DNA extraction protocol and careful primer design to target short DNA fragments can result in successful recovery of mtDNA data from such specimens.

INTRODUCTION

Island systems are well known for their remarkable biological diversity (e.g. Kier *et al.*, 2009; Mahler *et al.*, 2013). The lineages that successfully colonize oceanic archipelagos often diversify into a range of species with greater morphological and ecological diversity than their relatives from the mainland (Schluter, 2000, but see Gavrilets & Losos, 2009). Isolation and fragmented landscape are among the factors that promote this diversification, and these factors are also responsible for making island species more vulnerable to extinction. Therefore, although island systems are often viewed as great natural laboratories, their biotas are disproportionally represented on conservation lists.

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* (sometimes erroneously grouped under the genus *Bulimulus*; family Bulimulidae) represent the most species-rich adaptive radiation of these islands (Parent, Caccone & Petren, 2008). Whereas species belonging to *Naesiotus* on the mainland are mostly ground dwellers and are generally restricted to a narrow range of habitats, the Galápagos *Naesiotus* species have diversified in morphology and ecology, presumably adapting to a wide range of ecological conditions (Parent & Crespi, 2009). 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 (Coppois & Wells, 1987; Parent, personal observation), 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, as is the case for most invertebrate groups, well-preserved animal soft tissue, material which many would agree is a suitable source for extraction of genetic material, is rarely available. Nonetheless, 'empty' mollusc 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 mollusc shells is not a novel concept, previous studies have met with limited success, particularly when attempting extraction from weathered and/or subfossil shells (Caldeira *et al.*, 2004; Barsh & Murphy, 2008; Geist, Wunderlich & Kuehn, 2008; Andree & Lopez, 2013).

Recently, Andree & Lopez (2013) attempted the amplification of mitochondrial DNA (mtDNA) extracted from two shells collected 10 years earlier, morphologically identified as *Pomacea*

canaliculata (Table 1). One shell was in good condition, whereas the second showed clear signs of weathering. Amplification of a 300-bp fragment (as described by Andree & Lopez, 2013, the actual amplicon size was 279 bp; Table 1) of cytochome oxidase subunit I (COI) was successful only for the former sample. The authors used a redesigned primer (PomaR), instead of that initially used (HCO2198, Folmer *et al*., 1994), and the molecular sequence confirmed the morphological identification as *P. canaliculata*. Both samples failed to produce 700-bp amplicons (actual amplicon size 666 bp; Table 1). These results suggest that the DNA preserved in the specimens was significantly degraded with regard to strand length, similar to the classic observation for ancient DNA (aDNA) studies (Pääbo, 1989, 1990).

A few additional points about the Andree & Lopez (2013) study are worth noting. The first concerns their primer design and description of amplicon sizes produced from PCR. The authors were surprised that the sample identified as *P. canaliculata* failed to amplify with a 'species specific' forward primer (i.e. PcanCOI, Matsukara *et al*., 2008). The molecular identification was successful only with a forward primer designed for *P. insularum* (i.e. PinsCOI, Matsukara *et al*., 2008). While designing primers to be species specific is itself a challenging goal, Andree & Lopez (2013) failed to note that the primer designed by Matsukara *et al*. (2008) to amplify sequence of *P. canaliculata* anneals 302 bp upstream from the primer designed to amplify *P. insularum* (Matsukara *et al*., 2008: fig. 3). Thus, regardless of the specificity of the forward primer, their results demonstrate that amplification of a 555-bp fragment was not possible (PinsCOI-PomaR; Table 1), whereas amplification of 279 bp was successful (PcanCOI-PomaA; Table 1). The results of Andree & Lopez (2013), summarized in Table 1, are consistent with an expectation of an inverse relationship between fragment size and successful PCR amplification when working with degraded DNA (Pääbo, 1989, 1990; Cooper & Poinar, 2000).

Furthermore, it is important to note that the failure to amplify an amplicon, such as that observed by Andree & Lopez (2013), does not alone indicate the absence of template molecules for PCR. Co-extracted impurities present in DNA extracts can inhibit PCR, sometimes causing the reaction to fail entirely. The role that these PCR inhibitors play in the study of degraded DNA is largely underappreciated and should be considered (Kemp *et al*., 2014). The success of amplifying DNA from ancient and degraded specimens can be maximized when these PCR inhibitors are minimized (Grier *et al*., 2013; Kemp *et al*., 2014).

Following the low success rates of DNA recovery from snail shells by Andree & Lopez (2013) and others, we propose that the successful analysis of such DNA could be improved by following aDNA methodologies, as we believe DNA of sufficient quality for PCR amplification from snail shells can remain intact for long periods of time. Numerous reviews describe at length the typical characteristics of aDNA and the general methods utilized in their study (e.g. Pääbo *et al*., 2004; Gilbert *et al*., 2005; Gilbert, 2006; Der Sarkissian *et al*., 2014) and a full review is beyond the scope of this paper. In brief, aDNA has physical and chemical properties unlike DNA from still-living cells. Following death, DNA is subject to degradation and chemical modification from nucleases, microorganisms, hydrolysis, UV radiation and oxidation. When these forces fail to destroy the DNA completely, aged and degraded specimens may retain molecules in low copy number that are of short strand length and typically exhibit miscoding lesions which can appear as 'mutations' when, in fact, they are artifacts. Using aDNA methods involves paying close attention to contaminating exogenous DNA that can originate from modern (e.g. Barta, Monroe & Kemp, 2013) and ancient (e.g. Noonan *et al*., 2005) sources, and the co-extraction of PCR inhibitors (e.g. Kemp *et al*., 2014). As faithful replication can be key to authenticating aDNA results, it is particularly important that efforts to extract aDNA from mollusc shells employ independent amplification. For example, Andree & Lopez (2013) determined their first sample to be *P. canaliculata* based on amplification from two separate extracts that yielded identical sequences.

In this study, we performed DNA extraction 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 used two simple protocols for the extraction of DNA from weathered snail shells, and amplified and sequenced 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 of extant Galápagos *Naesiotus* species as a means to confirm their authenticity.

Table 1. PCR amplification results from Andree & Lopez (2013). See main text for discussion and references for each primer.

Amplicon size	Forward primer	Reverse primer	Successful amplification?	
			Sample 1	Sample 2
279	PinsCOI	PomaR	Yes	No
390	PinsCOI	HCO2198	No	No
555	PcanCOI	PomaR	No	No
666	PcanCOI	HCO2198	No	No

MATERIAL AND METHODS

Sample collection and preparation

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 (Table 2). All shells were collected from the ground and stored in plastic vials at room temperature until they were used in our study.

All extractions and PCR preparations were conducted in a dedicated aDNA facility at Washington State University (WSU), with amplification and post-PCR processing conducted in a laboratory located in a separate building. Each shell was weighed using an analytical balance and photographed (for an example, see Fig. 1). 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 evaluate both methods on the same samples.

Kemp et al. (2007) DNA extraction method

Each shell was rinsed once or twice with DNA-free water (Gibco) to remove particulates, then transferred to a 15 ml conical tube and demineralized by gentle rocking in 2 ml molecular grade 0.5 M EDTA (pH 8.0) for 2 d at room temperature. This process was sufficient for the complete dissolution of the shell. For every extraction set, an additional tube was added that contained no shell material, as an extraction negative control, which underwent the same extraction process as the samples. Approximately 60 U of Proteinase K (Invitrogen, Fungal Proteinase K, >20 U/mg) were added to each sample, followed by incubation at 65 °C for 3 h. DNA was first extracted by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to the EDTA, which was then vortexed briefly and centrifuged at 3,100 rpm for 5 min. The aqueous phase was removed and subsequently extracted

Table 2. Specimen reference, species, island, year collected, successful amplification (of any primer set), presence of PCR inhibition (detected through amplification of goose IPC) and number of additional silica purifications necessary to remove PCR inhibition for the 35 *Naesiotus* shells used in this study.

Specimen	Species	Island	Year collected	Successful amplification	Inhibited PCR	Number of additional silica purifications	Closest BLAST match
Kemp *et al.* (2007) method							
CP 1.1	*N. rabidensis* (Dall, 1917)	Rabida	2012	Yes	No	0	NA
CP 1.2	*N.* unidentified sp.	Sta Cruz	2012	No	No	0	NA
CP 1.3	*N.* unidentified sp.	Floreana	1965	Yes	Yes	2	*Bulimulus olla*
CP 1.4	*N. ustulatus* (Sowerby, 1833)	Floreana	2010	Yes	Yes	2	NA
CP 1.5	*N. adelphus* (Dall, 1917)	Sta Cruz	1965	No	No	0	NA
CP 1.6	*N. tortuganus* (Dall, 1893)	Isabela	1965	Yes	No	0	*B. perrus*
CP 1.7	*N.* unidentified sp.	Isabela	1966	Yes	No	0	*B. perrus*
CP 2.1	*N. duncanus* (Dall, 1893)	Pinzon	2012	No	No	0	NA
CP 2.2	*N. duncanus*	Pinzon	2012	No	No	0	NA
CP 2.3	*N. duncanus*	Pinzon	2012	Yes	Yes	1	NA
CP 2.4	*N. planospira* (Ancey, 1887)	Floreana	2010	No	Yes	1	NA
CP 2.5	*N. planospira*	Floreana	2010	No	Yes	1	NA
CP 2.6	*N. lycodus* (Dall, 1917)	Sta Cruz	1966	Yes	No	0	*Bulimulus* sp.
CP 2.7	*N. lycodus*	Sta Cruz	1966	Yes	No	0	*Bulimulus* sp.
WSU 'fast' method							
CP 3.1	*N. lycodus*	Sta Cruz	1966	Yes	Yes	1	*B. pinzonensis*
CP 3.2	*N. jacobi* (Sowerby, 1833)	Isabela	1965	No	Yes	1	NA
CP 3.3	*N. jacobi*	Isabela	1965	No	Yes	1	NA
CP 3.4	*N. albemarlensis* (Dall, 1917)	Isabela	1965	No	Yes	1	NA
CP 3.5	*N. albemarlensis*	Isabela	1966	No	Yes	1	NA
CP 3.6	*N.* unidentified sp.	Floreana	1966	No	Yes	1	NA
CP 3.7	*N.* unidentified sp.	Floreana	1966	No	Yes	1	NA
CP 4.1	*N.* unidentified sp.	Sta Cruz	2010	Yes	Yes	2	NA
CP 4.2	*N.* unidentified sp.	Sta Cruz	2010	Yes	No	0	NA
CP 4.3	*N.* unidentified sp.	Sta Cruz	2010	Yes	No	0	*B. pinzonopsis*
CP 4.4	*N. planospira*	Floreana	2010	No	Yes	2	Contamination (insect)
CP 4.5	*N. planospira*	Floreana	2010	Yes	Yes	2	NA
CP 4.6	*N. lycodus*	Sta Cruz	2010	Yes	Yes	2	*B. pinzonensis*
CP 4.7	*N. jacobi*	Isabela	2010	Yes	No	0	*B. pinzonensis*
Kemp *et al.* (2007) method							
CP 5.1a	*N. planospira*	Gardner	2004	No	No	0	Contamination (insect)
CP 5.2a	*N. planospira*	Gardner	2004	No	No	0	Contamination (insect)
CP 5.3a	*N. planospira*	Gardner	2004	No	No	0	NA
CP 5.4a	*N. planospira*	Gardner	2004	Yes	No	0	*B. pinzonensis*
CP 5.5a	*N. planospira*	Gardner	2004	Yes	No	0	*B. unifasciatus*
CP 5.6a	*N. planospira*	Gardner	2004	Yes	No	0	*B. nux*
CP 5.7a	*N. planospira*	Gardner	2004	No	Yes	1	NA
WSU 'fast' method							
CP 5.1b	*N. planospira*	Gardner	2004	No	Yes	2	NA
CP 5.2b	*N. planospira*	Gardner	2004	No	Yes	2	Contamination (insect)
CP 5.3b	*N. planospira*	Gardner	2004	No	Yes	2	*B. pinzonensis*
CP 5.4b	*N. planospira*	Gardner	2004	Yes	Yes	2	*B. pinzonensis*
CP 5.5b	*N. planospira*	Gardner	2004	Yes	Yes	2	*B. unifasciatus*
CP 5.6b	*N. planospira*	Gardner	2004	Yes	Yes	2	*B. nux*
CP 5.7b	*N. planospira*	Gardner	2004	No	Yes	2	NA

The closest BLAST match is also reported (note that *Naesiotus* was previously classified as a subgenus of *Bulimulus*). Gardner is the island near Floreana, not Española.

using phenol:chloroform:isoamyl alcohol (25:24:1), as just described. A third extraction was performed using an equal volume of chloroform:isoamyl alcohol (24:1), which was then vortexed briefly and centrifuged at 3,100 rpm for 3 min. DNA was precipitated from solution by adding one half volume of room temperature 5 M ammonium acetate and, to this combined volume, one volume of room temperature absolute isopropanol (as described in Hänni *et al.*, 1995), then storing the solution overnight at room temperature. The DNA was pelleted by centrifuging for 30 min at 3,100 rpm. The isopropanol was discarded and the sample air-dried for 15 min. The DNA was washed with 1 ml 80% ethanol by vortexing, pelleted again by centrifuging for

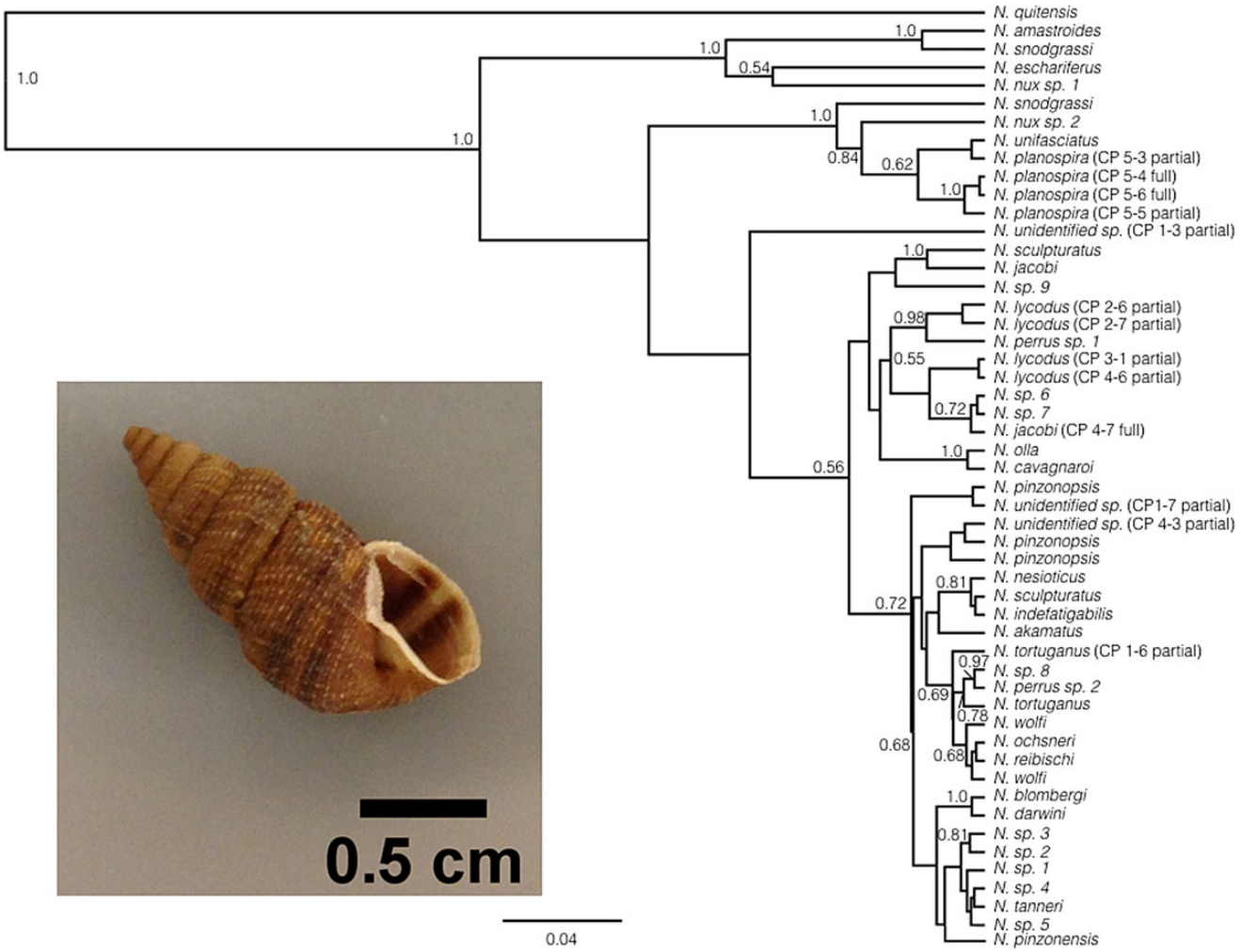

Figure 1. Maximum clade credibility phylogenetic tree of extant *Naesiotus* species from the Galápagos Islands, with sequences obtained in this study from shells of possibly extinct species. Complete or partial 472-bp sequences obtained from the three overlapping fragments are labeled as 'full' and 'partial', respectively, following their specimen number (denoted as CP). Clade support (posterior probability) is indicated above or below the nodes if greater than 0.5; branch length corresponds to median length of all trees retained in analysis. Figured shell is sample CP_3.7. (discoloration from weathering was characteristic of specimens used in this study).

30 min at 3,100 rpm and air-dried for 15 min after decanting the ethanol. The DNA was resuspended in 300 µl 60–65 °C DNA-free ddH_2O.

Resuspended DNA was further purified by silica extraction (Höss & Pääbo, 1993) following a modified protocol for the Wizard PCR Preps DNA Purification System (Promega). The Promega Wizard minicolumns were attached to 3 ml luer-lok syringe barrels (minus the plunger) and placed into a vacuum manifold. Columns were rinsed by running 3 ml of DNA-free ddH_2O before adding the samples. The DNA resuspension was mixed with 750 µl 2% celite in 6 M guanidine thiocyanate and 250 µl of 6 M guanidine thiocyanate, the tubes vortexed numerous times during a 2 min interval and the solution pulled through the columns. This was followed by washing the column with 3 ml 80% isopropanol. DNA was resuspended from the Wizard minicolumn in two serial 50 µl elutions of 60–65 °C DNA-free ddH_2O for a total of 100 µl.

WSU 'fast' DNA extraction method

The second method was designed to be simpler and faster. This 'fast' method, which has proved successful in previous studies (Cui *et al.*, 2013; Chatters *et al.*, 2014), is theoretically associated with less DNA loss because it employs a single extraction step (Barta *et al.*, 2014). Each shell was rinsed once or twice with DNA-free water (Gibco) to remove particulates, then transferred to a 2.0 ml microcentrifuge tube and demineralized by gentle rocking in 500 µl of molecular grade 0.5 M EDTA, pH 8.0, for 2 d at room temperature. For every extraction set, an extraction negative control was added, as above. Approximately 60 U Proteinase K (as above) were added to the sample, followed by incubation at 65 °C for 3 h. DNA was extracted directly following the silica-based method described above.

Controlling for co-extracted inhibition

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 consisted of 1.5 µl DNA extracted from ancient goose remains (Wilson *et al.*, 2011) which was added to every PCR reaction, as described below, and amplified using goose-specific primers instead of snail primers, as described in detail by Kemp *et al.* (2014). Samples scored as 'inhibited' (after the original silica extraction only) were

subjected to additional silica extractions, which has been demonstrated to reduce inhibition and increase PCR success. After each additional silica extraction, samples were tested for inhibition again, and the process repeated until inhibition of the goose IPC PCR reaction ceased.

PCR amplification and sequencing

Fragment size and successful PCR amplification are expected to have an inverse relationship (Pääbo, 1989, 1990; Cooper & Poinar, 2000). To test the efficiency of PCR for targets of different sizes we targeted fragments 244, 189 and 157 bp in length (henceforward the respective amplicons produced will be referred to as long, medium and short fragments).

To amplify a portion of the COI region, we performed 15 μl PCRs containing 0.32 mM dNTPs, 1X PCR Buffer, 1.5 mM $MgCl_2$, 0.24 μM primers (Table 3), 0.3 U of platinum Taq High Fidelity (Invitrogen) and 1.5 μl template DNA. PCR conditions were as follows: 94 °C for 3 min, 60 cycles of 15 s holds at 94, 55 and 72 °C, followed by a final 3 min extension period at 72 °C. Amplification success was determined by visualizing 5–6 μl of the amplicons on a 6% polyacrylamide gel stained with ethidium bromide under UV light.

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.

Amplicons were prepared for sequencing following Kemp *et al.* (2007) and sequenced in both directions at Elim BioPharm (Hayward, CA). We used the full mitochondrial genome of *Naesiotus nux* (NCBI GenBank acc. no. NC028553.1) as a reference to assemble the amplicons into an alignment. We used NCBI's BLAST tool on all amplicons to confirm taxonomic identity of the sequences. Because we expected our specimens to represent species that have never been sequenced before, we considered sequences whose closest match was in the genus *Naesiotus* (Table 2) to confirm that the aDNA was endogenous to the shell material.

The complete target sequence spans 472 bp, divided into three overlapping fragments. These 472 bp correspond to positions ~13,803 to ~14,275 in the *N. nux* reference genome. Complete sequences were deposited in GenBank (acc. nos KU575244–KU575246). To confirm sequence authenticity and eliminate the possibility of post-mortem damage, two independent rounds of PCR amplifications and sequencing were performed for all specimens from which we generated a complete 472-bp fragment.

Table 3. Primer sequence, annealing temperature (Tm) and product length of the three primer pairs used in this study.

Primer name	Sequence	Tm (°C)	Product length (bp)
Snail 1F	GCGGGTATATCTTCTATTTTGG	55.6	244
Snail 1R	CCCGGTAAAATTAAAATATAAACTTC	55.99	Long
Snail 2F	GGAGGATTTGGAAATTGATTAGTTCC	61.93	189
Snail 2R	TACCCGCTAAATGAAGAGAAAAA	58.01	Medium
Snail 3F	GRCTWTTACCTCCRGCTTTTAT	58.07	157
Snail 3R	ATWCCYGCTAAATGAAGWGAAA	57.14	Short

Amplicons of different lengths are referred to in the text as long, medium or short.

Phylogenetic analysis

We constructed a phylogenetic tree to determine the evolutionary relationship of the shell specimens relative to other Galapagos *Naesiotus* species. In addition to the 13 sequences we generated here, we used the partial mtDNA COI sequences of 37 Galapagos *Naesiotus* species (Parent & Crespi, 2006), with the same partial mtDNA COI sequence from *N. quitensis*, a species occurring on mainland Ecuador, as an outgroup (Parent & Crespi, 2006). We aligned the sequences using Seaview (Gouy, Guindon & Gascuel, 2010). We selected the best evolutionary model using JMODELTEST 2 (Darriba *et al.*, 2012) under the Bayesian information criterion. We performed Bayesian phylogenetic analyses in BEAST v. 1.8.3 (Drummond & Rambaut, 2007; Suchard & Rambaut, 2009) on CIPRES (Miller, Pfeiffer & Schwartz, 2010). All Markov chains were run for 10^8 generations and sampled every 10^4 generations to produce posterior distributions of 10,000 samples, and the first 1,000 trees were discarded as 'burn-in' from each run. We confirmed stationarity by visualizing the negative log-likelihood per generation in the program TRACER v. 1.6 (Drummond & Rambaut, 2007), as well as confirming satisfactory ESS statistics (>990). The analysis was replicated four times and compared to ensure Markov chain convergence by visually inspecting for similar likelihood scores and topologies. We used the resulting trees to determine a maximum clade credibility tree in TREEANNOTATOR v. 1.8.3 (Rambaut & Drummond, 2010).

RESULTS

Of the 35 shell samples processed in this study, 18 amplified successfully for at least one primer set (Table 2). Eight samples amplified for the longest fragment (244 bp), 11 for the intermediate fragment (189 bp) and 12 for the shortest fragment (157 bp) (Table 4). Five samples amplified for all three fragments. This is consistent with expectations for aDNA, that an inverse relationship exists between amplification success and size of targeted fragment (Pääbo, Gifford & Wilson, 1988; Pääbo, 1989). We generated sequence data for the complete 472-bp fragment for three individuals and partial sequence data for an additional ten individuals.

Comparing the Kemp *et al.* (2007) method with the WSU 'fast' method, both yielded a similar overall success rate (48%), but the former resulted in more samples that were free of PCR inhibitors (Table 2). It is notable, however, that up to two additional rounds of repeated silica extraction were necessary to remove inhibition completely from extracts in both methods.

In the direct comparison, where each of seven shells was split in half and extracted using both methods, the Kemp *et al.* (2007) method yielded a marginally higher number of amplifications (2 *vs* 1 amplifications for the long primer) with much lower inhibition (Tables 2, 4). A combination of the two methods can be used to maximize success, as some samples, for presently unknown reasons, gave successful results with one or other method, but not both (see e.g. Moss, Judd & Kemp, 2014).

Sequence identity for all sequences reported in Table 2 matched related snail species within Bulimulidae. Sequence identity for the three samples of which we generated a complete 472-bp sequence matched other *Naesiotus* species, although no exact matches were found with species already reported. Figure 1 illustrates the phylogenetic placement of 13 specimens processed from shell material relative to specimens of *Naesiotus* species processed from fresh animal tissue.

DISCUSSION

Our study capitalizes on readily-available shell material of terrestrial snail species that are rare or extinct to establish a

Table 4. Amplification success for the two extraction methods used in this study.

	Kemp *et al.* (2007) method		WSU 'fast' method		Both methods	
	Samples (*n*)	%	Samples (*n*)	%	Samples (*n*)	%
First set of specimens (28)*						
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 (244 bp)	2	14	3	21	6	21
Medium amplicon (189 bp)	5	36	4	29	9	32
Short amplicon (157 bp)	5	36	4	29	9	32
Direct comparison specimens (7)†						
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 (244 bp)	2	29	1	14	2	29
Medium amplicon (189 bp)	2	29	2	29	2	29
Short amplicon (157 bp)	3	43	3	43	3	43
All specimens (35)‡						
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 (244 bp)	4	19	4	19	8	23
Medium amplicon (189 bp)	7	33	6	29	11	31
Short amplicon (157 bp)	8	38	7	33	12	34

Amplification was attempted up to four times for each sample.

*Refers to the first 28 shells extracted, 14 for each method.

†Refers to the seven additional snail shells that were each split in half and tested using both methods.

‡Lists results for all 35 shells used in this study.

cost-efficient approach to generate sequence data. While a shell itself does not contain cells with mitochondria or nuclei (Marin & Luquet, 2004), we suspect dead cells from the mantle may become locked into the calciferous layers of the shell during the formation of the organic matrix of conchiolin, allowing for the preservation of small amounts genetic material, even in the absence of soft tissues (Caldeira *et al.*, 2004). All of the shells used in this study were collected from the ground and had therefore likely been exposed to UV radiation, oxygen radicals and hydrolysis, all causes of DNA damage. Our study demonstrates that DNA can survive in shells for at least 50 years after their collection. Survival for even longer periods remains a possibility, allowing for the recovery of genetic information from older museum specimens or weathered samples.

The reconstruction of the evolutionary history of the Galápagos *Naesiotus* species is beyond the scope of the present paper, but our novel approach to the sequencing of presumably extinct species will contribute to a more complete resolution of their phylogeny and to studies of the morphological and ecological diversification in this group. Knowledge of the phylogenetic relationship of these species will permit addressing previously unanswerable questions. For example, do the extinct and threatened species represent a random subsample of Galápagos *Naesiotus*, or are they phylogenetically clustered? When did these species diverge from their sister taxa? Are the extinct species morphologically and ecologically more similar to one another than expected by chance given their evolutionary history? Answers to these questions will contribute to our understanding of the determinants of extinction in Galápagos endemic snails and, importantly, will also inform conservation efforts aimed at protecting snail biodiversity on islands.

The contrast between the extremely low success rates of DNA recovery from snail shells in previous studies, and the high success rate employing aDNA protocols, is highly suggestive that 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 (in our study $\leq$244 bp), which is common in aDNA protocols.

The high inhibition rate associated with the samples studied here indicates that PCR inhibitors are co-extracted with shell DNA. The Kemp *et al.* (2007) method was found to be better suited to remove co-extracted PCR inhibitors. However, inhibitors were successfully removed from all samples after one or two additional rounds of silica extractions, regardless of which extraction method was employed.

Our recommendation for those conducting studies of DNA from empty mollusc shells is to implement aDNA or similar methods, including the use of primer pairs targeting short, overlapping fragments. We also advocate the proper monitoring and control of co-extracted PCR inhibitors. Using such methods, DNA from shells should be amenable for library builds from high-throughput sequencing. While some of these methods might seem excessive—extractions can take 4 d and require additional reagents—the benefits of successful DNA extractions from specimens of extinct or threatened species outweigh the increased time and financial costs.

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