Teaching an Old Shell New Tricks: Extracting DNA from Current, Historical, and Ancient Mollusk Shells

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The use of unconventional DNA sources has increased because the acquisition of traditional samples can be invasive, destructive, or impossible. Mollusks are one group for which novel genetic sources are crucial, but methodology remains relatively undeveloped. Many species are important ecologically and in aquaculture production. However, mollusks have the highest number of extinctions of any taxonomic group. Traditionally, mollusk shell material was used for morphological research and only recently has been used in DNA studies. In the present article, we review the studies in which shell DNA was extracted and found that effective procedures consider taxon-specific biological characteristics, environmental conditions, laboratory methods, and the study objectives. Importantly, these factors cannot be considered in isolation because of their fundamental, sometimes reciprocal, relationships and influence in the long-term preservation and recovery of shell DNA. Successful recovery of shell DNA can facilitate research on pressing ecological and evolutionary questions and inform conservation strategies to protect molluscan diversity.

Keywords: mollusk shells, shell DNA, historic DNA, noninvasive sampling

Over the past several decades, researchers have made increasing use of novel sources to obtain DNA (Payne and Sorenson 2002). In vertebrates, feathers, hair, fur, antlers, horn, scales, saliva, egg membranes and shells, urine, and feces serve as either noninvasive or nondestructive sources of DNA (e.g., Taberlet et al. 1999, Alpers et al. 2003, Idaghdour et al. 2003, Hedmark et al. 2004, Wisely et al. 2004, Carrol et al. 2018). In invertebrates, the exuviae (i.e., molt), dried bodies, frass (Feinstein 2004), and the fluid expelled by reflex bleeding from insects (Katoh et al. 2008), foot mucus (Armbruster et al. 2005, Palmer et al. 2008) and body swabs (Morinha et al. 2014) of mollusks, and the coelomic fluid secreted by annelids (Minamiya et al. 2011) have been used as novel DNA sources. Compared with traditional DNA sources such as blood and tissue, these unconventional sources of DNA pose unique challenges. For example, DNA is often present in lower quantity than tissue samples and may also be of lower quality because of degradation (Gerloff et al. 1995, Taberlet et al. 1996, Horváth et al. 2005, Harvey et al. 2006). However, there are also considerable advantages to using alternative samples for DNA-based studies (Taberlet et al. 1999). These sources can be obtained directly from the living organism without causing fatal injuries, obtained from the environment (eDNA), or collected from museum specimens. Furthermore, the acquisition of traditional DNA sources can be difficult or impossible, particularly from elusive, endangered, and extinct species or because of political or financial considerations.

Among the taxa with a dire need to expand the sources of accessible genetic material is the phylum Mollusca. Mollusks have the highest number of documented extinctions of any major taxonomic group, despite being the second most diverse animal phylum (Lydeard et al. 2004, Régnier et al. 2009). For many threatened species, the drastic documented declines have made it increasingly problematic to locate live individuals in the wild. Therefore, molluscan research has come to rely on museum collections for morphological, ecological, and molecular data on specimens. In the only study available on the present status of malacology collections, Sierwald and colleagues (2018) surveyed North American museums and found that roughly 87% of specimens are dry shell material, which is particularly challenging for molecular work that typically depends on available soft tissue. Although the morphological features

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and ornamentation of mollusk shells are traditionally used for taxonomic determinations (Skelton 1985), the use of mollusk shells in molecular research is not a novel concept. Early studies were focused predominately on biomolecular analysis of the calcareous structures—specifically, examining proteins responsible for biomineralization and to reconstruct geochronology (Weiner and Traub 1984, Miller and Brigham-Grette 1989, Penkman et al. 2007, Demarchi et al. 2011, Marin et al. 2013). Amino acid and protein analysis have been used in taxonomic identification of mollusk genera from shell material (Demarchi et al. 2014, Sakalauskaite et al. 2019, Sakalauskaite et al. 2020).

A variety of techniques have been implemented to extract DNA from these calcified structures. A thorough examination of the current methodology is needed to facilitate the development of effective shell DNA extraction procedures that consider the taxon of interest, the condition of the shell, the laboratory methodology, and the study objectives. The successful recovery of shell DNA is imperative to understanding the evolutionary history and genetic diversity of molluscan taxa and to facilitate research on mollusks as invasive species or of commercial interest.

In the present article, we provide a discussion of mollusk shell structure and their potential to contain DNA, summarize the factors that could affect the success of DNA extraction from mollusk shells, highlight the uses of shell DNA in research, and describe avenues for future shell DNA studies.

DNA preservation during shell formation

The calcified shells of mollusks are not known to contain living cells (Ponder and Lindberg 2008). However, during the process of biomineralization, it is possible that mantle epithelial cells (Hawk 2010) or haemocytes known to be involved in shell secretion (Ferreira et al. 2020) can become entombed within the shell matrix. Therefore, DNA may be trapped or absorbed within the layers of the shell during growth and persists within the shell postmortem (Der Sarkissian et al. 2017, Hawk and Geller 2018).

Mollusk shells are generally made up of two to five calcified layers and one organic layer (Marin et al. 2012). The organic layer, known as the periostracum, constitutes the outermost layer of the shell and can give the shell its coloration (Marin et al. 2012). In a simplified example, below the periostracum are the prismatic layer and, subsequently, the innermost and thickest layer of the shell. These mineralized shell layers are made up of one of three calcium carbonate polymorphs, most commonly aragonite or calcite and, very rarely, vaterite (Marin et al. 2012). The polymorphs can exhibit a wide variety of crystalline microstructures within the layers, including prismatic, spherulitic, crossed, homogenous (granular), helical, and laminar. The crossed-lamellar microstructure represents the most common microstructure produced across the phylum (Marin et al. 2012). Each of the microstructures has different mechanical properties that ultimately affect the toughness, flexibility, and durability of the shell (Marin et al. 2012).

Mollusk shell biomineralization is a biologically controlled process, where the shell formation is dictated by a series of genes (Marin et al. 2012). The mantle tissue has an external calcifying epithelium composed of a thin layer of cells (figure 1). Shell formation begins with the periostracum, which seals and delimitates a confined compartment between the mantle tissue and the shell, called the extrapallial space. The epithelial tissue secretes the calcium carbonate that forms the shell beneath the periostracum, which acts as a support where the calcium carbonate can deposit. The shell is formed along the margins, creating annual growth increments (figure 1). The process of mollusk shell formation and mineralization is described in detail in Marin and colleagues (2012).

Factors influencing DNA recovery from mollusk shells

Numerous studies have attempted to extract DNA from mollusk shells. Cumulatively, these studies highlight a number of factors that could affect the successful recovery of mollusk shell DNA. The factors can be broken down into three main categories: biological characteristics, environmental conditions, and laboratory methods.

Biological characteristics

The first category, biological characteristics, relates to factors that are taxon specific (table 1). These factors include shell biomineral microstructure and organic content, shell attributes including size or thickness, and shell growth patterns and reforming properties (Geist 2005, Geist et al. 2008, Der Sarkissian et al. 2017), which could influence the quantity and the quality of DNA preservation over time. These properties will most clearly differ between species, but could also vary, to some extent, within species among individuals sampled at different age stages, in different seasons or habitat, for example.

Shells that are composed of aragonite are known to have a higher organic content and denser structure than those composed of calcite (Marin et al. 2012). In two studies the same extraction and sequencing methods were used on a variety of mollusk taxa, and the researchers found that DNA extraction was most successful for ocean quahogs (*Arctica islandica*), clams, and abalones (Der Sarkissian et al. 2017, 2020). These species are characterized by shells with inner layers of homogenous or columnar aragonite, indicating that species with a high integrity of an aragonitic inner layer are the best candidates for DNA analysis. DNA extraction was less successful for scallops ($n = 5$) and oysters ($n = 6$), which have shells characterized by a foliated calcite inner structure (Der Sarkissian et al. 2017, 2020). No DNA was recovered from *Cernuella virgata* ($n = 2$), a terrestrial snail, or the limpet *Lottia gigantea* ($n = 1$; Der Sarkissian et al. 2020). Although the shells of *L. gigantea* contain layers of both calcite and aragonite, the aragonitic layers exhibit cross-laminar and microneedle prismatic microstructures (Marie et al. 2013), as opposed to the homogenous or

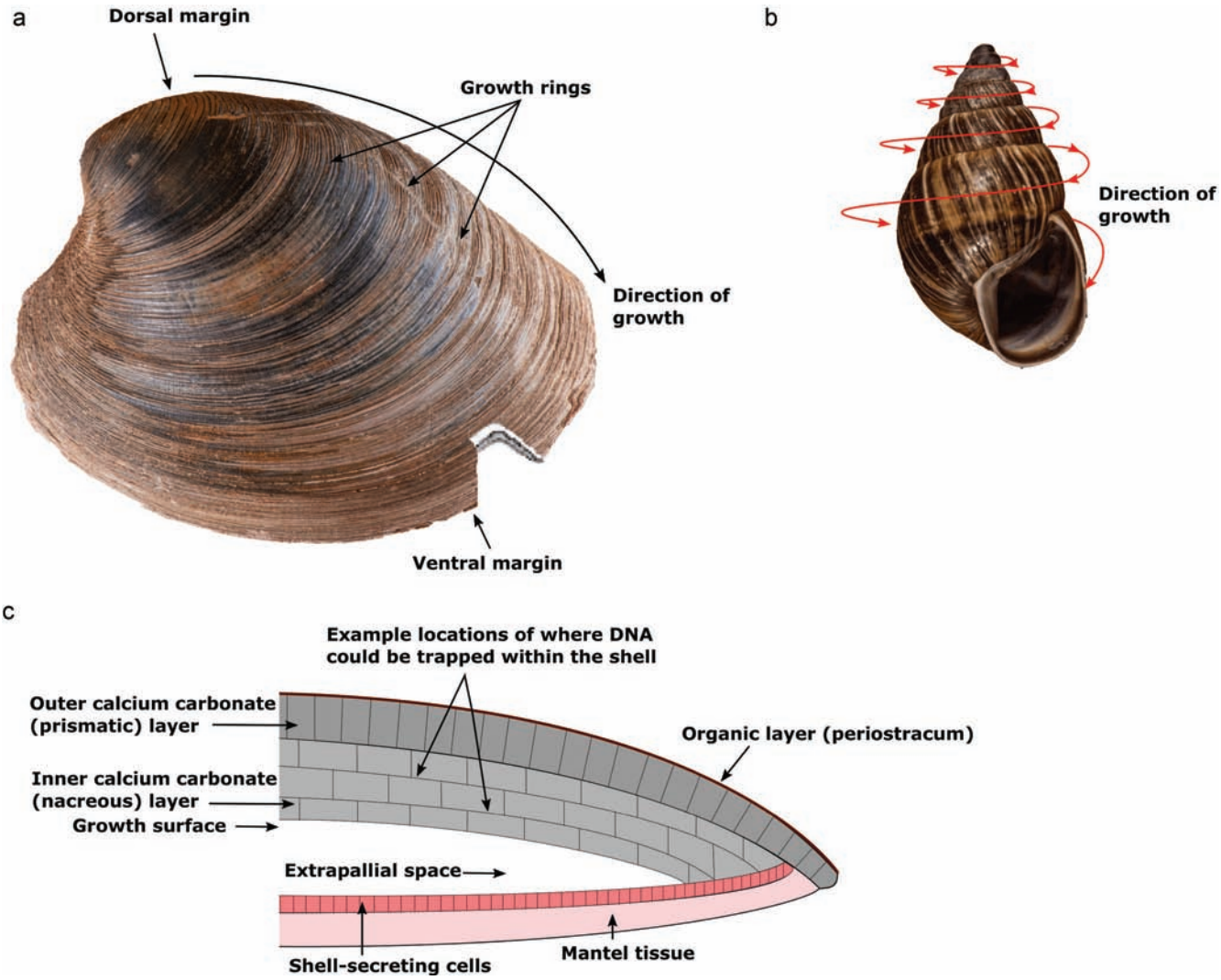

Figure 1. Important components of mollusk shells that are relevant for extracting shell DNA. (a) Select anatomical features of a bivalve shell indicating the direction of growth, growth rings, and the shell margins. Photograph: Christine Parent. (b) Direction of growth typical for gastropods (*Naesiotus nux*). Photograph: Christine Parent. (c) An illustration of a mollusk shell cross-section highlighting the shell layers: the organic layer (periostracum), the prismatic layer, and the inner nacreous layer.

columnar aragonite seen in quahogs, clams, and abalones, suggesting that both the calcium carbonate polymorph and microstructure could influence the success of DNA recovery from shell material.

Nacre is a type of crystalline microstructure found in a few species of mollusks, and it is considered the toughest material produced by the group (Marin et al. 2012). It is made up of mostly aragonitic tablets that are arranged as sheet, row stack, or columnar nacre. Importantly, despite its improved resistance, aragonite is more soluble than calcite (Marin et al. 2012) and can result in taphonomic alterations over time. The durability of nacre may reinforce the shell and provide increased protection of the DNA trapped inside the shell. In three separate studies, various extraction protocols and sequencing methods were used to examine abalone shells (*Haliotis* sp.), a gastropod categorized by a nacreous shell (Der Sarkissian et al. 2017, Hawk and Geller 2018, Der Sarkissian et al. 2020). Despite the differences in methodology, two of the studies had very high success rates (100% and 90.5%, respectively; Der Sarkissian et al. 2017, Hawk and Geller 2018), and the other study had a moderate success rate (40%; Der Sarkissian et al. 2020), regardless of the age of the specimen. In contrast, attempts to extract DNA from the freshwater pearl mussel, *Margaritifera margaritifera*, a species also categorized by a nacreous shell (Geist et al. 2008, Der Sarkissian et al. 2020), were unsuccessful. This suggests that factors beyond the shell structure and composition affect the recovery of shell DNA.

Table 1. Summary of the biological and environmental factors in the studies attempting to extract DNA from mollusk shells.

Species	Taxonomic class	Sample size (*n*)	Habitat	Shell fragment sampled	Shell condition	Sample Age	Protocol Referenced
Margaritifera margaritifera	Bivalvia	15	Freshwater (Europe)	Nonspecific fragment	Stream water exposure (hydrolysis)	Fresh–6 months	Geist et al. 2008
Crassostrea gigas	Bivalvia	10	Marine (China)	Ventral margin	Good condition	Fresh	Wang et al. 2012
Pomacea canaliculata	Gastropoda	2	Freshwater (Europe)	Nonspecific fragment	• Shell 1: Good condition • Shell 2: Photo-bleached (UV exposure)	Collected 10 years prior to study	Andree and Lopez 2013
Pinctada sp.	Bivalvia	74 (protocol A, 20; B, 18; C, 36)	Marine (South Pacific, UAE, Indonesia)	Whole pearl	Pearls were in good condition. One pearl had degraded organic matter interior	Harvested 2–10 years prior to study	Meyer et al. 2013
Naesiotus sp.	Gastropoda	35 (21 per protocol, seven samples shared)	Terrestrial (Galápagos Islands)	Whole shell	Shells were in fair condition, but exposed to UV radiation, oxygen radicals, and hydrolysis	Collected 6–50 years prior to study	Villanea et al. 2016
Ruditapes sp.	Bivalvia	18	Marine (Europe, Asia, South America)	• Modern samples: ventral margin • Historical samples: whole shell or fragment	Variable condition. Several clam shells exhibited damage from microbial infection	• Modern samples: Dated less than 60 years old • Historical samples: Dated 60–7000 years old	Der Sarkissian et al. 2017
Venerupis corrugata	Bivalvia	2					
Crassostrea sp.	Bivalvia	3					
Pecten maximus	Bivalvia	5					
Mytilus sp.	Bivalvia	2					
Arctica islandica	Bivalvia	3					
Haliotis tuberculata	Gastropoda	5					
Haliotis sorenseni	Gastropoda	95	Marine (California, Mexico)	Unspecified margin	Museum specimens were in good condition. Samples collected from sea floor were highly encrusted and eroded	Collected live and dead up to 79 years prior to study	Hawk and Geller 2018
Crassostrea gigas	Bivalvia	12 (six per protocol)	Marine (China)	• Dorsal margin · Middle • Ventral margin	Good condition	Fresh	Jiang et al. 2019
Perna canaliculus	Bivalvia	10,130 (aged; 16 cooked, four per treatment; 11 beach cast)	Marine (New Zealand)	• Nonspecific fragment • Dorsal margin (including ligament tissue) • Middle • Ventral margin	• Cooked (steam in salt water, cooked over firewood embers) • Beach-cast	• Fresh–13 months • Beach-cast (unknown age)	Ferreira et al. 2020
Mytilus sp.	Bivalvia	3	Marine (*n* = 7), Freshwater (*n* = 3), Terrestrial (*n* = 1); (Europe, Russia, North America)	Ventral margin	Variable condition. Three shells recovered from permafrost.	• Modern and Historical: Dated up to 7500 years old • Paleontological: Dated to 100,000 + years old	Der Sarkissian et al. 2020
Arctica islandica	Bivalvia	18					
Portlandia arctica	Bivalvia	1					
Crassostrea virginica	Bivalvia	3					
Dreissena polymorpha	Bivalvia	3					
Margaritifera margaritera	Bivalvia	3					
Cernuella virgata	Gastropoda	2					
Lottia gigantea	Gastropoda	1					
Haliotis spp.	Gastropoda	5					
Lymnaea stagnalis	Gastropoda	2					
Unidentified	Bivalvia	1					
Strombus pugilis	Gastropoda	18 (modern, 3; fresh, 5; archaeological, 5; paleontological, 5)	Marine (Panama)	Outer lip shell segment	Variable condition. Paleontological shells exhibited color bleaching and brittle textures due to UV exposure.	• Fresh/modern • Archaeological: 984–1258 BP • Paleontological: 5711–7187 BP	Sullivan et al. 2020

Biomineral microstructure and organic content have also been shown to be important for DNA extraction in other molluscan calcified structures, such as oyster pearls. Pteriidae pearls are composed of nacreous aragonite, which is secreted by the mantle tissue. The pearls are formed by the same processes that produce the nacreous layer in the shell. Meyer and colleagues (2013) tested DNA extraction methods on marine pearls collected from three oyster species (*Pinctada margaritifera*, *Pinctada maxima*, and *Pinctada radiata*). DNA amplification was least successful for *P. maxima* pearls (66%). Meyer and colleagues (2013) attributed their low success rate to the organism's observed pearl characteristics including less visible organic matter and thinner nacreous layers.

Shell attributes, such as size and thickness, may also affect the ability to recover DNA from mollusk shells (Geist 2005, Geist et al. 2008). Species with large, thick shells, such as abalones or conchs, may contain larger quantities of DNA within the shell matrix and have increased protection from degradation than do smaller species with a thin shell structure. Shell attributes can also greatly vary among individuals of the same species. For example, one study found that DNA was not recoverable from juvenile *Haliotis sorenseni* shells (Hawk and Geller 2018). Although Hawk and Geller (2018) could not attribute the failed DNA amplification to the age of the specimen, it is probable that juvenile mollusks, which are still growing to their adult size, do not contain sufficient amplifiable DNA. In contrast, individuals actively growing their shells might have more recent DNA trapped within their shell matrix, which could increase the chance of successful DNA extraction. The effect of age of the individual on the successes of DNA recovery deserves further study.

In addition, the location on the shell where material is removed can influence the recovery of DNA. As shelled mollusks grow, their mantle tissue secretes proteins and minerals to form the shell (Marin et al. 2012). Therefore, the shells grow by adding material at the margins that form annual growth increments (figure 1). The ventral margin of the shell represents the newest growth and the dorsal margin, such as the umbo in bivalves or the protoconch in gastropods, is the oldest part of the shell. In long-lived species, the difference in age between the margin and umbo shell material can be significant. In abalones, for example, the umbo is excreted more than 30 years before the ventral margin (Andrews et al. 2013). One study found no connection between shell sampling location in *Perna canaliculus* and DNA extraction success (Ferreira et al. 2020). In another study, the amount of DNA present in three distinct parts of *Crassostrea gigas* shells was examined (Jiang et al. 2019). In contrast, Jiang and colleagues (2019) found that the ventral margin of the shells contained the highest DNA content whereas the dorsal margin contained the lowest. Similarly, Sullivan and colleagues (2020) found that the most recently deposited material on the outer lip of the shell aperture had the greatest DNA yield in *Strombus pugilis*. These results suggest that in the oldest part of the shell the DNA has more time to degrade than the newer ventral margin does. Shell samples obtained from newer growth may increase the success of obtaining amplifiable DNA, particularly in long-lived species. Therefore, the sampling location should be considered as a factor of the lifespan, growth patterns, and time since excretion.

The shell repair process may also influence DNA recovery. Mollusks can repair their shells following damage from predators and microorganisms by generating new shell material (Marin et al. 2012). The process of shell regeneration is beyond the scope of the present article and has been studied at length for a variety of taxa including bivalves (e.g., Chen et al. 2018), gastropods (e.g., Taylor 2016), and cephalopods (e.g., Meenakshi et al. 1974). It is possible that isolating fragments from repaired sections of mollusk shells may increase the potential of recovering DNA, much like that of samples taken from the ventral margin.

Environmental conditions

The second category of factors influencing success of DNA extraction are environmental factors that can affect the quantity and quality of the DNA within the shell during the organism's lifetime or postmortem, either *in situ* or in museum collections (table 1). Field and storage conditions are known to negatively affect DNA quality in noninvasive and nondestructive samples (Jeffery et al. 2007, Vili et al. 2013, Sirois and Buckley 2019). These factors include hydrolysis and dissolution, UV exposure, encrustation by microorganisms, exposure to high heat, fluctuations in temperature and humidity, oxygen radicals, preservation condition, global location, and habitat (Geist 2005, Geist et al. 2008, Villanea et al. 2016, Der Sarkissian et al. 2017, 2020).

Exposure time to stream water in the freshwater mussel *M. margaritifera* decreased the success of DNA extraction from 89% (fresh samples) to 8% after 1 month postmortem and 0% after 3 months (Geist et al. 2008). Geist and colleagues (2008) concluded that the acidity of the water led to increased hydrolysis and dissolution of the calcium carbonate. Similarly, Der Sarkissian and colleagues (2017) found that shells which experienced dissolution of the inner aragonite layer yielded lower amounts of DNA. In a study on the New Zealand greenshell mussel, *P. canaliculus*, Ferreira and colleagues (2020) found that beach-cast shells were brittle, had areas of breakage and the outer layers were eroded. The significant environmental degradation of this sample of shells resulted in the lowest DNA yields in the study. Other environmental factors, such as UV exposure, can also decrease the success of recovering shell DNA. A photobleached *Pomacea canaliculata* shell failed to yield DNA as a result of prolonged sun exposure (Andree and Lopez 2013). Similarly, paleontological shells of *S. pugilis* exhibited color bleaching and brittle textures as a result of UV damage and yielded the lowest amount of DNA in the study (Sullivan et al. 2020).

Mollusk shells are also subject to encrustation, boring, and infection by other organisms during their life and postmortem. Hawk and Geller (2018) found that of the abalone

shells that failed to yield amplifiable DNA, several were heavily infested with encrusting species from remaining on the sea floor prior to collection. Despite the shell condition, Hawk and Geller (2018) were unable to attribute the failed DNA amplification to the shell encrustation. Two other studies found that disease state could lead to variable success rates across samples (Der Sarkissian et al. 2017) and, in some cases, decrease DNA yield (Ferreira et al. 2020). Living mollusks can respond to microbial attack through increased mineralization via encapsulation (e.g., Trinkler et al. 2010) or recruitment of hemocytes in the tissue or extrapallial space (Paillard et al. 1996). It has been hypothesized, albeit untested, that mollusk defense mechanisms can increase DNA content in shell material (Der Sarkissian et al. 2017). Infection and encrustation by other organisms affect shell integrity, which is an important factor influencing the success of shell DNA recovery.

In some cases, researchers are interested in mollusk species of commercial interest (e.g., Ferreira et al. 2020) or that have been historically exploited for human consumption. In archaeological settings, shells are found deposited in trash piles, or middens, after being processed and cooked. Ferreira and colleagues (2020) found that DNA can be amplified from shells exposed to high heat (steamed in salt water or cooked over fire), but this included the shell with the lowest DNA yield across the entire study. Similarly, ongoing research on *S. pugilis* has shown that DNA can be amplified from processed shells found in archaeological middens (Sullivan et al. 2020).

Other potential environmental factors that could affect DNA recovery are postmortem conditions, such as fluctuations in temperature and humidity and oxygen radicals. Temperature and humidity swings, which can occur during long-term storage of shell samples, can lead to shell cracking, shattering, or brittleness (Sturm 2006). Exposure of the interior shell matrix may increase or facilitate DNA degradation. In some cases, the combination of temperature, humidity, and acidity can cause the calcium carbonate of the shell to decompose (Byne's disease; Tennent and Baird 1985). It is also possible that exposure to oxygen radicals can lead to DNA damage in shell material (Villanea et al. 2016). Finally, the preservation environment can influence DNA recovery, particularly of historical and ancient shell material. Der Sarkissian and colleagues (2020) found that Siberian permafrost marine sediment preserves the calcium carbonate shell matrix and promotes DNA preservation by minimizing water and microbial damage across significant timescale (see Pedersen et al. 2015 and the references therein).

Mollusk shell size is known to have a global latitudinal gradient where polar species have proportionally smaller shells than tropical species (Watson et al. 2017). As was described in the previous section, species with larger shells may contain larger quantities of DNA within the shell matrix and have increased protection from degradation than smaller species (Geist et al. 2008). This suggests that global latitudinal location may influence DNA quantity in shell material. Furthermore, habitat may affect DNA quantity and quality. Shelled mollusks occupy marine, freshwater, and terrestrial environments. In aquatic settings, mollusks are constantly battling shell dissolution, which is increasing because of ocean acidification (e.g., Rodolfo-Metalpa et al. 2011). To combat the dissolution rate, aquatic mollusks can increase their calcification rate (Rodolfo-Metalpa et al. 2011). It is possible that the high calcification rates seen in aquatic mollusks may trap larger amounts of DNA or the entombed DNA may be less degraded because it is refreshed more often than terrestrial taxa.

Laboratory methods

The last category of factors determining success of shell DNA extraction includes laboratory methods (table 2) such as processing techniques (e.g., grinding methods, chemical treatment), extraction methodology, and sequencing methods (Geist 2005, Geist et al. 2008).

Processing techniques. Pre-extraction chemical treatments may influence the success of extracting shell DNA. The use of chemical treatments prior to extraction can reduce the risk of possible contamination. Hydrochloric acid, formalin, and bleach have been used as a pre-extraction chemical treatment (Hawk and Geller 2018). Hydrochloric acid caused both color and mass loss, bleach whitened the shells, and the formalin treatment resulted in no change of color or mass. Although all three treatments were effective at decontaminating shells, bleach was determined to be the most effective and safest option (Hawk and Geller 2018). In other studies in which bleach was used as a pre-extraction treatment, the researchers found that it did not increase the damage of DNA or negatively affect DNA content (Der Sarkissian et al. 2017, 2020). The lack of a pre-extraction decontamination step may have contributed to the higher levels of nonmollusk contamination seen in one study (Villanea et al. 2016).

A designated pre-extraction chemical treatment is important for preventing contamination but also for ensuring that the recovered DNA is contained inside the mollusk shell as opposed to residual tissue. For example, in several studies, the researchers attempted to extract DNA from the residue adhering tissue of mollusk shells (Caldeira et al. 2004, Strugnell et al. 2006) and cells remaining on bivalve hinge ligaments (Doherty and Was 2007, Gardner et al. 2012). Other studies did not include a pre-extraction chemical treatment, and the true origin (entombed within the shell, or on adhering tissue) of the amplified DNA is unclear (Geist et al. 2008, Andree and Lopez 2013, Villanea et al. 2016). Several studies have incorporated processing methods to ensure that the source of the DNA was from inside the mollusk shells (Der Sarkissian et al. 2017, Hawk and Geller 2018, Jiang et al. 2019, Der Sarkissian et al. 2020, Ferriera et al. 2020). For researchers wishing to amplify DNA contained within mollusk shells, the shells should be thoroughly cleaned of soft tissue and subjected to a pre-extraction chemical treatment to remove exogenous DNA.

Table 2. Summary of the laboratory methods applied in the studies attempting to extract DNA from mollusk shells.

Processing techniques					
Chemical treatment	**Grinding intensity**	**Demineralization**	**Extraction method: DNA binding and purification**	**Amplification method: Sequencing**	**Protocol referenced**
None	Tested three grinding intensities (fine, medium, coarse)	None	Phenol–chloroform method	• Single gene amplicons: COI (mtDNA, 543 bp) • Microsatellite markers	Geist et al. 2008
None	Ground to fine powder	EDTA solution with Tris-HCl and Proteinase-K	Phenol–chloroform method	Single gene amplicons: CO1 (mtDNA, approximately 200 bp), Beta-actin (approximately 300 bp)	Wang et al. 2012
None	Crushed to small pieces	None	DNeasy Blood and Tissue Kit (Qiagen)	Single gene amplicon: CO1 (mtDNA, 300 bp)	Andree and Lopez 2013
Bleach	Tested two grinding intensities (intact pearl, fine powder)	EDTA solution	Fast DNA Spin Kit for soil (MP Biomedicals)	Single gene amplicons: 16S (rRNA, 444–524 bp), CO1 (mtDNA, 149–575 bp), ITS1 (rRNA, 226–675 bp), ITS2 (rRNA, 221–591 bp)	Meyer et al. 2013
None	Tested two grinding intensities (intact shell, fine powder)	EDTA solution with Proteinase-K	Phenol and chloroform:isoamyl alcohol extractions. Suspended DNA mixed with celite particles in GuSCN buffer and purified using Wizard PCR Preps DNA purification System (Promega)	Single gene amplicon: CO1 (mtDNA; overlapping fragments of 244,189, 157 bp)	Villanea et al. 2016, Kemp et al. 2007
None	Tested two grinding intensities (intact shell, fine powder)	EDTA solution with Proteinase-K	On celite particles in GuSCN buffer and purified using Wizard PCR Preps DNA purification System (Promega)	Single gene amplicon: CO1 (mtDNA; overlapping fragments of 244,189, 157 bp)	Villanea et al. 2016 (WSU "fast" method)
Three chemical treatments (strong acid, bleach, formalin)	Fragment removed from shell	EDTA solution	DNeasy Blood and Tissue Kit (Qiagen)	Single gene amplicons: CO1 (mtDNA, 539 bp), Histone H3 (nDNA, 256 bp)	Hawk and Geller 2018
None	Ground to powder	EDTA solution with Tris-HCl and Proteinase-K	Phenol–chloroform method	Single gene amplicons: CO1 (mtDNA, 227 bp), 28S (rRNA, 482 bp)	Jiang et al. 2019
None	Ground to powder	Guanidine lysis buffer method (EDTA solution with guanidine thiocyanate, Tris-HCl, TE buffer, TritonX-100)	Single gene amplicons: CO1 (mtDNA, 227 bp), 28S (rRNA, 482 bp)	Jiang et al. 2019	
None	Crushed to small pieces	EDTA solution with Tris-HCl	Extracted following the salting out method. Dilution of DNA extracts with DNase-free water to prevent PCR inhibitors.	Single gene amplicons: COI (mtDNA, 191 bp), Pcan (mtDNA, 305 bp), Chitin synthase (CS1, nDNA, 198 bp)	Ferreira et al. 2020
Bleach	Ground to powder	EDTA solution with N-laurylsarcosyl and Proteinase-K	On the spin column of the MinElute PCR Purification Kit (Qiagen)	Illumina HiSeq4000 platform	Der Sarkissian et al. 2017, 2020, Yang et al. 1998, Gamba et al. 2014, 2016 ("Y1" method)
None	Ground to powder	EDTA solution with sodium dodecyl sulfate and Proteinase-K	On the spin column using a modified version of the QIAquick PCR Purification Kit (Qiagen)	NextSeq500 High-Output platform	Sullivan et al. 2020

Postmortem preservation of the periostracum may be an important part of successful DNA extraction for fresh, well-preserved shells. As aforementioned, mantle tissue secretes calcium carbonate that forms the shell beneath the periostracum, potentially trapping DNA between the layers. One of the main functions of the periostracum is to protect the shell against dissolution (Marin et al. 2012). Two studies showed that the removal of the periostracum from fresh and well-preserved shells (Geist et al. 2008), and the loss of the periostracum during cooking of fresh shells (Ferreira

et al. 2020) decreased the yield of DNA in the extractions. However, for older mollusk shells, the periostracum is often broken because of desiccation (Morton 2006) and is subject to microbial damage, such as brown ring disease (Paillard and Maes 1995), infestation, and decay (e.g., Byne's disease; Tennent and Baird 1985). In addition, in some species, following the formation of the calcified layers, the periostracum is quickly eroded and is absent on the shell. The presence of microorganisms in this layer increases the risk of DNA contamination and could warrant its removal prior to extraction, as was done in Jiang and colleagues (2019) and Wang and colleagues (2012). In both cases, the exterior periostracum of mollusk shells was removed prior to extraction and DNA was successfully amplified.

Previous studies have shown that grinding intensity can affect the success of DNA amplification (Geist 2005). Grinding the shell material into a fine powder can increase the surface area on which the lysis buffer can act, but it can also increase adsorption or damage the DNA (Geist 2005). The successful recovery of shell DNA likely relies on a balance between coarsely and fine ground shell material (Ferreira et al. 2020). Furthermore, exposing the interior of the shell is important for recovering DNA. Oyster pearls that were left intact during extraction yielded no DNA (Meyer et al. 2013). Pearls that were broken open and from which inner material was used for extraction yielded 92% success from direct polymerase chain reaction (PCR) amplification for the nDNA ITS2 marker (Meyer et al. 2013). In addition, inner material removed nondestructively via drilling a hole into the pearl resulted in 81% success for the ITS2 marker (Meyer et al. 2013). These results suggest that DNA can be obtained when the interior portion of the shell is exposed. One study showed no association between the amount of shell material used and successful DNA recovery (Der Sarkissian et al. 2020). However, Hawk (2010) found that PCR success rates increased with increasing shell fragment size.

Extraction method. The extraction method could influence the DNA yield from shell material. First, in order to access the DNA trapped within the shell, a mild decalcifying agent (ethylenediaminetetraacetic acid, EDTA) is used to remove calcium and expose the internal matrix of the shell. The demineralization solution might contain only EDTA (Meyer et al. 2013, Hawk and Geller 2018) or include additional digestion chemicals, such as N-laurylsarcosyl, Proteinase K, sodium dodecyl sulfate (SDS), Tris-HCl, or a combination (Geist et al. 2008, Villanea et al. 2016, Der Sarkissian et al. 2017, Jiang et al. 2019, Der Sarkissian et al. 2020, Ferreira et al. 2020, Sullivan et al. 2020). The SDS detergent was found to be more effective than N-laurylsarcosyl in breaking down *S. pugilis* shell matrix at room temperatures or higher (Sullivan et al. 2020). The length of time that the shell remains in the demineralization buffer will depend on the thickness and size of the shell. Species with thick shells, such as abalones or conchs, require longer demineralization and digestion time (Sullivan et al. 2020) between 5 and 20 days (Hawk and Geller 2018), and thinner shells require between 1 and 3 days. While they are in solution, the shells should be incubated at 37–65 degrees Celsius to facilitate demineralization. Following decalcification, the shell material is subject to DNA extraction.

In several studies, researchers have attempted to extract shell DNA using manufacturer kits (table 2; Geist et al. 2008, Meyer et al. 2013, Andree and Lopez 2013, Der Sarkissian et al. 2017, Hawk and Geller 2018, Der Sarkissian et al. 2020, Sullivan et al. 2020). Geist and colleagues (2008) found that the NucleoSpin Tissue Kit (Machery-Nagel) and QIAamp Stool Mini Kit (Qiagen) failed to produce sufficient DNA yields. Similarly, ongoing research has found limited success in amplifying shell DNA with the NucleoSpin Tissue Kit (Machery-Nagel; Chris Hobbs, Pacific Center for Molecular Biodiversity, Bernice Pauahi Bishop Museum, personal communication, 11 August 2019). In contrast, the DNeasy Blood and Tissue Kit (Qiagen) produced high quality DNA yields for 90.5% ($n = 95$) of abalone shells (Hawk and Geller 2018) and 50% ($n = 2$) of *P. canaliculata* shells (Andree and Lopez 2013). Der Sarkissian and colleagues (2017, 2020) completed extractions on a variety of mollusk taxa (clams, abalones, oysters, scallops, mussels, and ocean quahogs) using the MinElute PCR Purifcation Kit (Qiagen) as part of the "Y1" extraction method (table 2; Yang et al. 1998, Gamba et al. 2014, Gamba et al. 2016). Sullivan and colleagues (2020) compared the DNA yields between the QIAquick and MinElute PCR Purifcation Kits (Qiagen) from *S. pugilis* shells and found that the QIAquick PCR Purifcation Kit produced higher yields. Shell DNA was successfully recovered from Pteriidae pearls using the Fast DNA Spin Kit for soil (MP Biomedicals; Meyer et al. 2013).

Phenol–chloroform extraction methods have been shown to be successful in obtaining DNA from mollusk shells (Geist et al. 2008, Wang et al. 2012, Jiang et al. 2019). Compared with other tested methods, two studies showed that success rates were highest using phenol–chloroform extractions (Geist et al. 2008, Jiang et al. 2019). Despite these claims, in no study has the extraction of shell DNA been attempted with the EZNA Mollusc DNA Kit (Omega Bio-tek), a chloroform-based kit designed for invertebrates. In some cases, the use of toxic substances in DNA extraction is unattractive or the methods are not cost effective. Ferreira and colleagues (2020) used a simple, cheap, and nontoxic salting out DNA extraction method to obtain high quality DNA from *P. canaliculus* shells (Gemmel and Akiyama 1996).

Other researchers have proposed that shell DNA be treated as ancient DNA (aDNA; Villanea et al. 2016) and that DNA extraction can benefit by implementing aDNA techniques, specifically when using older, more degraded samples (Villanea et al. 2016, Der Sarkissian et al. 2017, 2020, Sullivan et al. 2020). The physical and chemical damage sustained by aDNA, as a result of environmental exposure or microbial attack, required the development of specialized protocols to maximize DNA recovery. A

thorough review of these methodologies is beyond the scope of the present article, and a more detailed history of the aDNA field, including characteristics of aDNA, applications to new fields, and methodological challenges can be found in papers such as Green and Speller (2017), Willerslev and Cooper (2005), Pääbo and colleagues (2004), and Hofreiter and colleagues (2001). The methodologies from the aDNA field have been commandeered for studies attempting to extract DNA from a variety of novel materials considered to contain low DNA yields and be of low quality (Green and Speller 2017), including mollusk shells.

In summary, key aDNA extraction techniques that have been implemented in shell DNA studies include conducting experiments in specialized facilities to minimize the risk of exogenous DNA contamination and employing steps, such as preforming multiple, reproducible, and independent extractions and including extraction and PCR controls, to detect or reduce the amount of contamination and impurities in the DNA extracts (Villanea et al. 2016, Der Sarkissian et al. 2017, 2020, Sullivan et al. 2020). Exogenous DNA contamination and coextracted impurities are thought to inhibit downstream enzymatic reactions (e.g., library building, PCR amplification) and result in failed reactions. These techniques can be used in conjunction with modified manufacturer kits (Der Sarkissian et al. 2017, 2020, Sullivan et al. 2020) or as part of a do-it-yourself protocol (table 2; Villanea et al. 2016). Extraction protocols that incorporated aDNA techniques, combined with high-throughput sequencing, enabled the recovery of shell DNA from modern samples (less than 50 years old) up to specimens dating from 7000 (Der Sarkissian et al. 2017, Sullivan et al. 2020) to 100,000 years old (Der Sarkissian et al. 2020). The amplified DNA from the 100,000 years old *Portlandia arctica* and *Mytilus trossulus* represents the oldest shell DNA recovered. These results indicate that aDNA methodology and high-throughput sequencing can make it possible to recover DNA data from archaeological and paleontological mollusk shells.

Several authors have suggested that an inability to amplify DNA could be a result of PCR inhibitors. During the extraction process, it is possible that impurities can be coextracted with DNA and can cause the PCR reaction to fail. Therefore, it is necessary to minimize these inhibitors to increase the successful recovery of shell DNA. Villanea and colleagues (2016) acknowledged the role of PCR inhibitors in DNA amplification failure and incorporated silica extractions to mitigate their effect in recovering DNA from Galápagos Island *Naesiotus* shells. The Kemp and colleagues (2007) method resulted in more samples with less PCR inhibitors than the WSU "fast" method (Chatters et al. 2014), but an additional two rounds of silica extractions were able to remove all inhibitors from the extracts from both protocols. The extra rounds of silica extractions allowed DNA to be amplified from an additional seven shells. Lendvay and colleagues (2020) did not detect PCR inhibitors in their coral DNA extractions in either the WSU "fast" or "Y1" method (Lendvay et al. 2020). Another method used to remove PCR inhibitors from DNA extracts is through dilution with DNase-free water. Ferreira and colleagues (2020) found that the dilution of the DNA template enabled the amplification of an additional 76 *P. canaliculus* samples (18%).

Lendvay and colleagues (2020) aimed to extract DNA from worked precious coral fragments by testing five different extraction protocols. Three of the five protocols have been used on mollusk shells: the WSU "fast" method (Villanea et al. 2016), the "Y1" method (Der Sarkissian et al. 2017, 2020) and a protocol tested on Pteriidae pearls (Meyer et al. 2013). The phenol–chloroform method was not tested. The "Y1" method successfully amplified and sequenced DNA from all 25 coral samples, whereas the protocol used in Meyer and colleagues (2013) and the WSU "fast" method successfully obtained DNA from 21 and 13 samples, respectively (Lendvay et al. 2020). These results suggest that different extraction methods result in different amplification success rates.

Sequencing methods and targets. The most common sequencing method employed across all mollusk shell DNA studies is targeting single gene amplicons (e.g., Wang et al. 2012, Andree and Lopez 2013, Villanea et al. 2016, Hawk and Geller 2018, Ferreira et al. 2020) and microsatellite markers (Geist et al. 2008). Ferreira and colleagues (2020) reported higher amplification success of *P. canaliculus* shell DNA for mitochondrial DNA (mtDNA) than for nuclear DNA (nDNA). The two mitochondrial amplicons, cyctochrome c oxidase subunit I (COI) and NADH4/ATP8 (Pcan) genes, successfully amplified 96.5% of shell material extracted from fresh shells up to 13 months after death (Ferreira et al. 2020). Comparatively, only 47.5% of those shells were amplified for the nuclear gene, chitin synthase (CS1), where success was highest (80%) for fresh shells (0–1 month after death) and lowest (10%) for older shells (6 and 13 months after death; Ferreira et al. 2020). The amplification success for the mtDNA genes was also higher for cooked shells (Pcan, 93.75%; COI, 93.75%) and beach-cast shells (Pcan, 25%; COI, 54.2%), than was the nDNA (75% and 4.2%, respectively; Ferreira et al. 2020). The success rate was higher for the COI gene from shell material extracted from fresh shells up to 13 months after death (97.7%) and beach-cast shells (54.2%) than that of the Pcan gene (95.3% and 25%, respectively; Ferreira et al. 2020). In contrast, Meyer and colleagues (2013) found the nuclear ITS2 gene to be more successful than the mitochondrial CO1 and 16S rRNA genes when amplifying DNA from oyster pearls. However, the least successful molecular marker across the entire study was the nuclear ITS1 gene (Meyer et al. 2013). Other studies were successful in amplifying both mtDNA and nDNA from mollusk shells (Hawk and Geller 2018, Jiang et al. 2019). This suggests that both nuclear and mitochondrial loci can be used to amplify shell DNA and researchers should target multiple loci, if possible, to optimize their amplification success given their study goals.

One important consideration for using mtDNA or nDNA is the length of the target fragment. Villanea and colleagues

(2016) were successful in amplifying short fragments (157,189, and 244 base pairs [bp]) of mtDNA, but had the highest success with the shortest fragment size. Similarly, ongoing research showed that amplification of shell DNA from *Lissachatina fulica* was only possible with mtDNA fragments of less than 200 bp (Chris Hobbs, Pacific Center for Molecular Biodiversity, Bernice Pauahi Bishop Museum, personal communication, 11 August 2019). These studies show that the DNA preservation in shells, particularly older and degraded shells, is also degraded in regard to length (Villanea et al. 2016), resulting in an inverse relationship between fragment length and successful PCR amplification also seen in other studies amplifying ancient DNA (Pääbo et al. 1988). Because of its much higher copy number, lack of sequence ambiguities from heterozygous genotypes, and faster rate of mutation, targeting mtDNA for extraction presents several advantages over nDNA (Rasmussen and Morrissey 2008). However, in some mollusks high rate of hybridization and double uniparental inheritance of mtDNA can negate some of these benefits.

The development of next-generation sequencing has greatly improved the ability to recover degraded and fragmented genetic information, particularly from older samples. High-throughput sequencing methods are able to generate billions of short sequencing reads and characterize the size, chemical degradation, and contamination of genetic material. Three studies have sequenced shell DNA from various molluscan taxa via an Illumina HiSeq4000 (Der Sarkissian et al. 2017, 2020) and NextSeq500 High-Output platforms (Sullivan et al. 2020). Der Sarkissian and colleagues (2020) were able to reconstruct the phylogenetic tree for *Mytilus* sp. using the complete mitochondrial genome, increase by twice to sixfold the number of complete mitochondrial genome sequences available for various molluscan taxa, and identify population affinities on the basis of whole-genome data. High-throughput sequencing allowed researchers to characterize the types of damage to molluscan shell DNA as depurination, cytosine deamination resulting in nucleotide misincorporation, and high fragmentation (Der Sarkissian et al. 2017, Sullivan et al. 2020). The observed degradation patterns seen in the ancient shell DNA are those patterns typical of aDNA (Dabney et al. 2013). Importantly, the DNA sequences obtained from mollusk shells should be verified to confirm the taxonomic identity of the sequences. Researchers should preform multiple independent shell DNA extractions and amplifications to ensure that the extracts yield identical sequences (Andree and Lopez 2013) or authenticate results against published sequences (Villanea et al. 2016, Der Sarkissian et al. 2017, 2020).

Utility of mollusk shell DNA

DNA analyses of mollusk shells have allowed scientists to capitalize on the remarkable malacology collections in museums worldwide. Traditionally, shell material has been used for morphology research. The successful recovery of DNA from mollusk shells has allowed researchers to address previously unanswerable questions.

The DNA can be used to make temporal scale comparisons between ancient, historical, and present populations. Specifically, it would allow researchers to track changes in genetic diversity, measure changes in community assemblages throughout time (Hawk and Geller 2018), and identity population affinities between ancient and modern populations (Der Sarkissian et al. 2020). For example, Hawk and Geller (2018) used museum shell collections of *H. sorenseni*, an endangered species for which little soft tissue exists and no historical genetic data is available, to measure genetic diversity over spatial and temporal scales. The study concluded that the genetic diversity of this species has been historically low and that the current low genetic diversity is attributed to factors prior to human exploitation. This finding would not have been possible without the successful extraction of DNA from historical shell material (Hawk and Geller 2018). In addition to tracking genetic diversity change over time, shell DNA enables investigation of past environmental conditions, tracking of invasive species, reconstruction of the evolutionary history of microbial communities and molluscan pathogens (Der Sarkissian et al. 2017), and assessing species adaptive responses. Findings can potentially help researchers predict future species responses to the changing climate.

The recovery of DNA from mollusk shells can facilitate the reconstruction of a more complete phylogeny through increased taxon sampling (Villanea et al. 2016, Der Sarkissian et al. 2020). Notably, it can improve our understanding of the evolutionary history for many molluscan species for which only shell representatives exist, including the sequencing of extinct species. More resolved phylogenies can help answer questions regarding systematics, biogeographic patterns, and extinctions.

Shell DNA can also be of relevance to species of commercial interest (Meyer et al. 2013, Hawk and Geller 2018, Jiang et al. 2019). Mollusks rank as the second most important taxa in aquaculture production, just behind fish (Astorga 2014). In particular, many marine mollusks are harvested or cultivated for human exploitation. Intensive collection has contributed to a decline in species numbers and, in some cases, a reduction in their natural range (e.g., Hobday et al. 2000). Shell DNA can be used to examine the effect of exploitation by comparing genetic diversity through time; identifying the genetic basis for phenotypic change (Sullivan et al. 2020); detecting the presence of inbreeding, admixture, or hybridization (Astorga 2014); and reconstructing the history of pathogens responsible for past mortality (Der Sarkissian et al. 2017). Recovering DNA from pearls allowed one study to identify the oyster species that produced the pearls as an initial step toward identifying geographic origin, as is relevant to the pearl industry (Meyer et al. 2013). Using shell DNA in aquaculture is an important tool especially when the collection of live tissue from stock populations results in high mortality rates (Jiang et al. 2019). Most

importantly, information obtained from shell DNA studies can inform conservation and management strategies.

Future directions

When attempting to extract DNA from mollusk shells, researchers need to consider taxon-specific characteristics, the extent and type of environmental degradation, the appropriate laboratory methods, and the objectives of the study. These factors cannot be considered in isolation because of their inherent interconnectedness and comparable influence in the long-term preservation and potential recovery of shell DNA. We recommend testing multiple extraction methods to find the most effective protocol for a given mollusk taxon with consideration for the aims of the study and the downstream applications for the recovered DNA. We emphasize the need for the publication of more studies in which extraction methods are compared using the same source material. When working with historical, ancient, and degraded samples, researchers should be mindful to follow aDNA best practices (Cooper and Poinar 2000), specifically avoiding exogenous DNA contamination and authenticating results, even if specialized aDNA protocols are not used for DNA extraction (e.g., Hawk and Geller 2018).

One important consideration for researchers attempting to extract shell DNA is the shell microstructure. Compared with other microstructures, nacre is considered the toughest shell material (Marin et al. 2012). The strength of the nacre could explain the high success of DNA recovery from abalone shells (Der Sarkissian et al. 2017, Hawk and Geller 2018, Der Sarkissian et al. 2020) and oyster pearls (Meyer et al. 2013). Future work should target other nacreous species, which are widespread among mollusks, including families of snails (other Haliotidae species, Trochidae, Turbinidae), cephalopods (Nautilidae), bivalves (Pteriidae, Margaritiferidae) and the monoplacophoran *Veleropilina zografi* (Checa et al. 2009) to test this hypothesis.

The majority of the mollusk shell DNA research has focused on aquatic gastropods and bivalves. The future of shell DNA research should include terrestrial mollusks and previously underrepresented molluscan taxa, such as monoplacophorans, scaphopods, polyplacophorans (chitons), and cephalopods. Studies should expand to include other calcified molluscan structures including the cephalopod cuttlebone, gastropod operculum, calcified epiphragm and clausilium, or gastropod gypsobelum (love dart). In addition, there is a need to test the efficacy of extracting shell DNA from samples previously stored in ethanol or formalin.

A major constraint for the use of mollusk shells in DNA research is the destructive nature of the sampling process. In several studies, the whole shell was crushed and used in the extraction procedure (e.g., Villanea et al. 2016, Der Sarkissian et al. 2017). In other studies, only a small piece of the shell was removed and subjected to extractions (e.g., Hawk and Geller 2018). However, even partial sampling of a shell is a problem for small species for which even minor excision would result in significant morphological damage. Furthermore, such small samples may be unlikely to contain sufficient amplifiable DNA (Mulligan 2005). Destructive specimen sampling is particularly unappealing for rare or extinct species, and specimens of scientific value (i.e., type specimens). This concern is echoed in other taxonomic groups, such as insects, using novel sources of DNA (Gilbert et al. 2007, Thomsen et al. 2009). Investigators should record morphological information prior to extraction via photography, photogrammetry, CT (computed tomography) scanning, or X-ray imaging of the shells.

One possible solution is to extract DNA from the periostracum (i.e., the organic outer layer), of live, fresh, or well-preserved specimen (Armbruster et al. 2005). Similarly, DNA can be targeted from residual adhering tissue on mollusk shells (Caldeira et al. 2004, Strugnell et al. 2006) or from the hinge ligament in bivalves, which contains a protein–calcium carbonate matrix, and leaves the remaining shell material undisturbed (Doherty et al. 2007, Gardner et al. 2012). The importance of the DNA origin (entombed within the shell, or on adhering tissue) will depend greatly on the study objectives. In some cases, particularly for old shells or those of conservation importance, excluding adherent tissue may result in the loss of meaningful data. These considerations should be addressed before undertaking a shell DNA study.

Another potential nondestructive option is to immerse the shells in a digestion buffer and extract DNA from the resulting solution. This method has successfully extracted amplifiable DNA in arthropods (e.g., Gilbert et al. 2007, Rowley et al. 2007, Thomsen et al. 2009), vertebrates (Rohland et al. 2004), and foraminifera (Lyu et al. 2016). Jiang and colleagues (2019) tested the efficacy of a guanidine lysis buffer on extracting DNA from *C. gigas* shell powder. Although the method was successful, Jiang and colleagues (2019) found that the extracted DNA contained more impurities than another tested method. It is unclear if a digestion buffer will be able to access the DNA trapped within the shell without premediated grinding or complete dissolution. In a study examining oyster pearls, Meyer and colleagues (2013) were unable to extract DNA from intact pearls that were incubated in an EDTA solution. Using a guanidine lysis buffer or another comparable solution, such as a sodium deoxycholate or cetyltrimethylammonium bromide buffer (Pawlowski 2000) may be effective at extracting DNA from intact mollusk shells, but further investigation is needed.

In some cases, DNA samples need to be collected from live organisms without causing fatal injury. For instance, if there is no historical collection available, in aquaculture production, to compare modern and historical genetic diversity or to prevent population loss. Nonlethal sampling is particularly important for species of conservation concern. In mollusks, nonlethal sampling has focused on obtaining DNA from foot mucus (Kawai et al. 2004, Armbruster et al. 2005, Palmer et al. 2008), haemolymph (Geist and Kuehn 2005), body swabs (Henley et al. 2006, Morinha et al. 2014), and the periostracum (Armbruster et al. 2005). Mantle clipping has

also been employed as a nonlethal technique for extracting DNA (e.g., Berg et al. 1995), however it can result in shell deformity (Henley et al. 2006). Two studies showed that shell fragments yielding DNA could be removed from live *C. gigas* specimens without resulting in mortality (Wang et al. 2012, Jiang et al. 2019). Future shell DNA studies should implement this noninvasive shell sampling procedure to explore its utility for other molluscan taxa.

Conclusions

The growing list of publications seeking to amplify DNA from mollusk shells highlights the increasing need to identity novel sources of genetic information from this threatened group. Obtaining DNA from dry shell material has several notable advantages to traditional tissue sampling and other noninvasive sampling methods. Shell fragments can be obtained directly from the living organism without causing fatalities and collected from ancient, historical, or museum specimens for which only shell representatives exist. A variety of methods have been implemented to extract DNA from the calcified structures of mollusks. We found that effective shell DNA extraction procedures consider the taxon of interest, the condition of the shell, the laboratory methodology, and the study objectives. The successful recovery of shell DNA has enabled investigation into new avenues of the ecological relationships, evolutionary history, and genetic diversity of molluscan taxa. Future work in this field will be critical in informing conservation and management strategies to ensure molluscan diversity endures in perpetuity.

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References cited

Alpers DL, Taylor AC, Sunnucks P, Bellman SA, Sherwin WB. 2003. Pooling hair samples to increase DNA yield for PCR. Conservation Genetics 4: 779–788.

Andree KB, Lopez MA. 2013. Species identification from archived snail shells via genetic analysis: A method for DNA extraction from empty shells. Molluscan Research 33: 1–5.

Andrews AH, Leaf RT, Rogers-Bennett L, Neuman M, Hawk H, Cailliet GM. 2013. Bomb radiocarbon dating of the endangered white abalone (*Haliotis sorenseni*): Investigations of age, growth and lifespan. Marine and Freshwater Research 64: 1029–1039.

Armbruster G, Koller B, Baur B. 2005. Foot mucus and periostracum fraction as non-destructive source of DNA in the land snail *Arianta arbustorum*, and the development of new microsatellite loci. Conservation Genetics 6: 313–316.

Astorga MP. 2014. Genetic considerations for mollusk production in aquaculture: Current state of knowledge. Frontiers in Genetics 5: 435.

Berg DJ, Haag WR, Guttman SI, Sickel JB. 1995. Mantle biopsy: A technique for nondestructive tissue-sampling of freshwater mussels. Journal of the North American Benthological Society 14: 577–581.

Caldeira RL, Jannotti-Passos LK, Lira PM, Carvalho OS. 2004. Diagnostic of *Biomphalaria* snails and *Schistosoma mansoni*: DNA obtained from traces of shell organic materials. Memorias Do Instituto Oswaldo Cruz 99: 499–502.

Carroll EL, Bruford MW, DeWoody JA, Leroy G, Strand A, Waits LP, Wang J. 2018. Genetic and genomic monitoring with minimally invasive sampling methods. Evolutionary Applications 11: 1094–1119.

Chatters JC, et al. 2014. Late Pleistocene human skeleton and mtDNA link Paleoamericans and modern Native Americans. Science 344: 750–754.

Checa AG, Ramírez-Rico J, González-Segura A, Sánchez-Navas A. 2009. Nacre and false nacre (foliated aragonite) in extant monoplacophorans (= Tryblidiida: Mollusca). Naturwissenschaften 96: 111–122.

Chen Y, Liu C, Li S, Liu Z, Xie L, Zhang R. 2018. Repaired shells of the pearl oyster largely recapitulate normal prismatic layer growth: A proteomics study of shell matrix proteins. ACS Biomaterials Science and Engineering 5: 519–529.

Cooper A, Poinar HN. 2000. Ancient DNA: Do it right or not at all. Science 289: 1139–1139.

Dabney J, Meyer M, Pääbo S. 2013. Ancient DNA damage. Cold Spring Harbor Perspectives in Biology 5: a012567.

Demarchi B, Williams MG, Milner N, Russell N, Bailey G, Penkman K. 2011. Amino acid racemization dating of marine shells: A mound of possibilities. Quaternary International 239: 114–124.

Demarchi B, O'Connor S, Ponzoni AD, Ponzoni RDR, Sheridan A, Penkman K, Hancock Y, Wilson J. 2014. An integrated approach to the taxonomic identification of Prehistoric shell ornaments. PLOS ONE 9: e99839.

Der Sarkissian C, et al. 2017. Ancient DNA analysis identifies marine mollusc shells as new metagenomic archives of the past. Molecular Ecology Resources 17: 835–853.

Der Sarkissian C, Möller P, Hofman CA, Ilsøe P, Rick TC, Schiøtte T, Sørensen MV, Dalén L, Orlando L. 2020. Unveiling the ecological applications of ancient DNA from mollusk shells. Frontiers in Ecology and Evolution 8: 1–21.

Doherty S, Gosling E, Was A. 2007. Bivalve ligament: A new source of DNA for historical studies. Aquatic Biology 1: 161–165.

Feinstein J. 2004. DNA sequence from butterfly frass and exuviae. Conservation Genetics 5: 103–104.

Ferreira S, Ashby R, Jeunen GJ, Rutherford K, Collins C, Todd EV, Gemmell NJ. 2020. DNA from mollusc shell: A valuable and underutilised substrate for genetic analyses. PeerJ 8: e9420.

Gamba C, et al. 2014. Genome flux and stasis in a five millennium transect of European prehistory. Nature Communications 5: 5257.

Gamba C, Hanghøj K, Gaunitz C, Alfarhan AH, Alquraishi SA, Al-Rasheid KAS, Bradley D, Orlando L. 2016. Comparing the performance of three ancient DNA extraction methods for high-throughput sequencing. Molecular Ecology Resources 16: 459–469.

Gardner JP, Boesche C, Meyer JM, Wood AR. 2012. Analyses of DNA obtained from shells and brine-preserved meat of the giant clam *Tridacna maxima* from the central Pacific Ocean. Marine Ecology Progress Series 453: 297–301.

Geist J. 2005. Conservation genetics and ecology of European freshwater pearl mussels *(Margaritifera margaritifera* L.). PhD dissertation, Technische Universität München, Munich, Germany.

Geist J, Kuehn R. 2005. Genetic diversity and differentiation of central European freshwater pearl mussel (*Margaritifera margaritifera* L.) populations: Implications for conservation and management. Molecular Ecology 14: 425–439.

Geist J, Wunderlich H, Kuehn R. 2008. Use of mollusc shells for DNA-based molecular analyses. Journal of Molluscan Studies 74: 337–343.

Gemmell NJ, Akiyama S. 1996. An efficient method for the extraction of DNA from vertebrate tissues. Trends in Genetics 12: 338–339.

Gerloff U, Schlötterer C, Rassmann K, Rambold I, Hohmann GA, Fruth B, Tautz D. 1995. Amplification of hypervariable simple sequence repeats (microsatellites) from excremental DNA of wild living bonobos (*Pan paniscus*). Molecular Ecology 4: 515–518.

Gilbert MTP, Moore W, Melchior L, Worobey M. 2007. DNA extraction from dry museum beetles without conferring external morphological damage. PLOS ONE 2: e272

Green EJ, Speller CF. 2017. Novel substrates as sources of ancient DNA: Prospects and hurdles. Genes 8: 180.

Harvey MG, Bonter DN, Stenzler LM, Lovette IJ. 2006. A comparison of plucked feathers versus blood samples as DNA sources for molecular sexing. Journal of Field Ornithology 77: 136–140.

Hawk HL. 2010. Historic genetic diversity of the endangered white abalone (*Haliotis sorenseni*). Master's thesis. California State University Monterey Bay, Seaside, California, United States.

Hawk HL, Geller JB. 2018. DNA entombed in archival seashells reveals low historical mitochondrial genetic diversity of endangered white abalone *Haliotis sorenseni*. Marine and Freshwater Research 70: 359–370.

Hedmark E, Flagstad Ø, Segerstr öm P, Persson J, Landa A, Ellegren H. 2004. DNA-based individual and sex identification from wolverine (*Gulo gulo*) faeces and urine. Conservation Genetics 5: 405–410.

Henley WF, Grobler PJ, Neves RJ. 2006. Non-invasive method to obtain DNA from freshwater mussels (Bivalvia: Unionidae). Journal of Shellfish Research 25: 975–977.

Hobday AJ, Tegner MJ, Haaker PL. 2000. Over-exploitation of a broadcast spawning marine invertebrate: Decline of the white abalone. Reviews in Fish Biology and Fisheries 10: 493–514.

Hofreiter M, Serre D, Poinar HN, Kuch M, Pääbo S. 2001. Ancient DNA. Nature Reviews Genetics 2: 353–359.

Horváth MB, Martínez-Cruz B, Negro JJ, Kalmár L, Godoy JA. 2005. An overlooked DNA source for non-invasive genetic analysis in birds. Journal of Avian Biology 36: 84–88.

Idaghdour Y, Broderick D, Korrida ANDA. 2003. Faeces as a source of DNA for molecular studies in a threatened population of great bustards. Conservation Genetics 4: 789–792.

Jeffery KJ, Abernethy KA, Tutin CE, Bruford MW. 2007. Biological and environmental degradation of gorilla hair and microsatellite amplification success. Biological Journal of the Linnean Society 91: 281–294.

Jiang Q, Wei L, Gai C, Yu W, He C, Chen M, Zhang Z, Song H, Wang W, Wang, X. 2019. An improved extraction method reveals varied DNA content in different parts of the shells of Pacific oysters. Aquatic Living Resources 32: 5.

Kawai K, Shimizu M, Hughes RN, Takenaka O. 2004. A non-invasive technique for obtaining DNA from marine intertidal snails. Journal of the Marine Biological Association of the United Kingdom 84: 773–774.

Kemp BM, 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.

Katoh T, Furukawa K, Katakura H. 2008. DNA analysis of insects using reflex bleeding. Japanese Journal of Entomology 11: 25–31.

Lendvay B, Cartier LE, Gysi M, Meyer JB, Krzemnicki MS, Kratzer A, Morf NV. 2020. DNA fingerprinting: An effective tool for taxonomic identification of precious corals in jewelry. Scientific Reports 10: 1–12.

Lydeard C, et al. 2004. The global decline of nonmarine mollusks. BioScience 54: 321–330.

Lyu M, Lei YL, Li TG. 2016. Efficiency of DNA preservation and extraction from benthic hyaline foraminifera of *Ammonia* spp.: A methodological comparison. Oceanologia et Limnologia Sinica 47: 346–353.

Marie B, Jackson DJ, Ramos-Silva P, Zanella-Cléon I, Guichard N, Marin F. 2013. The shell-forming proteome of *Lottia gigantea* reveals both deep conservations and lineage-specific novelties. FEBS Journal 280: 214–232.

Marin F, Le Roy N, Marie B. 2012. The formation and mineralization of mollusk shell. Frontiers in BioScience 4: 1099–1125.

Marin, F, et al. 2013. "Shellome": Proteins involved in mollusk shell biomineralization-diversity, functions. Pages 149–166 in Watabe S, Maeyama K, Nagasawa H, eds. Recent Advances in Pearl Research. Terra Scientific Publishing Company.

Meenakshi VR, Martin AW, Wilbur KM. 1974. Shell repair in *Nautilus macromphalus*. Marine Biology 27: 27–35.

Meyer JB, Cartier LE, Pinto-Figueroa EA, Krzemnicki MS, Hanni HA, McDonald BA. 2013. DNA fingerprinting of pearls to determine their origins. PLOS ONE *8*: e75606.

Miller GH, Brigham-Grette J. 1989. Amino acid geochronology: Resolution and precision in carbonate fossils. Quaternary International 1: 111–128.

Minamiya Y, Ohga K, Hayakawa H, Ito K, Fukuda T. 2011. Coelomic fluid: A noninvasive source of DNA in earthworms. Molecular Ecology Resources 11: 645–649.

Morinha F, Travassos P, Carvalho D, Magalhães P, Cabral JA, Bastos E. 2014. DNA sampling from body swabs of terrestrial slugs (Gastropoda: Pulmonata): A simple and non-invasive method for molecular genetics approaches. Journal of Molluscan Studies 80: 99–101.

Morton J. 2006. The effect of relative humidity on mollusc periostracum and the use of coatings to prevent loss. Collection Forum 20: 67–82.

Mulligan CJ. 2005. Isolation and analysis of DNA from archaeological, clinical, and natural history specimens. Pages 87–103. Methods in Enzymology, vol 395. Academic Press.

Pääbo S, Gifford JA, Wilson AC. 1988. Mitochondrial-DNA sequences from a 7000-year old brain. Nucleic Acids Research 16: 9775–9787.

Pääbo S, Poinar H, Serre D, Jaenicke-Despres V, Hebler J, Rohland N, Kuch M, Krause J, Vigilant L, Hofreiter M. 2004. Genetic analyses from ancient DNA. Annual Review of Genetics 38: 645–679.

Paillard C, Maes P. 1995. The brown ring disease in the Manila clam, *Ruditapes philippinarum*: II. Microscopic study of the brown ring syndrome. Journal of Invertebrate Pathology 65: 101–110.

Paillard C, Ashton-Alcox KA, Ford SE. 1996. Changes in hemolymph and extrapallial fluid parameters in the American oyster, *Crassostrea virginica* affected by the Juvenile oyster disease. Aquatic Living Resources 9: 145–148

Palmer AN, Styan CA, Shearman DC. 2008. Foot mucus is a good source for non-destructive genetic sampling in Polyplacophora. Conservation Genetics 9: 229–231.

Pawlowski J. 2000. Introduction to the molecular systematics of foraminifera. Micropaleontology 46: 1–12.

Payne RB, Sorenson MD. 2002. Museum collections as sources of genetic data. Bonner Zoologische Beiträge Band 51: 97–104.

Pedersen MW, et al. 2015. Ancient and modern environmental DNA. Philosophical Transactions of the Royal Society B 370: 20130383.

Penkman KEH, Preece RC, Keen DH, Maddy D, Schreve DC, Collins MJ. 2007. Testing the aminostratigraphy of fluvial archives: The evidence from intra-crystalline proteins within freshwater shells. Quaternary Science Reviews 26: 2958–2969.

Ponder W, Lindberg DR, eds. 2008. Phylogeny and Evolution of the Mollusca. University of California Press.

Rasmussen RS, Morrissey MT. 2008. DNA-based methods for the identification of commercial fish and seafood species. Comprehensive Reviews in Food Science and Food Safety 7: 280–295.

Régnier C, Fontaine B, Bouchet P. 2009. Not knowing, not recording, not listing: Numerous unnoticed mollusk extinctions. Conservation Biology 23: 1214–1221.

Rodolfo-Metalpa R, et al. 2011. Coral and mollusc resistance to ocean acidification adversely affected by warming. Nature Climate Change 1: 308–312.

Rohland N, Siedel H, Hofreiter M. 2004. Nondestructive DNA extraction method for mitochondrial DNA analyses of museum specimens. Biotechniques 36: 814–821.

Rowley DL, Coddington JA, Gates MW, Norrbom AL, Ochoa RA, Vandenberg NJ, Greenstone MH. 2007. Vouchering DNA-barcoded specimens: Test of a nondestructive extraction protocol for terrestrial arthropods. Molecular Ecology Notes 7: 915–924.

Sakalauskaite J, et al. 2019. "Palaeoshellomics" reveals the use of freshwater mother-of-pearl in prehistory. Elife 8: e45644.

Sakalauskaite J, Marin F, Pergolizzi B, Demarchi B. 2020. Shell palaeoproteomics: First application of peptide mass fingerprinting for the rapid identification of mollusc shells in archaeology. Journal of Proteomics 227: 103920.

Skelton PW. 1985. Bivalvia-Rostroconchia. Pages 81–101 in Murray JW, Longman, The Palaeontological Association, London, eds. Atlas of Invertebrate Macrofossils. Wiley.

Sierwald P, Bieler R, Shea EK, Rosenberg G. 2018. Mobilizing mollusks: Status update on mollusk collections in the USA and Canada. American Malacological Bulletin 36: 177–215.

Sirois SH, Buckley DH. 2019. Factors governing extracellular DNA degradation dynamics in soil. Environmental Microbiology Reports 11: 173–184.

Strugnell J, Norman M, Cooper A. 2006. DNA from beach-washed shells of the ram's horn squid, *Spirula spirula*. Bulletin of Marine Science 78: 389–391.

Sturm CF. 2006. Archival and curatorial methods. Pages 45–57 in Sturm CF, Pearce TA, Valdés A, eds. The Mollusks: A Guide to Their Study, Collection, and Preservation. Universal Publishers.

Sullivan AP, Marciniak S, O'dea A, Wake TA, Perry GH. 2020. Modern, archaeological, and paleontological DNA analysis of a human-harvested marine gastropod (*Strombus pugilis*) from Caribbean Panama. BioRxiv: 2020.08.26.269308.

Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, Waits LP, Bouvet J. 1996. Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic Acids Research 24: 3189–3194.

Taberlet P, Waits LP, Luikart G. 1999. Noninvasive genetic sampling: Look before you leap. Trends in Ecology and Evolution 14: 323–327.

Taylor D. 2016. Impact damage and repair in shells of the limpet *Patella vulgata*. Journal of Experimental Biology 219: 3927–3935.

Tennent NH, Baird T. 1985. The deterioration of mollusca collections: Identification of shell efflorescence. Studies in Conservation 30: 73–85.

Thomsen PF, Elias S, Gilbert MTP, Haile J, Munch K, Kuzmina S, Froese DG, Sher A, Holdaway RN, Willerslev E. 2009. Non-destructive sampling of ancient insect DNA. PLOS ONE 4: e5048.

Trinkler N, Labonne M, Marin F, Jolivet A, Bohn M, Poulain C, Bardeau JF, Paillard C. 2010. Clam shell repair from the brown ring disease: A study of the organic matrix using Confocal Raman micro-spectrometry and WDS microprobe. Analytical and bioanalytical chemistry 396: 555–567.

Villanea FA, Parent CE, Kemp BM. 2016. Reviving Galapagos snails: Ancient DNA extraction and amplification from shells of probably extinct endemic land snails. Journal of Molluscan Studies 82: 449–456.

Vili N, Nemesházi E, Kovács S, Horváth M, Kalmár L, Szabó K. 2013. Factors affecting DNA quality in feathers used for non-invasive sampling. Journal of Ornithology 154: 587–595.

Weiner S, Traub W. 1984. Macromolecules in mollusk shells and their functions in biomineralization. Philosophical Transactions of the Royal Society B 304: 425–434.

Willerslev E, Cooper A. 2005. Ancient DNA. Proceedings of the Royal Society B 272: 3–16.

Wisely SM, Maldonado JE, Fleischer RC. 2004. A technique for sampling ancient DNA that minimizes damage to museum specimens. Conservation Genetics 5: 105–107.

Yang DY, Eng B, Waye JS, Dudar JC, Saunders SR. 1998. Improved DNA extraction from ancient bones using silica-based spin columns. American Journal of Physical Anthropology 105: 539–543.

Wang X, Song X, Li L, Zhang G. 2012. An improved method of DNA extraction from the shell of the Pacific oyster, Crassostrea gigas. Israeli Journal of Aquaculture Bamidgeh 64: 1–4.

Watson SA, Morley SA, Peck LS. 2017. Latitudinal trends in shell production cost from the tropics to the poles. Science Advances 3: e1701362.

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