

Opportunities for reconstruction of pre-Contact native oyster distribution and population structure in north Puget Sound

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Managing for a successful recovery of native oysters (*Ostrea lurida*) requires an understanding of their “natural” diversity and population meta-structure. Establishing a baseline population model is confounded by the intensity of human exploitation of oyster populations prior to European contact and settlement, and commercial manipulation and ultimate destruction of oyster populations within a few decades following settlement and industrialization. Native oysters were cultivated throughout the Salish Sea, and growers not only transplanted brood stock, but also built networks of dikes to expand the oysters’ shallow sub-tidal habitat. Relic populations today have presumably also been influenced by widespread restoration out-plantings of hatchery seed, and possible hybridization with the repeatedly introduced European flat oyster.

There is no scarcity of fossil shell from archaeological sites and entombed oyster reefs in the San Juan Archipelago from 100-500 years before present. Ethnographic accounts and Coast Salish oral history identify additional sites that could be tested for fossil reefs. The question has been whether useable, uncontaminated DNA could be recovered from oyster shells from archaeological deposits and sediments.

Recent research on the process by which oysters build and repair their shells has shown it likely that cells remain trapped within shell layers during the life of the animal, and may persist for some time in sediments or archaeological deposits after the animal’s death.

Mollusk shell is mainly composed of a prismatic layer and a foliated or nacreous layer, both composed of calcium carbonate, although the prismatic and foliar are usually calcite and the nacreous aragonite (for reviews, see Marin and Luquet 2004; Zhang and Zhang 2006). The mechanical properties of shells depend primarily on the organic matrix upon which calcium carbonate crystallizes; the matrix consists of rich calcium-binding proteins that comprise barely five percent of the shell’s weight. Different shell matrix proteins are secreted from the mantle edge to enlarge the shell, and from the pallium to strengthen the myostracum. The shell matrix proteins and underlying cDNA sequences for *Crassostrea gigas* have already been partly characterized (Wheeler et al 1987; Miyamoto et al. 2002; Lee et al. 2006), but less is known about shell proteins in the genus *Ostrea*.

Mount et al. (2004) have now demonstrated that hemocytes also appear to be involved in the re-modeling of shell layers during growth in *Crassostrea virginica*. We predict that this is a highly conserved mechanism in the Ostreidae, and that it ensures the presence of trapped hemocytes—and nuclear and mitochondrial DNA—in shells of *Ostrea* as well as *Crassostrea*. In this paper we report preliminary success in extracting DNA from native oyster shell collected in 2005 from a living outplanted population, and from the entombed century-old Lopez Island reef. Questions that remain include the size and integrity of the

DNA fragments, i.e. whether suitable for making species- or population-level distinctions between individuals; possible differences in the integrity of fossil versus archaeological DNA; and persistence of useable DNA over longer periods of time. Yang et al. (2004) have recovered species-level genetic markers from salmon vertebrae in Pacific Northwest archaeological sites several thousand years old, but conditions of chromatin preservation presumably differ between vertebrate bone and mollusk shell.

Archaeological and ethnographic reports provide a starting point for understanding the pre-industrial distribution of native oysters. Some heavily exploited species, such as the red sea urchin, *Strongylocentrotus franciscanus*, are well represented in archaeological sites and, since they spoil rapidly, they are likely to have been consumed relatively near the waters where they were harvested (Barsh 2005). Pacific salmon (*Oncorhynchus spp*) were exploited using fixed gear in the San Juan Islands, leaving physical evidence in the form of anchor-stone piles (Easton 1985) as well as oral histories providing locations and ownership histories of individual fishing sites (Suttles 1951).

Two considerations give pause to relying on archaeology for the pre-Contact distribution of native oysters. Unshelled oysters keep longer than sea urchins and could conceivably have been consumed at a greater distance from harvesting sites. Ethnographic accounts indicate that oysters were eaten fresh where found, but generally shucked and dried for shipment and trade (Suttles 1951). If these practices survived unchanged from previous centuries, the correlation of archaeological oyster shell sites and harvesting sites should be close.

Native oysters also pose taphonomic issues for archaeologists. A possible bias may arise from the poor preservation of oyster shells in Pacific Northwest shell middens (stratified deposits of household refuse). Shells readily split along lamellar planes after the animal dies, and the thin lamellae are extremely fragile. This has resulted in some debate about the antiquity of human consumption of oysters in Puget Sound, since *Ostrea* remains are generally reported only from the most recent horizons of shell middens. Whether this is an artifact of preservation, or evidence of the relatively recent addition of oysters to the Coast Salish diet, is itself an important research question that may finally be resolved if oyster DNA survives in otherwise unrecognizable shell fragments in shell middens.

It must also be recognized that a majority of known north Puget Sound archaeological sites have not yet been explored in more than cursory fashion; and that many more are probably awaiting discovery. In short, there are many sources of false negatives.

Figure 1 shows existing data on the pre-1900 distribution of native oysters in the San Juan Islands. The circle marks the oyster reef sampled by naturalist C.B.R. Kennerly in 1858; the star marks the site of a reef entombed in mud and clay approximately 125 years ago; and the squares are archaeological sites containing oyster shell (500-1500 years old). It should be noted that any archaeological sites that may have been associated with either the Kennerly reef or the entombed Lopez Island reef, have long since been obliterated by development.

We do not include the recently extirpated Shoal Bay Lagoon native oyster population on Lopez Island, which was included in the presentation by Stick et al. (these proceedings). This population is almost certainly not endemic. The lagoon was constructed less than a half-century ago to serve as a swimming pool and was subsequently planted with oysters. Unfortunately, no record survives of the source of the introduced oysters; however, Stick et al. concluded that they were most closely related to the Discovery Bay population on the Olympic Peninsula. The history of the lagoon resolves this apparent phylogeographic anomaly.

Figure 2 shows two representative paired oyster shells from the shell midden on Fidalgo Bay, Fidalgo Island, WA (45SK43), blackened with charcoal and ash but otherwise intact and apparently not calcined by high cooking heat. Intact shells such as these were largely restricted to the site's San Juan Phase (see Stein 2000), i.e. approximately 200-500 years before present. We have recovered numerous articulated shells in comparable condition from the entombed Lopez Island reef, although they are more fragile when removed from the waterlogged clay matrix and must be dried slowly prior to handling.

Preservation of DNA in oyster shell is presumably affected by handling, e.g. whether the oysters were roasted, and by the chemistry of the depositional environment including pH (acidity resulting in depurination of DNA) and salinity (=calcium and magnesium salts). Shells may sometimes be better preserved in sediments because shells were not processed with heat; or worse preserved, due to long immersion in salt water. The entombed Lopez Island reef may present the "best case" where shells were deposited naturally in clay-rich sediment that appears to have protected them from abrasion and chemical degradation. In future, where possible, we will compare results from paired natural sediments and nearby archaeological sites to identify the nature and direction of possible taphonomic biases.

Methods

DNA extraction was attempted on four paired specimens of *O. lurida* shells: specimens 1 and 2 were collected from an entombed reef beneath Port Stanley Lagoon (Lopez Island, WA) estimated from historical sources to be 125 years old; specimens 3 and 4 were collected from a living population in 2006, a native oyster restoration site in Fidalgo Bay (Anacortes, WA). Specimens were processed with different sets of acid-washed and autoclaved tools to minimize the risk of cross-contamination.

Four fragments of approximately 1 cm² were removed from each specimen for different pre-extraction decontamination treatments as follows:

- (1) Sonicated for 10 minutes in a tube with 20-30 mL de-ionized water, then rinsed twice in de-ionized water and once in 100% ethanol, crushed with a glass rod and shaken gently overnight in Qiagen DNeasy lysis buffer before extraction in accordance with the Qiagen Dneasy animal tissue protocol (labeled *OCA*).

- (2) Rinsed for 10 minutes in de-ionized water, rinsed for 1 minute in 1 M NaOH, and rinsed again in de-ionized water; crushed with a glass rod and shaken gently overnight in

Qiagen DNeasy lysis buffer before extraction in accordance with the Qiagen Dneasy animal tissue protocol (marked *OCB*).

(3) Rinsed for 10 minutes in de-ionized water, rinsed for 1 minute in 1-2% NaClO, rinsed for 1 minute in 1 M HCl and neutralized for 1 minute in 1 M NaOH, rinsed in de-ionized water again and then exposed to UV for approximately 20 minutes; crushed with a glass rod, and shaken gently overnight in Qiagen DNeasy lysis buffer before extraction in accordance with the Qiagen Dneasy animal tissue protocol (marked *OCC*).

(4) Rinsed for 10 minutes in de-ionized water, then gently shaken overnight at 55° C in a decalcifying solution of 0.5 M EDTA (pH 8.0), 0.5% SDS, and 100 µM proteinase K (Yang et al. 2004); after which they were crushed and shaken an for additional 4 hours at 55° C. We then added 400 µl Qiagen DNeasy buffer AL and 400 µl 100% ethanol to the crushed shell, centrifuged the mixture at 2000 g for 5 minutes, removed the supernatant and centrifuged it at 16000 g for 5 minutes before extraction in accordance with the Qiagen Dneasy animal tissue protocol (marked *OCD*). Both the decalcified residue and the used decalcification solutions were separately amplified.

Aliquots (1 µl) of extracted DNA were run on 1% agarose gels containing 2 µl ethidium bromide per 100 ml. No bands were observed. Extracted DNA was then amplified using three sets of primers for related species in the absence of publicly available sequences for *Ostrea lurida*.

(1) Primers for 5S rDNA that amplify in Ostreidae and other bivalves including *Mytilus galloprovincialis*, *Ostrea edulis*, *Ostrea stentina*, *Crassostrea angulata*, and *Crassostrea gigas* (Cross and Redbordinos 2006): MT1 (5'-CGTCCGATCACCGAAGTTAA), MT2 (5'-ACCGGTGTTTTCAACGTGAT).

(2) Primers designed to amplify a portion of the non-transcribed spacer in the 5S rDNA region of one closely related species, *Ostrea edulis* (Cross and Redbordinos 2006): ED1 (5'-GACTTGCCATTTTAGAGGGTCT); ED2 (5'-TGTTTAATTGGTGATAACGATGA).

(3) Forward and reserve primers for two highly polymorphic *Ostrea edulis* microsatellites (Launey et al. 2002): Oedu H15-F (5'-TTTTGACTCTGTGATATCGAC); Oedu H15-R (5'-TAATGATTTTCGTTTCGTTGAC); Oedu J12-F (5'-GCTGTATTTCCATCAATTTCGAG); Oedu J12-R (5'-TCGTCACCTCCCTCTCAGAG).

PCR reactions were initiated with 9 µl Invitrogen Platnum PCR Super Mix, 1 µl template DNA, 0.05 µl 10 mM forward primer and 0.05 µl 10 mM reverse primer. PCR reactions with MT and ED primers were run on a Biometra thermocycler and cycled at 94° C for 5 minutes, followed by 56 cycles at 94° C for 45 seconds, 59° C for 45 seconds, and 72° C for 1 minute, with a final elongation step at 72° C for 10 minutes. PCR reactions with microsatellite primers were run on a Biometra thermocycler and cycled at 94° C for 2 minutes, followed by 56 cycles of 94° C for 1 minute, 50° C for 1 minute, 72° C for 75 seconds, with a final elongation step of 72° C for 5 minutes. All PCR products were run on 1% agarose gels containing 2 µl ethidium bromide per 100 ml in 0.5X TBE at 100V, and visualized using a Biorad ChemiDoc XRS and Quantity One software. OCA, OCB

and OCC were run for 30 minutes (Figures 3a and 4a); OCD were run for 15 minutes (Figures 3b and 4b).

Results

DNA that can be amplified using the MT and ED primers was extracted from both recent and 100 year-old shell (Figure 3). Microsatellite primers developed for *Ostrea edulis* did not consistently produce bands from the extracts, however (Figure 4). Bands amplified with MT and ED primers were smaller than reported in *Ostrea edulis*. Decontamination methods appear to have affected the amplification; sonication alone and NaOH alone produced the most amplification, but may have allowed non-target DNA to be extracted. It is possible that *Ostrea* shell is too translucent to expose to UV without penetrating the inner shell lamellae and destroying target DNA. Decalcification prior to extraction did not improve amplification.

A balance must be maintained between removing contamination and preserving target DNA. Method OCC without UV may represent a reasonable compromise. Future work will require more specific primers (such as those developed by Stick et al and Wright et al. in these proceedings). Our results show that the recovery of DNA from *Ostrea* shell is feasible, but that further work to optimize extraction and recover useful genetic data is necessary.

Acknowledgments

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FIGURES

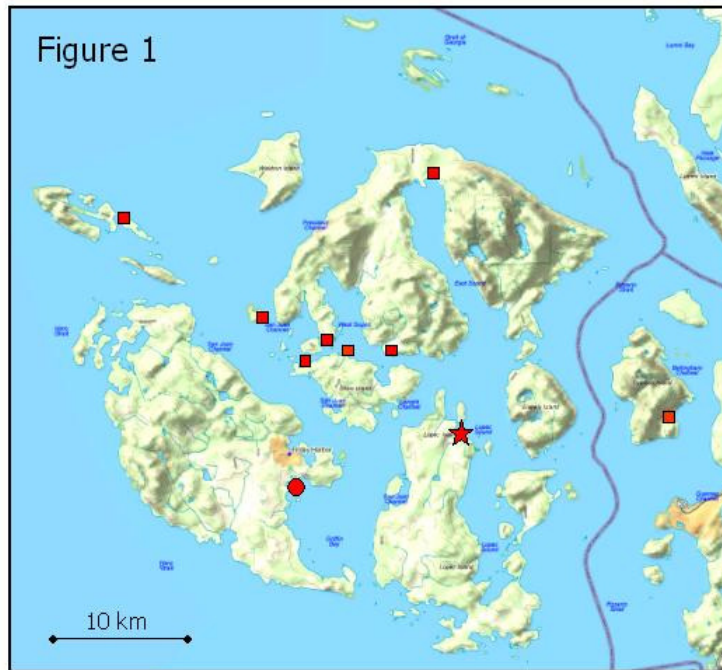


Figure 1. Locations of archaeological sites in the San Juan Islands, WA, with oyster shell 500-1500 years old (squares); also the oyster reef sampled by naturalist C.B.R. Kennerly in 1858 (circle) and a reef entombed in mud and clay approximately 125 years ago (star).

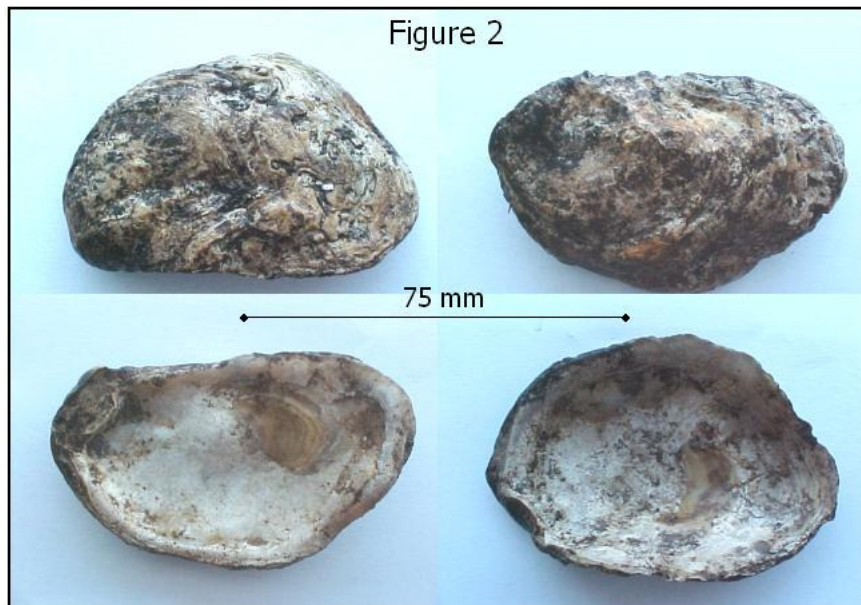


Figure 2. Two paired valves of native oysters (*Ostrea lurida*) recovered from the Fidalgo Bay archaeological site (Anacortes, WA) in 2004.

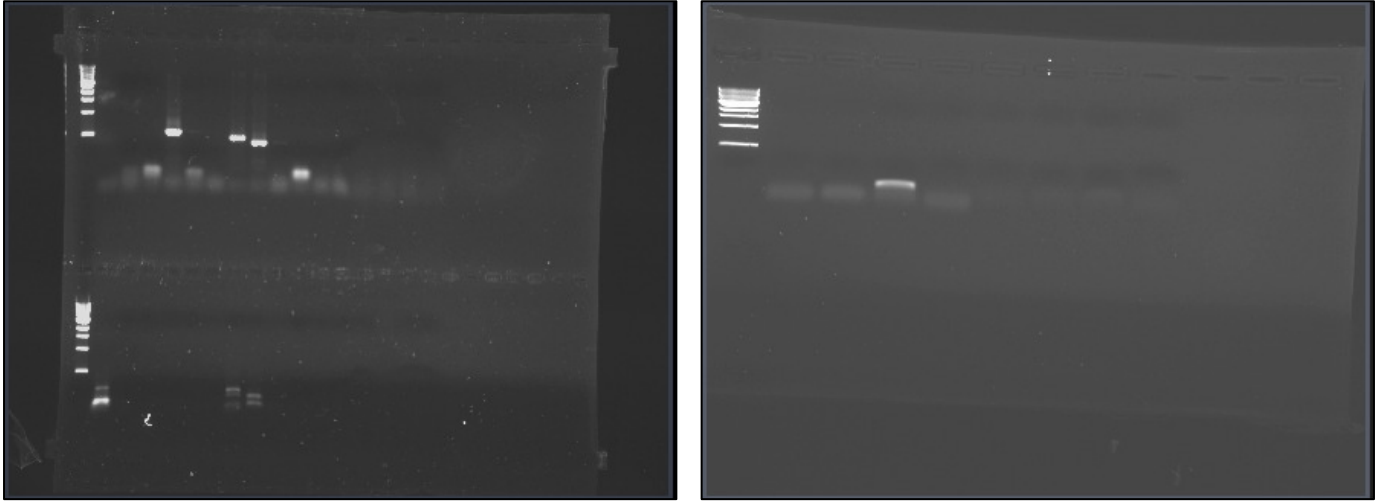


Figure 3. (a) PCR of *Ostrea lurida* shell specimens *OCA*, *OCB*, *OCC* and *OCD* with MT primers (upper lanes) and ED primers (lower lanes). (b) PCR of shell specimen *OCD* with MT primers (lanes 2 to 5) and ED primers (lanes 6 to 9). The leftmost lane in both images is a 10 µl 1-kb ladder. Amplification with the MT primers can be seen in *OC3B*, *OC3D*, *OC4A* and *OC4B* (recent shell), *OC2C* (fossil shell) and possibly *OC3A* (recent) and *OC1B* (fossil). Amplification with ED primers can be seen in *OC1A* (fossil) and in *OC3B* and *OC4B* (recent).

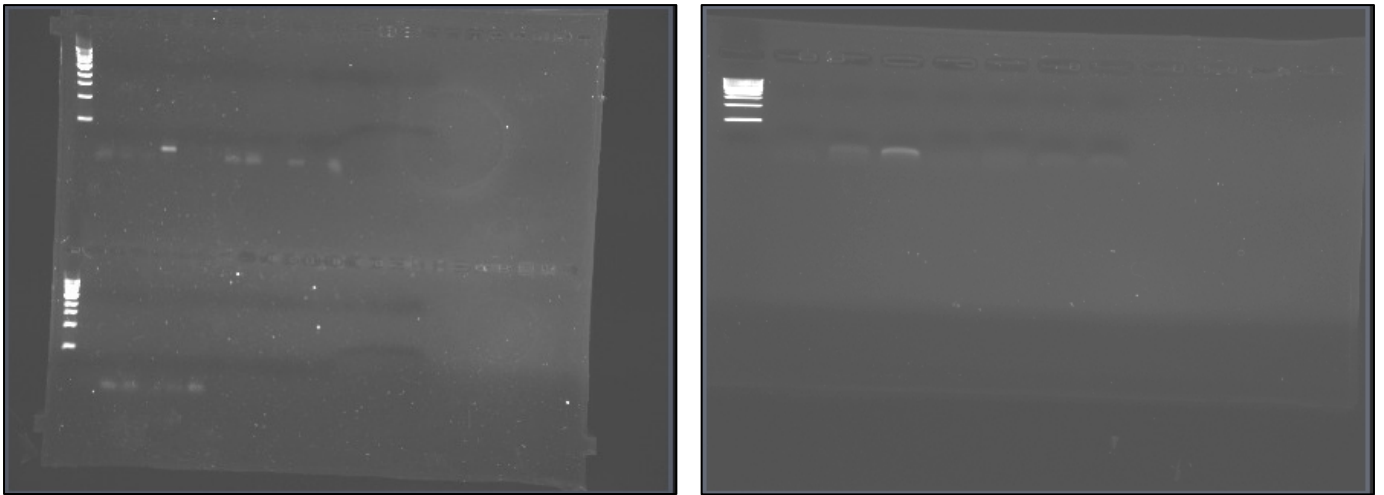


Figure 4. (a) PCR of *Ostrea lurida* shell specimens *OCA*, *OCB*, *OCC* and *OCD* with Oedu H15 primers (upper lanes) and Oedu J12 primers (lower lanes). (b) PCR of shell specimen *OCD* with Oedu H15 primers (lanes 2 to 5) and Oedu J12 primers (lanes 6 to 9). The leftmost lane in both images is a 10 µl 1-kb ladder. Amplification with the Oedu H15 primers can be seen in *OC4A* (recent shell), and possibly in *OC2C* (fossil shell) and *OC3B*, *OC4B* and *OC3D* (recent). Amplification with Oedu J12 is possibly seen in *OC2A* and *OC2B* (fossil) and *OC3A* (recent).