Bivalve ligament—a new source of DNA for historical studies

Sandra Doherty[1], Elizabeth Gosling[1,*], Anna Was[1,2]

[1]Molecular Ecology Research Group, Department of Life Sciences, Galway-Mayo Institute of Technology, Dublin Road, Galway, Ireland

[2]Sea Fisheries Institute Gdynia, Kollataja 1, 81-332 Gdynia, Poland

ABSTRACT: Bivalves are aquatic animals enclosed in 2 rigid shells that are held together by a hinge ligament. Features of the shell valves are important in taxonomy, and museums hold global collections of catalogued fossil, historical and contemporary specimens. Museum collections of plants and animals are routinely used for the retrieval of DNA, but to date this has not been extended to bivalves. We show that DNA can be extracted from the ligament of the bivalve shell. The use of this tissue for DNA extraction allows for the non-destructive sampling of archived specimens, as once a portion of the ligament has been removed, the remaining shell plus shell ligament scar, essential features in classical taxonomic identification, are left intact. We extracted DNA from the ligament of individual mussel shells collected in 1970, and compared population genetic structure using a nuclear DNA marker with a sample of recent (2006) shells from the same location. We present a new source of genetic data that can be used to expand many areas of bivalve taxonomy, population history and phylogeography.

KEY WORDS: Bivalve shell ligament · DNA retrieval · Archived specimens

INTRODUCTION

Recent developments have greatly increased the efficiency of DNA recovery from ancient and preserved specimens (Nicholls 2005, Gilbert & Willerslev 2007). The application of such methodologies to specimens archived in laboratories and museums has provided new opportunities for the study of genetic changes in populations over long periods of time and thus provides a rich pool of information that can be applied to a wide variety of conservation and evolutionary questions.

The inevitable degradation of DNA by endogenous nucleases after death presents problems. Bacteria, fungi and insects that feed on macromolecules can further degrade DNA. However, degradation is slowed down under conditions of rapid desiccation of tissue after death, or if the DNA becomes absorbed into a mineral matrix such as bone or teeth (Pääbo et al. 2004). Natural degradation results in the reduction in the size and quantity of DNA molecules, but the problem can be overcome by using primers that can amplify short fragments of <200 to 300 base pairs (bp) (Taberlet et al. 1999).

Traditionally, studies of temporal changes in populations of aquatic organisms have been carried out using fresh or frozen tissue samples. With the development of rapid extraction methods to recover DNA from ethanol-preserved tissue and bony structures such as fish scales (Yue & Orban 2001) and otoliths (Hutchinson et al. 1999), it is now possible to compare historical and contemporary populations of fish (Hutchinson et al. 2003, Hoarau et al. 2005). For example, Hutchinson et al. (2003) identified 2 significant population declines associated with intense exploitation over the period 1954 to 1998, using a temporal series of archived otoliths from North Sea cod, currently listed as a threatened species (IUCN 2002).

Bivalves are aquatic animals enclosed in 2 rigid shells and number between 15 000 (Pojeta et al. 1987) and 30 000 species (Bivalvia 2007, http://en.wikipedia.org/wiki/bivalves, accessed 11 July). The shell is a carbonate material secreted by the mantle, a thin layer of tissue surrounding the animal, and the hinge ligament

*Corresponding author. Email: elizabeth.gosling@gmit.ie

attaches the 2 valves. We hypothesize that in the construction of the ligament, some cells, along with their DNA, become embedded and preserved in the protein-calcium carbonate matrix. Alternatively, a few cells may remain attached to the ligament after death, and serve as a source of DNA. The use of the ligament for DNA extraction also allows for the non-destructive sampling of archived specimens because once the ligament has been removed, the remaining shell is left intact.

MATERIALS AND METHODS

Samples. Mussel shells from Kilmore Quay, Co. Wexford, Ireland (52° 10′ N 6° 35′ W), collected in the summer of 1970 for a morphometric study (Seed 1974), and subsequently deposited with the Belfast Natural History Museum, were analysed together with a recent sample collected in November 2006 from the same location. Shells from each sample (N = 50) were washed in sterile deionised water and dried in an incubator at 100°C for 4 h to remove any adhering cells, either from the mussel's own (native) tissue or contaminating cells from another individual. For each shell valve, any remaining ligament on the shell was removed and placed into a 1.5 ml Eppendorf tube and the tissue crushed with a metal rod. This procedure was performed under sterile conditions.

DNA extraction and analysis. DNA was extracted using the QIAGEN DNEASY® Blood and Tissue Kit (Qiagen). The manufacturer's protocol was followed, except digestion time was increased from 5 to 6 h to approximately 30 h. To compare results from shell ligament and body tissue in the 2006 sample, DNA was extracted from the posterior adductor of the same 50 ind. using a standard phenol-chloroform method. DNA was quantified using a Cary 50 scan UV visible spectrophotometer that uses Cary WinUV software.

In Ireland, mussels are comprised of an interbreeding mixture of *Mytilus edulis* and the Mediterranean mussel *M. galloprovincialis* (Gosling 1992). To identify the 2 species and their hybrids we used the DNA marker *Me15/16*, located within the nuclear gene encoding a polyphenolic adhesive protein, a key component in forming the byssal threads that attach mussels to the substrate. PCR amplification produces a species-specific band of about 180 bp for *M. edulis* and a 126 bp band for *M. galloprovincialis*; individuals with both bands are considered to be hybrids (Inoue et al. 1995). We are aware that the use of the term 'hybrid' is a simplification, because if hybrids are fertile both the two-banded and single banded genotypic classes will also contain some F2 backcross individuals.

Amplification of *Me15/16* was carried out in a 15.5 µl reaction mix containing 6.9 µl of extracted DNA, 0.6 mM of each dNTP, 2.5 mM of $MgCl_2$, 6 µM of forward and reverse primers, 0.6 U of RedTaq™ polymerase, PCR buffer (Sigma) and sterile deionised water. Protocols for thermocycling and electrophoresis followed Coghlan & Gosling (2007). Negative and positive controls, plus a molecular weight ladder (HyperLadder V, Bioline), were included on each gel.

Because only 2 alleles and 3 genotypes are observed at the *Me15/16* locus, it is possible that the single or 2-banded patterns on the gel may represent amplified DNA from more than one individual. Therefore, to test for possible contamination between individual mussels in the historical sample we used a dinucleotide repeat microsatellite marker, *Mgµ7*, for which 14 alleles have been reported (Presa et al. 2002) with potentially over 100 genotypes expected. Amplification was carried out in a 10 µl reaction mix containing approximately 1 µl (5 to 60 ng) of template DNA, 0.2 mM of each dNTP, 1.5 mM of $MgCl_2$, 10 pmol of forward and reverse primer, 0.5 U of RedTaq™ polymerase, PCR buffer (Sigma) and sterile deionised water. The thermocycling protocol consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 35 cycles at 55°C for 40 s, 35 cycles at 72°C for 1 min, and a final extension at 72°C for 15 min. PCR products were diluted (1:10) with deionised water; this mixture was again diluted (1:10) with loading buffer. Amplification products were separated on 6.5% polyacrylamide gels using a Li-COR 4300 automated sequencer.

RESULTS

DNA was successfully extracted and amplified from 25 of the 50 ind. from the historical (1970) sample. The genotype frequencies were: *Mytilus edulis* = 0.64, *M. galloprovincialis* = 0.08, hybrids = 0.28 (Fig. 1). DNA was also extracted from the shell ligament and adductor muscle tissue of 40 of the 50 ind. from the recent (2006) sample, and in every case there was an exact match between shell ligament and adductor muscle tissue genotype, and genotype frequencies did not differ significantly from those in the 1970 sample: *M. edulis* = 0.65, *M. galloprovincialis* = 0.05, hybrids = 0.30 (*G* = 0.22, df = 2, ns, Sokal & Rohlf 1994). The quantity of DNA obtained from the shell ligament in the 2006 sample (85 to 500 ng µl^{-1}) was considerably variable, as was that from the shell ligament in the 1970 sample (4.25 to 235 ng µl^{-1}). By comparison, the quantity of DNA (600–700 ng µl^{-1}) obtained from adductor muscle tissue in the 2006 sample was far greater than that obtained from either the 1970 or 2006 shell ligament tissue.
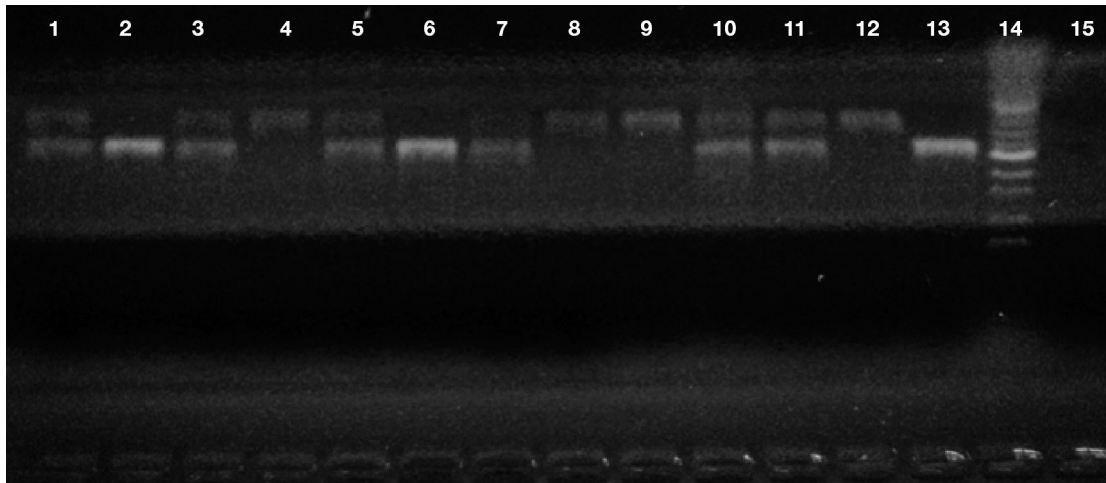
Fig. 1. *Mytilus* spp. *Me15/16* DNA markers from posterior adductor tissue sampled in 2006 and shell ligaments sampled in 2006 and 1970. Lanes 1 to 4: tissue from 2006; lanes 5 to 8: shell ligaments from same individuals as in 1–4; lanes 9 to 12: 1970 shell ligaments; lane 13: positive control (*M. galloprovincialis*, Madeira Island); lane 14: ladder (higher intensity bands indicate 200 and 100 bp fragments); lane 15: negative control. Single-banded genotypes 126/126 and 180/180 are *M. galloprovincialis* and *M. edulis*, respectively; double-banded genotypes 126/180 are hybrid individuals

Because of the low variability of *Me15/16*, there is the possibility that single-banded genotypes on the gel may represent amplified DNA from more than one individual of M*ytilus edulis* or *M. galloprovincialis*, or, in the case of the 2-banded pattern, may include individuals from both taxa. The only way we could realistically test for possible contamination was to use a highly variable microsatellite marker developed for *M. galloprovincialis*. A total of 17 alleles and 21 different *Mgμ7* genotypes were observed when muscle tissue from 39 ind. in the 2006 sample were analysed (Table 1). Shell ligament DNA from these 39 ind. was also assayed, and in every case there was an exact match between tissue and shell ligament genotype. In the 1970 sample 10 alleles and 12 different genotypes were observed for 22 ind. that successfully amplified during PCR.

In the 2006 sample, 36 of the 39 ind. were 2-banded *Mgμ7* heterozygotes and the other 3 were single-banded homozygotes, while in the 1970 sample, 15 of the 22 ind. were heterozygotes and 7 were homozygotes. If there was contamination in the 1970 sample, some of the 'heterozygotes' could in fact comprise 2 or more individuals homozygous for 2 different alleles. However, tests for deviations from Hardy-Weinberg equilibrium (Raymond & Rousset 1995) showed no observable significant excess (or deficit) of heterozygotes in either the old (p = 0.917, ns) or new (p = 0.886, ns) samples. Conversely, some homozygotes might comprise 2 or more individuals homozygous for the same allele. The very low homozygosity in the 2006 sample, and to a lesser extent in the 1970 sample, makes it unlikely that contaminating individuals, if

Table 1. *Mytilus* spp. *Mgμ7* microsatellite genotypes from tissue of individuals collected in 2006 (N = 39), and from the ligament of shells collected in 1970 (N = 22). No: number of individuals with the genotype. Species composition of individuals with the genotype: Me, *M. edulis*; Mg, *M. galloprovincialis*; H, hybrid, all identified using *Me15/16*

2006			1970		
Genotype	No.	Species	Genotype	No.	Species
147/147	1	Me	147/147	1	Me
147/149	2	2 H			
147/161	2	2 Me	147/161	5	H, 4 Me
147/163	2	Me, Mg			
147/167	5	4 Me, H			
147/169	2	2 Me			
147/177	2	2 Me			
147/195	2	H, Me			
149/167	2	Me, H			
			149/177	1	Mg
161/161	1	Me	161/161	5	3 Me, 2H
161/163	1	Me	161/163	3	3 Me
			161/165	1	H
			161/167	1	H
161/169	2	Mg, Me			
161/175	2	2 H			
			161/177	1	Me
			161/179	1	Me
			163/173	1	H
167/209	1	Me			
167/211	1	Me			
			169/173	1	Mg
169/179	2	Me, H			
169/185	2	H, Me			
171/179	2	H, Me			
			173/173	1	Me
173/205	2	2 Me			
177/179	2	2 H			
197/197	1	Me			

present, would appear as homozygotes for the exact same allele. Alternatively, in view of the high heterozygosity of the 2006 (0.92) and 1970 (0.68) samples, one might expect to see *Mgμ7* genotypes with 3 or more alleles if contamination was an issue. While most individuals were either 1- or 2-banded, some had additional bands in both the old (N = 2) and new (N = 7) samples. Since the same banding pattern was observed for tissue and shell ligament of the 7 ind. in the 2006 sample, we think that the additional bands were not caused by contamination but were non-specific products of PCR amplification, probably related to the fact that *Mgμ7* was specifically developed for *M. galloprovincialis*.

DISCUSSION

We have shown that it is possible to extract DNA from bivalve shell ligaments, previously not considered subjects for genetic analysis. Historical shell collections abound in museums worldwide and have the advantage of being individually catalogued with collection date and place. Another source of ancient and historical samples are shell middens, archaeological deposits of food refuse found in coastal zones all over the world, mostly consisting of the remains of edible molluscs. Radio carbon dating of shells has shown that the age of middens ranges from the Mesolithic period (~8000 to 4000 BC) to the 19th century (Cunha et al. 2002).

Our study has unlocked a new source of genetic data that can be used to expand many areas of bivalve taxonomy, population history and phylogeography. Biological research is dependent on species identification, and DNA sequences in conjunction with morphological and ecological information can be useful, particularly for closely related or poorly differentiated species. A 650 bp fragment of the *5′* end of the mitochondrial gene cytochrome *c* oxidase I (COI) has been designated as the barcode region for animals (Hebert et al. 2003), although other variable sequences can be used. For specimens with degraded DNA, shorter COI fragments (100 to 200 bp) have been shown to be effective for identification of species in >90% of cases (Hajibabaei et al. 2006). Currently, barcode library construction focuses on DNA analysis from recently collected, well-preserved specimens, and to date about 2000 bivalve specimens have been barcoded, the majority from unspecified regions[1]. The retrieval of DNA sequences from

archived shells would provide a cost-effective way of building barcode libraries with a broader geographical coverage of individual taxa than has been possible to date. In addition, retrieving DNA from shell ligament may allow comparison of sequences from extinct species of bivalves with those of extant species, via molecular phylogenies using sequence data with sufficient phylogenetic signal to resolve evolutionary relationships e.g. 18S rRNA, 28S rRNA (Hajibabaei et al. 2007).

It may be possible to track changes in bivalve populations over time through analysis of the DNA from preserved museum specimens, either retrieved by archaeologists at a single site, or collected from a single locality by earlier generations of scientists. We used *Me15/16* and *Mgμ7* to compare genetic structure between a 37 yr old mussel sample and a present-day one, both collected from the same location. We found no significant difference in allele frequency between old and new samples at the *Me15/16* locus. There appeared to be differences using *Mgμ7*, but in view of the high polymorphism of this locus and the relatively small sample sizes analysed, we did not attempt a statistical comparison. So far, DNA temporal studies have focused on just a few commercially important marine fish species and most have provided convincing evidence, from archived otoliths or scales, of genetic changes through time, e.g. reduced heterozygosity and loss of alleles resulting from population decline linked to fishing pressure (Hauser et al. 2002, Hutchinson et al. 2003, Hoarau et al. 2005), or temporal heterogeneity due to immigration from genetically differentiated populations elsewhere (Purcell et al. 1996). In addition, effective population size (N_e), often several orders of magnitude less than census population size, can be estimated from a decline in heterozygosity (Nei et al. 1975), or from fluctuations in allele frequencies through time (Waples 1989), either over short intervals, e.g. several generations in fishery-based studies, or over a much longer timescale where large environmental fluctuations, such as glaciations, may affect N_e.

Genetic data combined with radio carbon dating of shells could be a powerful tool in determining how species ranges have changed over time. For example, mussels in the southern hemisphere are thought to have arisen as the result of a migration event from the northern hemisphere, via the Atlantic Ocean, during a Pleistocene ice age (Hilbish et al. 2000). Evidence for this has been based on an analysis of mitochondrial DNA sequence data from contemporary samples of preserved mussel tissue. Perhaps a more comprehensive view of events would unfold if DNA sequence data from museum collections or archaeological sources had been available for analysis.

---

[1]BOLD (2007) Barcode of Life Data Systems, www.barcodinglife.com, accessed July 11

LITERATURE CITED

Coghlan B, Gosling EM (2007) Genetic structure of hybrid mussel populations in the West of Ireland: two hypotheses revisited. Mar Biol 150:841–852

Cunha E, Umbelino C, Cardosa F (2002) New anthropological data on the Mesolithic communities in Portugal: the shell middens from Sado. Hum Evol 17:187–197

Gilbert MT, Willerslev E (2007) Rescuing ancient DNA. Nature Biotechnol. 25: 872–874

Gosling EM (1992) Genetics. In: Gosling EM (ed) The mussel *Mytilus*: ecology, physiology, genetics and culture. Elsevier Science, Amsterdam, p 309–382

Hajibabaei M, Smith MA, Janzen DH, Rodriguez JJ, Whitfield JB, Hebert PDN (2006) A minimalist barcode can identify a specimen whose DNA is degraded. Mol Ecol Notes 6:959–964

Hajibabaei M, Singer GAC, Hebert PDN, Hickey DA (2007) DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. Trends Genet 23:167–172

Hauser L, Adcock GJ, Smith PJ, Ramirez JHB, Carvahlo GR (2002) Loss of microsatellite diversity and low effective population size in an overexploited population of New Zealand snapper (*Pagrus auratus*). Proc Natl Acad Sci USA 99:11742–11747

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identification through DNA barcodes. Proc R Soc Lond B Biol Sci 270:313–321

Hilbish TJ, Mullinax A, Dolven SI, Meyer A, Koehn RK, Rawson PD (2000) Origin of the antitropical distribution pattern in marine mussels (*Mytilus* spp.): routes and timing of transequatorial migration. Mar Biol 136:69–77

Hoarau G, Boon E, Jongma DN, Ferber S and others (2005) Low effective population size and evidence for inbreeding in an overexploited flatfish, plaice (*Pleuronectes platessa* L.). Proc R Soc Lond B Biol Sci 272:497–503

Hutchinson W, Carvalho G, Rogers S (1999) A non-destructive technique for the recovery of DNA from dried fish otoliths for subsequent molecular genetic analysis. Mol Ecol 8:893–894

Hutchinson W, van Ousterhout C, Rogers S, Carvalho G (2003) Temporal analysis of archived samples indicates marked genetic changes in declining North Sea cod (*Gadus morhua*). Proc R Soc Lond B Biol Sci 270:2125–2132

Inoue K, Waite JH, Matsouka M, Odo S, Harayama S (1995) Interspecific variations in adhesive protein sequences of *Mytilus edulis, M. galloprovincialis*, and *M. trossulus.* Biol Bull 189:370–375

IUCN (2002) Red List of threatened species. International Union for Conservation of Nature and Natural Resources, Gland

Nei M, Maruyama T, Chakraborty R (1975) The bottleneck effect and genetic variability in populations. Evolution 29:1–10

Nicholls H (2005) Ancient DNA comes of age. PloS Biol. 3:e56

Pääbo S, Poinar H, Serre D, Jaenicke-Després V and others (2004) Genetic analyses from ancient DNA. Annu Rev Genet 38:645–679

Pojeta J, Runnegar D, Peel JS, Gordon M (1987) Phylum Mollusca. In: Boardman RS, Cheetham AH, Rowell AJ (eds) Fossil invertebrates. Blackwell Scientific Publishing, Palo Alto CA, p 270–435

Presa P, Perez M, Diz AP (2002) Polymorphic microsatellite markers for blue mussels (*Mytilus* spp.). Conserv Genet 3:441–443

Purcell MK, Kornfield I, Fogarty M, Parker A (1996) Interdecadal heterogeneity in mitochondrial DNA of Atlantic haddock (*Melanogrammus aeglefinus*) from Georges Bank. Mol Mar Biol Biotechnol 5:185–192

Raymond M, Rousset F (1995) GENEPOP Version 1.2.: population genetics software for exact tests and ecumenicism. J Hered 86:248–249

Seed R (1974) Morphological variations in *Mytilus* from Irish coasts in relation to occurrence and distribution of *M. galloprovincialis* Lmk. Cah Biol Mar 15:1–25

Sokal RR, Rohlf FJ (1994) Biometry: the principles and practice of statistics in biological research. W. H. Freeman, New York

Taberlet P, Waits L, Luikart G (1999) Non-invasive genetic sampling: Look before you leap. Trends Ecol Evol 14:323–327

Waples RS (1989) A generalised approach for estimating population size from temporal changes in allele frequency. Genetics 121:379–391

Yue GH, Orban L (2001) Rapid isolation of DNA from fresh and preserved fish scales for polymerase chain reaction. Mar Biotechnol 3:199–204