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A REVIEW OF CURRENT KNOWLEDGE OF TECHNIQUES TO EXTRACT AND AMPLIFY DNA FROM 'DIFFICULT' WHALE SAMPLES

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Abstract

DNA analysis methods currently used for whale product identification are dependent on extraction and PCR amplification of cetacean nucleic acids, but certain product types and intensive processing may restrict the amount of DNA recovered or degrade the DNA and inhibit amplification. Newly developed methods developed for "ancient DNA" and human forensic analysis may provide for more robust extraction from and amplification of cetacean tissues, including: (1) bone demineralization procedures which can release 10X more DNA than standard methods, (2) optimized silicon-capture techniques that more effectively isolate and purify DNA, (3) post-extraction purification that can remove inhibitors from degraded and chemically treated DNA isolated from "processed" samples, (4) whole genome amplification methods which increases the amount of DNA template material prior to PCR amplification, and (5) use of a variety of different DNA polymerases which can amplify cetacean DNA with high specificity, even in a background containing degradation products and other inhibitors.

Introduction – DNA Extraction and PCR are critical steps

DNA analysis methods currently used for whale product identification are dependent on PCR amplification of genetic components from purified tissue extracts. Amplified mitochondrial fragments are commonly used for species identification of products from a huge variety of animal products (see review by Rasmussen and Morrissey 2009) including cetaceans (e.g. Baker *et al*. 1996, Cipriano and Palumbi 1999a, Baker *et al.* 2006). After successful amplification the resulting PCR product is purified, used as template in a secondary "cycle sequencing" reaction, and the resulting products from that reaction then separated electrophoretically to produce DNA sequence data. Microsatellite analysis uses PCR amplification of nuclear DNA regions containing microsatellite repeats (also called "short tandem repeats" or STRs), followed by electrophoretic separation of the fluorescently labelled PCR products, to determine the number of repeat units in both alleles of each locus analyzed (typically ten or more), and is similarly dependent on effective tissue purification methods that permit successful PCR. Processing of commercial products may make it difficult to extract and amplify DNA from those products, because DNA degradation is associated with three general problems: (i) reduced quantity of DNA, which makes those samples particularly sensitive to exogenous contamination, (ii) breakage of DNA strands, which reduces the lengh of sections that can be amplified, and (iii) DNA damage which can lead to the identification of false mutations (Leonard 2008). In addition, tissue degradation and heat/chemical processing can result in the presence of chemical inhibitors that prevent operation of the polymerases used to amplify sections of DNA.

Processing can involve physical shearing/grinding, heat, high pressure, and a variety of chemical treatments. Poor preservation of unprocessed tissues may also result in samples that are difficult or impossible to PCR. Similar difficulties face researchers attempting to analyze "ancient DNA" (aDNA) from specimens of teeth, bones, museum skins, naturally mummified tissues, archeological and paleontological remains ranging in age from tens of years to about a hundred thousand years old (Lindahl 1993) and forensic investigators also routinely deal with tiny specimens containing scant and degraded DNA, often accompanied by inhibitors and contaminants. Although expensive and tedious, advanced methodologies now being used with <200 year old materials archived in museums (e.g. Wandeler *et al.* 2007), truly ancient DNA samples collected from ancheological and paleontological sites (e.g. Leonard *et al.* 2000) and forensic samples (e.g. Giardina *et al.* 2009) may further extend the type of samples that

can be routinely analyzed in the future. In addition, the use of such techniques to analyze modern and historical samples at the population level holds great promise for many lines of research critical to wildlife management (Leonard 2008), such as estimating past population sizes, levels of gene flow, and relatedness between populations before the perturbations caused by humans in the last centuries (Ramakrishnan *et al.* 2005). Because biases inherent in estimating population parameters are exaggerated when populations are small or have been heavily impacted, the inclusion of samples from past populations represented by historical samples ameliorates many of the difficulties that undermine conclusions based only on samples from extant populations (Leonard 2008). Initial attempts to analyze historical samples at the population level (Borge *et al.* 2007, Nichols *et al.* 2007) illustrate the potential value of such samples in our interpretation of population parameters critical for management decisions, such as effective population size and connectivity (i.e. many of the components of "stock structure").

Alternate Extraction Methods for Different Cetacean Tissue Types

Previous experience with canned whale meat and highly processed products indicated that at least one problem with such samples is that DNA strands are short and fragmented, and chemicals used in the processing inhibit PCR (e.g. Cipriano and Palumbi 1999b). Similar problems have been encountered by researchers working with "ancient DNA" and by forensic scientists working with human remains that have been degraded by decomposition or damaged by heat, chemicals, burning, and explosions (e.g. Weber *et al.* 2000, Esslinger *et al.* 2004, Ye *et al.* 2004). Tissue types represented by different kinds of whale products include: fresh, frozen, dried, salted or parboiled red meat; fresh, frozen, dried, salted or parboiled blubber; canned and stewed meat and organs; sliced, dyed and chemically treated muscle tissue, organs, skin, blubber and baleen; all of the preceeding may be found in a variety of packaging types and accompanied by a variety of sauces. Unfortunately, while automated methods for routine extraction of DNA from fresh, frozen, or chemically preserved cetacean tissue specimens are fast, require only tiny amounts of tissue, yield very "clean" DNA containing few inhibitors, and allow very high throughput of hundreds of specimens extracted per day, they are not effective on many of the tissue and product types mentioned above, so techniques for some market products require more laborious and time-intensive "manual" methods not yet available in an automated format.

Here we present alternative methods already in use by cetacean researchers for analysis of different types of market samples, including some that are known to be difficult to extract and amplify (Fig. 1). We will also briefly review some of the developing methodologies being used by the forensic and ancient DNA communities that may also prove useful in the identification analysis of market products. Genetic studies using historical specimens from natural history collections have similar difficulties, because DNA extracted from such specimens is often degraded, analyses of such samples is technically demanding, and many potential pitfalls exist; discussion of this and general guidelines for study design and laboratory methods for use with such samples are provided by Wandeler *et al.* (2007).

Many of the surveys of whale products sold in Korean and Japanese markets have taken advantage of one of the simplest DNA extraction techniques, the use of Chelex 100 resin (BioRad, Hercules CA) following the protocol of Walsh, Metzger and Higuchi (1991) as described in Baker *et al.* (1996), which is amazingly effective on a variety of tissue types given its simplicity (the protocol consists of adding a tiny speck of tissue to a tube containing 5-10% Chelex suspension in water, and incubating at 95^{o}C for 20 minutes). Chelex extraction is very effective for isolating DNA from fresh, frozen or parboiled cetacean tissue and blood samples, but canned whale products are often difficult to extract and amplify, presumably because the canning process includes high heat and pressure which fragments the DNA (Cipriano 2005). Chelex extractions also have a relatively low success rate with pure blubber samples that contain no skin or muscle tissue. Improvement in the analysis of canned samples was achieved with use of a glass milk suspension for capturing DNA from lysed tissues, sequestering the bound DNA on a DNEasy (Qiagen Inc.) column, rinsing the bound DNA to remove inhibitors, and use of closely separated PCR primers to target small sections of the fragmented strands (Cipriano 2005).

Techniques for extracting DNA from bone and tooth samples have been well developed to take advantage of the availability of such specimens in museum collections (e.g. Morin *et al.* 2006, Borge et

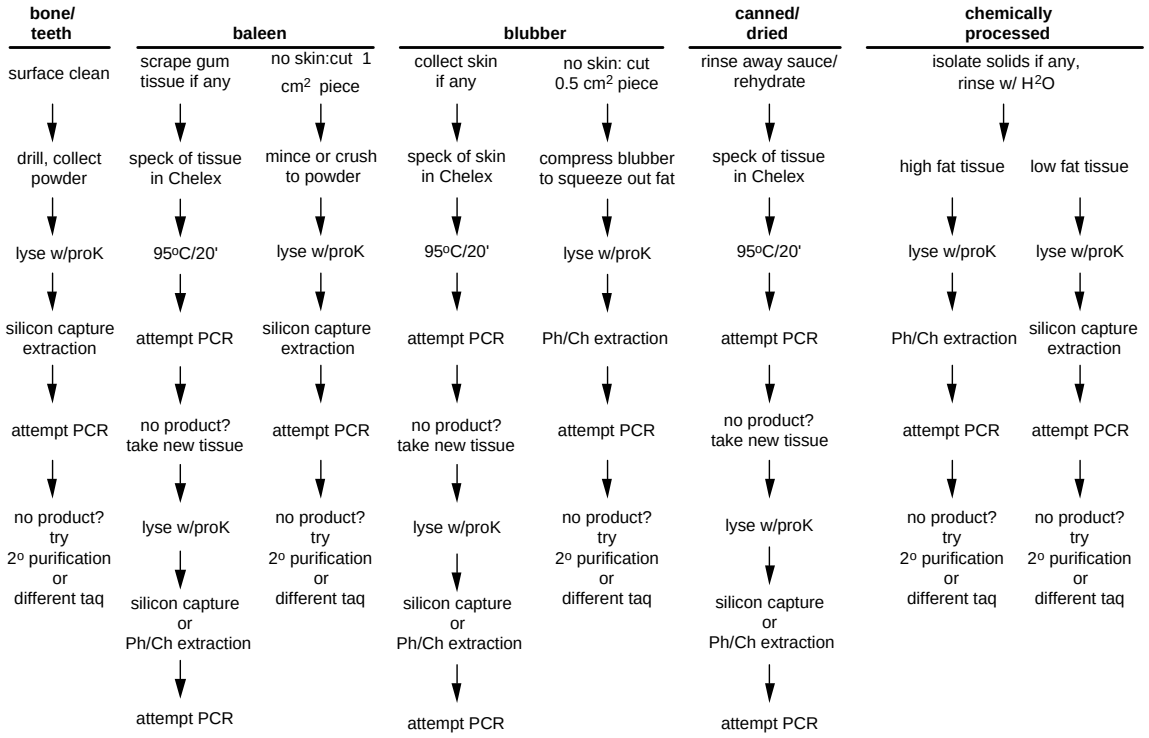

Figure 1. Flow chart showing various extraction/PCR options for 'difficult' cetacean product types

al. 2007, Nichols *et al.* 2007) and recent improvements in some of those techniques may be applicable to other tissue types including commercial whale products. Extensive optimization and testing of extraction techniques for use with aDNA samples suggested that silicon capture using a glass milk suspension was more effective than "silica membrane" columns for routine DNA extraction available from a variety of manufacturers (e.g. DNEasy from Qiagen, PureLink from Invitrogen) and also suggested that a variety of chemicals routinely added to DNA extractions (e.g. surfactants such as SDS and various non-ionic detergents, BSA, PVP, PTB) had no positive effect on DNA yield, at least not from the tissue types analyzed (Rohland and Hofreiter 2007). Phenol-chloroform extractions were slightly less effective for extraction of DNA from cave bear skeletal material (Rohland and Hofreiter 2007), but are likely to be more effective for extracting from lipid-rich tissues such as blubber samples, given the high solubility of lipids in organic solvents and the effectiveness of two-phase organic/aqueous suspensions in sequestering DNA into the aqueous-soluble layer.

DNA can often be extracted from baleen samples if dried gum tissue still attached at the margins can be scraped off, otherwise small sections of baleen plates should be cut into tiny pieces or ground in a ball mill, and well-incubated in lysis buffer to initiate extraction (pers. comm. Kelly Robertson, SWFSC). The ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) in various cetacean tissue types was analyzed by Morin *et al.* (2007), who found that fresh preserved tissue samples had a of mtDNA:nDNA ratio of about 300:1, while historical bone and tooth samples had ratios about 60-fold higher, and baleen almost 8000-fold higher than in preserved fresh tissue, indicating that nDNA may be degrading more quickly or starts at lower quantities in these hard tissues. Thus it may be very difficult to amplify nuclear loci from baleen samples, although analysis of mtDNA for species identification from baleen is fairly routine.

Improved PCR Techniques for 'Difficult' Samples

Degraded DNA is often fragmented so that larger amplicons cannot be generated successfully. Cipriano (2005) used closely situated primers CTR5F-CTR3R to amplify a small (~ 155 bp) portion of the control region for species identification from highly degraded samples, but some closely related species (e.g. Bryde's vs. sei whales) could not be distinguished using such a small fragment alone. Additional sequence fragments, generated using sets of primers adjacent in position to the CTR5F-CTR3R fragment

to increase the total amount of sequence data would help with Bryde's and sei whale-derived products (Cipriano 2005).

Specialty *taq* polymerases designed for amplification from low copy number templates can be very useful with "ancient DNA" and other degraded tissue extractions (or tiny organisms such as copepods and mites), e.g. Expand High Fidelity Plus (Roche) and Platinum Taq DNA Polymerase High Fidelity (Invitrogen). New types of engineered polymerases have also shown some success in amplifying degraded and low copy number DNA from ancient samples (Shapiro 2008), including 4,000-60,000 cave bear specimens (d'Abbadie *et al.* 2007), so as these enzymes are further developed they may be very helpful with amplification from highly processed cetacean products. The addition of the D-glucose disaccharide trehalose greatly improves DNA efficiency in GC rich templates (Spiess *et al.* 2004) and may be similarly effective in amplification from degraded or inhibitor-containing DNA extracts. The addition of trehalose to PCR reactions may enhance reaction efficiency by decreasing the melting temperature of double-stranded DNA to make priming sites more accessible for annealing and extension of the copied product (Spiess *et al.* 2004). Secondary purification of DNA extracts using ion exchange columns improved PCR success in amplifications from 500-3,300 year old human bone samples, probably by the removal of PCR inhibitors (Kim *et al.* 2008), and such additional purification methods may also be useful in amplification from 'difficult' cetacean products.

Whole genome amplification (WGA) techniques used to increase the amount of template material prior to PCR, have been used to improve the recovery of DNA sequence data from ancient and degraded DNA samples, such as sub-fossil material and evidence from crime scenes (e.g. Ballantyne *et al.* 2007, Giardina *et al.* 2009). Recent improvements (including the addition of trehalose) to the multiple displacement amplification (MDA) approach, which uses Φ29 DNA polymerase and random exonuclease-resistant primers for whole genome amplification, allowed highly specific amplification of complex DNA pools without bias or the production of excessive amounts of template-independent products (Pan *et al.* 2008). WGA approaches may be helpful in the analysis of certain cetacean product types with extremely limited amounts of DNA or extremely degraded DNA (e.g. products treated with bleach or lye, or subjected to intensive heat/pressure as in canned products). Nested PCR (in which two rounds of amplification are used, first with "outer" less-specific primers, followed by another round of PCR using "inner" more-specific primers) may also be useful in amplifying from product samples containing very small amounts or highly degraded DNA.

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