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NEW MATERIALS AND METHODS FOR STREAMLINING WHALE PRODUCT IDENTIFICATION ANALYSIS

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Abstract

Whale product identification analysis methods 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 materials for streamlining the PCR process were tested for easier and more robust amplification from cetacean tissue extracts, including: (1) pre-aliquoted and dried PCR "MasterMix" (including taq polymerase, buffer, dNTPs and $MgCl_2$) and (2) sterile gel excision tips for cutting out bands from agarose gels for recovery of purified PCR product. Initial trials using these new approaches were tested during a recent (Feb. 2011) survey of whale products in Japan, and proved to be faster, more efficient, and better able to amplify and then identify DNA sequences from cetacean products of several types.

Introduction – DNA Extraction, PCR, and Reamplification from Agarose Gel Bands

DNA analysis methods used for whale product identification are dependent on PCR amplification of genetic components from purified tissue extracts. Amplified mitochondrial control region fragments are commonly used for species identification of products from cetaceans (e.g. Baker et al. 1996, Baker et al. 2000). After successful amplification the resulting PCR product is purified, used as template in a secondary amplification, then “cycle sequenced", and the resulting products from that reaction run on a capillary sequencer. Although it is relatively easy to extract and amplify DNA from fresh, frozen, or chemically preserved tissue samples, extensive processing of some commercial products may result in highly degraded DNA, and such products may also contain inhibitors that prevent or limit amplification. Poor preservation of unprocessed tissues may also result in samples that are difficult or impossible to PCR. 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. Butler et al. 2003, Esslinger et al. 2004, Ye et al. 2004). PCR products are typically run out on a 1.8% agarose gel in TBE buffer for size-separation and visualization, and then PCR-product "bands" from such gels are excised using a flame-sterilized scalpel blade, placed into a sterile, labeled tube for return to a home laboratory for re-amplification, sequence analysis, and species identification. However, such methods using sharp scalpel blades are unsafe, require multiple steps including flame sterilization, may lead to variable results, and are slow and tedious.

Procedural Approach and Objectives

Previous experience with highly processed whale products indicated that at least one problem with such samples is that DNA strands are short and chemicals used in the processing inhibit PCR (e.g. Cipriano and Palumbi 1999). In order to test whether pre-aliquoted PCR amplification kits ("Bioneer tubes") were effective for amplifying market products (including a variety of cetacean tissue types) on site in the country of origin using a portable laboratory, a set of cetacean tissue samples were used in experiments comparing standard materials with Bioneer tubes for PCR amplification. The samples tested included "fresh" (non-preserved or processed) cetacean soft tissues and canned cetacean products. PCR-product bands were then excised using rectangular band shaped disposable tips, 6.5 mm x 1 mm, molded from polypropylene, which speeds up the gel-excision process, and eliminates the potential for cross contamination between samples.

Materials and Methods

Extractions were performed using Chelex suspended in ultra-pure water, following standard methods (e.g. Cipriano and Palumbi 1999). Approximately 10 mg of tissue was transferred to an incubation tube containing 125 µl of 10% Chelex suspended in ultrapure water, using 3X-flame-sterilized forceps and scalpel blade, and the tube was then heated to 95°C for 20 minutes using a thermal cycler. Following the extraction, PCR amplifications from purified extract were then attempted using 5’ control region primers (dlp1.5 - dlp5 targeting an approximately 515 bp fragment following two procedures: (1) standard PCR using individual stocks of ultrapure water, 10X PCR buffer, 10 mM (total) dNTPs, 25 mM $MgCl_2$, and taq polymerase combined into a "MasterMix" to which extracted DNA template is added, (2) amplification using Bioneer tubes (Bioneer, Inc., Alameda, Calif.) which contain most pre-aliquoted and

dried components, so that only ultrapure water and the two primers must be added before template addition. Resulting PCR products were then run out on an agarose mini-gel using standard protocols, amplified products ("bands") were excised using GeneCatcher disposable gel excision tips (GelCompany, San Francisco, CA) and the excised bands returned for BigDye 3.1 sequencing (Applied Biosystems, Foster City, Calif.) and sequence identification analysis using phylogenetic methods as described by Baker et al. (1996).

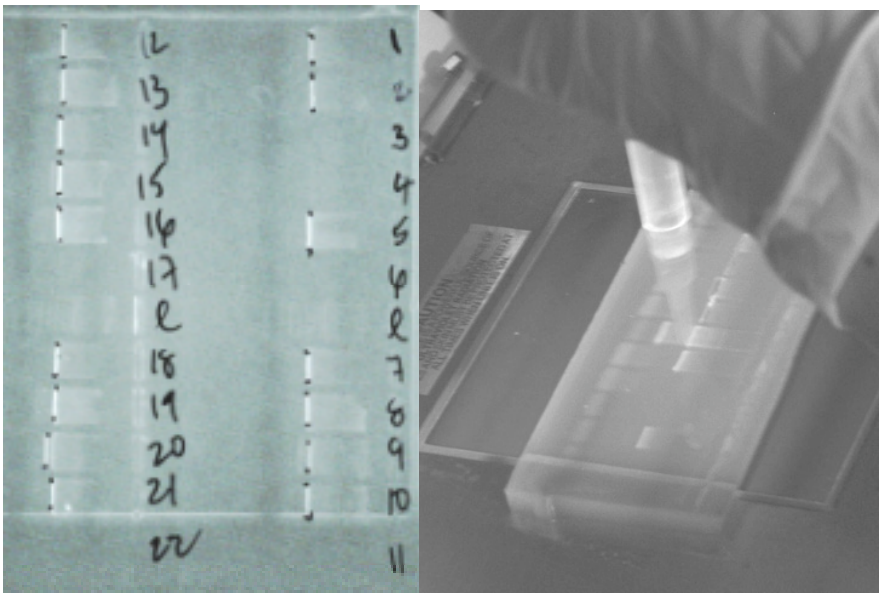

Figure 1a (left panel): Comparison of PCR results from paired samples amplified using standard Master Mix (samples 1-11) and Bioneer tubes (samples 12-22) from the same tissue extracts, note that samples 3 and 4 failed to amplify using standard Master Mix, but produced bright products using Bioneer tubes (lanes 14 and 15). Sample 6/17 failed to amplify using either method; lanes 11/22 are negative controls. **Figure 1b (right panel):** Cutting out a PCR product band from an agarose gel using a GeneCatcher gel excision tip mounted on a 1000 µl pipet.

Results and Conclusions

More products were successfully amplified using Bioneer tubes (17/20 attempted) compared to PCR using standard Master Mix (14/20 attempted) in two separate paired-sample experiments (experiment 1 shown in Fig. 1a above). Because the chemical components in Bioneer tubes are strictly quality controlled and stable at room temperature for months, there are also fewer concerns about storage or transporting of materials for use in whale product surveys. GeneCatcher gel excision tips proved to be much faster and easier to use than a scalpel for cutting out gel bands, and since they are sterile as supplied and used only once, the possibility for cross-contamination was eliminated.

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