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RAPID GENOTYPING TECHNIQUES for IDENTIFICATION of SPECIES and STOCK IDENTITY in FRESH, FROZEN, COOKED and CANNED WHALE PRODUCTS

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Abstract

DNA sequencing methods used for whale product identification are limited because such methods are expensive and time consuming. As a result, the number of samples that can be processed and analyzed in each survey is too small given the need for genetic surveys of current and emerging whale product markets. Using high-throughput genetic typing technology, we are developing more rapid and cost-efficient assay procedures to identify cetacean products. We designed molecular probes for use in one such technique and have begun testing this method for use in large-scale surveys of commercial whale markets. Such techniques for whale product identification will allow international programs to accurately monitor the identity of commercial whale products, and allow any future increase in commercial whaling to be accomplished with minimal impact on threatened species and populations.

We tested molecular probes with products from a new (1999) survey of Japanese commercial whale markets (N=130) and with products from additional species collected in previous surveys. Protected great whales detected in the 1999 survey included two products derived from different humpback whales, two products derived from sperm whales, and one product derived from a Bryde's whale. Subsamples of whale products identified in the 1999 survey were also subjected to pollutant analysis, so that pollutant loads associated with commercial food products could be related to species of origin and advertised identity. Eighteen products were advertised as derived from one species while containing products from another, while nineteen unidentified or misidentified"whale" products were derived from odontocetes. Eleven products correctly advertised as odontocete and seven of the un- or mis-advertised odontocete-containing products which were analyzed for pollutants contained at least one pollutant type at a level of concern for human consumers.

Introduction

Genetic testing of commercial whale products has been used in a series of market surveys to determine the species identity of products available to consumers in Korea and Japan, and the results submitted to the Scientific Committee of the International Whaling Commission (IWC) and published in peer-reviewed scientific journals (reviewed in Baker et al. 1996b, Cipriano and Palumbi 1997, Lento et al. 1998a). These studies relied on sequencing of the 5' portion of the mitochondrial control region, which contains a number of diagnostic fixed differences between cetacean species (Baker and Palumbi 1994), making it well-suited for species identification of wildlife products (Cronin et al. 1991). After the first market survey in 1993 (Baker and Palumbi 1994) the Fisheries Agency of the Government of Japan began compiling a mitochondrial control region database for whale product identification (Goto and Yagi 1995), and has conducted market surveys of its own using control region sequences (e.g. Government of Japan 1997). Control region sequencing was part of the genetic profile for a DNA database registry proposed for the Norwegian commercial catch (Olaisen 1997), and its inclusion was recommended by the working group which reviewed the proposal (IWC Scientific Committee, 1998). However, even

with advances in “automated” sequencing and a decrease in the cost per sample, control region sequencing is a necessarily expensive, relatively slow, and complex undertaking, which requires individual inspection of base calls for each sequence and statistical analysis of each sample with reference to a database of known-identity sequences for species identification (Baker et al. 1996). Market surveys to date have included a maximum of around 100 individual products, due to the cost and time involved in DNA sequencing, making it difficult to accurately describe the total market or to detect trends in the availability of different species. Market surveys are the only way to insure that products from protected species are not sold under the cover of meat available from legal sources. Genetic profiling of individual legal whales as soon as they are killed (including incidentally caught animals and documentation of frozen stockpiles), in an widely accessible database would also make it possible to detect smuggling of whale products. Much larger surveys, including thousands of samples annually, will be needed to accurately supervise whale markets with the resumption of even limited commercial whaling.

A variety of rapid genetic typing techniques (methods collectively called “high throughput screening”) have been developed for typing of many individuals in population genetic studies, development of human forensic databases, and diagnosis of genetic diseases . In order to meet the demands for speed and accuracy in these applications, they are typically PCR based (DNA is first PCR-amplified to provide enough template for detection), are highly specific (accurately distinguish particular target sequences), and are appropriate for automated sample manipulation and computerized analysis. In many cases diagnostic target sequences are first detected through a DNA sequencing study, and then sequence information is used to design a method for detecting particular sequence variants. We already use one of these methods, species-specific PCR, to detect southern minke whale samples in our market surveys and thereby prioritize the remaining products for sequence analysis (Cipriano and Palumbi, 1997). Since southern minkes comprise 50-60% of the sample, this reduces our sequencing load significantly.

In order to further develop a high-throughput screening method for identification of whale products, we reviewed the literature on the rapidly increasing number of genetic typing techniques (Table 1). We considered methods appropriate for whale product identification on several criteria, including speed, specificity and robustness of the method (insensitivity to minor variations in laboratory technique), as well as the ease in transferring such technology to other laboratories, so that such testing could be widely used and easily standardized. We also considered the availability of analysis equipment and cost per analysis, especially in relation to DNA sequencing. Finally, we insisted on a method that was based on control region sequences and that allowed supplemental sequencing of the same control region products used in earlier surveys. This will allow spot-checking of the accuracy of the method, sequence analysis of products not identified by the method (due to rare mutations, inclusion of new species, or unusual species being sold as whale products), and sequencing of individual products when more information, such as individual or population distinctions are needed. As its utility in many market surveys and

population studies has demonstrated, control region sequencing is the accepted gold standard for whale product identification, and is also useful for discriminating some population differences.

Table 1. Genetic Typing Techniques, all PCR-based and relatively fast and specific electrophoretic detection

Technique / Detection Method*	Advantages	Disadvantages	References
PCR/Sequencing / fluorescence/electrophoresis (polyacrylamide, "auto"-sequencer)	highly specific	multiple steps, costly, requires extended analysis per sequence	Palumbi et al. 1996 Bowen 1995 Baker et al. 1996
species-specific PCR / ethidium bromide/electrophoresis (agarose mini-gel)	quick, no additional procedures, easily transferred technology	1 reaction per species, sensitivity must be tested	Takata et al. 1996 Cipriano and Palumbi, 1997
species-specific restriction analysis / (RFLP/AMP-FLP) ethidium bromide/electrophoresis (agarose mini-gel)	quick, reliable, easily transferred technology	1 reaction and lane per site, limited number of sites examined, new mutations undetected	Replogle et al. 1994 Budowle et al. 1995 Johnson and Kotowski, 1996
mis-match RFLP / ethidium bromide/electrophoresis (agarose mini-gel)	more sites screened than standard RFLP	additional PCR's required	Cotton et al. 1988 Ganguly and Prockop 1990
SSCP/DGGE/TGGE/CSGE / silver staining/electrophoresis (polyacrylamide gel)	1 lane for many sites, no additional reaction, can detect new mutations	short fragments only, very sensitive to conditions, difficult optimization, difficult to interpret, difficult to transfer technology	Orita et al. 1989 Sheffield et al. 1993 Rehbein et al. 1997 Körkkö et al. 1998
multiplex microsatellite analysis (“STR”s) / fluorescence/electrophoresis (polyacrylamide, "auto"-sequencer)	highly specific, can be extended for individual analysis, easily transferred	untested for species i.d., extensive development and testing, multiple PCR and electrophoresis runs	Budowle et al. 1997 Isenberg et al. 1998 Mansfield et al. 1998
oligonucleotide ligation assay with sequence-coded separation/ two-probe ligation, length and fluorescence-coded reporter probes	highly specific, insensitive to hybridization conditions, simple sample preparation, multiplexed assay using all species-specific probes at once, automatic gel analysis using GeneScan software	multiple annealing, ligation, elution, steps; many expensive fluorescently-labelled and PEO-tailed probes	Grossman et al., 1994 Baron et al. 1996

Table 1. Genetic Typing Techniques, cont'd.

non-electrophoretic detection

Technique / Detection Method*	Advantages	Disadvantages	References
single-gene hybridization probes / membrane dot blot or slot blot/ colorimetric or fluorescent ELISA detection of species-specific probes	highly specific, simple sample preparation	multiple hybridization/detection/ elution steps, sensitive to hybridization conditions, separate probe/hybridization per species	Keller and Manak 1989 Narayanan 1992 Klevan et al. 1995
oligonucleotide ligation assay / two-probe ligation, colorimetric or fluorescent ELISA detection of species-specific reporter probes	highly specific, insensitive to hybridization conditions, simple sample preparation, suitable for 96 well plates and some automation, two species reporters per well	multiple annealing, ligation, elution, detection steps, separate binding and reporter probes per species or groups of species	Delahunty et al. 1996 Tobe et al. 1996 Chakravarty et al. 1997 Edelstein et al. 1998
peptide nucleic acid probes / species-specific probes of different molecular weights, detected with laser-desorption mass spectrometer	highly specific, insensitive to hybridization conditions, simple sample preparation	untested for species detection, expensive equipment??, unknown speed or cost/sample	Koster et al. 1996 Griffin et al. 1997 Ross et al. 1997
molecular beacons / hairpin-shaped oligonucleotides with terminal fluorophore and quencher, when annealed to the target sequence the fluorophore emits colored light	highly specific - one base discrim- ination, many different color fluorophores available, can be multi-plexed, also used in quantitative PCR	untested for species detection, complicated synthesis, not commercially available, unknown cost/sample	Matayoshi et al. 1990 Tyagi et al. 1998 Giesendorf et al., 1998 Marras et al. 1999
multiplex microsatellite analysis ("STR"s) / detected with MALDI-TOF MS	highly specific, extremely fast analysis, can be computerized and extended for individual analysis	untested for species i.d., extensive development and testing, limited number of analysis facilities	Ross et al. 1998
PinPoint Assay / multiple primers extended by single dideoxy-nucleotide residue, identity of added nucleotides detected by MALDI-TOF MS mass measurements	highly specific, rapid analysis, can be automated, can be multiplexed, no labelling required,	untested for species i.d., extensive development and testing, limited number of analysis facilities	Haff and Smirnov 1997a and 1997b Ross et al. 1998
short oligonucleotide mass analysis (SOMA) / PCR product targets endonuclease-fragmented, then detected with electrospray ionization mass spectrometry	simple and cheap primers, highly specific, PCR products detected directly, rapid analysis which can be automated, can be multiplexed, no labels required	untested for species i.d., extensive development and testing, expensive and limited number of analysis facilities	Laken et al. 1998
high-density oligonucleotide array ("DNA chip") / fluorescent RNA fragments hybridized/fragmented/hybridized and detected with confocal microscope or MALDI-TOF MS.	highly specific, simple sample preparation and hybridization, very rapid scanning and computerized sequence or sequence-specific pattern analysis, detection of new polymorphisms possible	expensive and proprietary hardware and software, complex array and analysis design, limited number of array construction and analysis facilities	Chee et al. 1996 Hacia et al. 1996 Kozal et al. 1996 Little et al. 1997 Ramsay 1998

***Technique abbreviations:** PCR - polymerase chain reaction; RFLP - restriction fragment length polymorphism; AMP-FLP - amplified fragment length polymorphism; SSCP - single-strand conformational polymorphism; DGGE - denaturing gel gradient electrophoresis; TGGE - temperature gel gradient electrophoresis; CSGE - conformation sensitive gel electrophoresis; STR - short tandem repeat (=microsatellite); OLA - oligonucleotide ligation assay; PEO - pentaethylene oxide; ELISA - enzyme-linked immunosorbent assay; MALDI-TOF MS - matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Technology Development - Oliognucleotide Ligation Assay for Whale Product Identification

We selected oligonucleotide ligation assay (OLA) for development of a high-throughput screening method for whale product identification. OLA probes could be designed to detect species-specific loci in the same control region fragment used in our previous sequence-based identification surveys. Sample handling, DNA extraction, and PCR amplification steps are unchanged, and purified artifical DNA copies can be returned to the laboratory for both OLA analysis and sequencing. By using OLA probes labelled with fluorescent dyes, results can be analyzed using the same automated electrophoresis equipment used for DNA sequencing, and software available for microsatellites analysis (e.g. GeneScan, PE Biosystems), can be used to score results.

OLA involves a very simple biochemical reaction (compared to DNA sequencing) that links, or "ligates" together two short artificial DNA probes (oligonucleotides) that together exactly match a particular DNA sequence. The two probes are designed to anneal, side-by-side, to a DNA region that contains diagnostic differences unique to the species, or population, or allele, of interest (Fig. 1). The ligated product can be detected and its size measured accurately by a variety of analytic methods. If the correct template sequence is not present, no ligation product is formed. Activity of the enzyme that links together the two probes is so specific that even a single base mismatch at the juncture will prevent ligation. OLA methodology was developed for detecting single base mutations associated with human genetic diseases, identification of viral strains, and human forensic identification (refs. in Table 1).

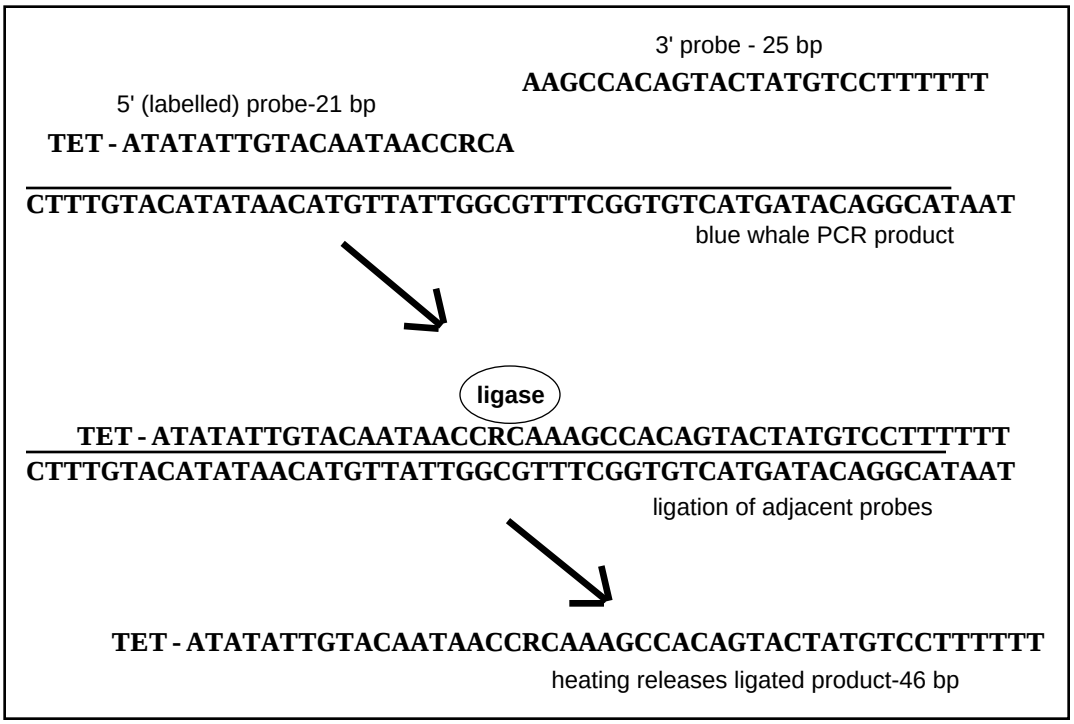

Figure 1. Oligonucleotide Ligation Assay produces a known-size ligation product only when both diagnostic probes match a DNA template (in this case, amplified DNA from a blue whale).

Using the large sequence database developed in earlier surveys, we designed sets of molecular probes for OLA that distinguish between whale species. We can distinguish between several species by designing a single common probe that matches all the species at a particular locus (the "left" probe in Figure 1), while the adjacent region contains species-specific differences allowing only one of a variety of probes to anneal and ligate (the "right" probe). By labelling common probes at a series of loci with different color fluorophores, and adjusting the size of the species-specific probes with varying number of thymine residues ("T-tails"), a number of species can be recognized and each ligation product (differing in total size and color) can be distinctly identifiable . These probes musted be tested singly and in combination to insure that a multiplexed analysis will reliably identify any of the candidate species. Here we demonstrate the reliability of OLA analysis using PCR products from reference samples, and confirm utility of OLA probes for species identification using samples collected in commercial market surveys from 1994-1999.

Materials and Methods

Whale products (n=130 samples) were purchased throughout Japan in February 1999, from Sendai to Nagasaki. As in previous collaborative surveys, a variety of commercial outlets were visited by independent wildlife agents acting as ordinary consumers. Two or three packages of whale meat products were purchased from each vendor, and no more than one of each type of sample is purchased in any one shop to limit the number of duplicate samples from the same whale. Types of products were similar to previous surveys, including sashimi, salted and partially cooked meat, and whale bacon and sliced blubber strips with attached skin. In addition, collectors purchased a variety of canned whale products and cooked whale meat, and visited a small number of whale meat restaurants in order to broaden the scope of the sample.

As in previous surveys of commercials markets, all DNA extractions from whale tissue and subsequent PCR amplifications were performed on site using a portable laboratory (Baker and Palumbi 1994; Baker et al. 1996b). We attempted amplifications of every sample for both control region and cytochrome *b* fragments, so that cytochrome *b* sequences could be used for confirmation of identifications or when control region fragments would not amplify. Control region amplifications included a southern minke-specific primer that produced an additional band only if the product contained DNA from a southern minke whale, thus immediately identifying such samples as derived from southern minkes. All amplified products were gel-purified to remove template DNA and gel cuts transported to our laboratory for direct DNA sequence analysis. DNA extraction, amplification, and isolation methods were otherwise as described by Baker et al. (1996a).

We sequenced 400-500 bp from the 5' half of the mitochondrial control region for identification analysis, as in previous studies. Sequence identification analysis was performed using the phylogenetic methods described by Baker and Palumbi (1994) and Baker et al. (1996). Briefly, identification of "test" sequences was performed using phylogenetic reconstruction methods based on parsimony (PAUP, Swofford 1993) and neighbour-joining genetic distance algorithms available in the computer program MEGA (Kumar et al. 1993). Each test sequence was analysed individually with a suite of type sequences to make an initial species assignment. Type sequences of the mtDNA control region were available from 97 individuals representing regional populations or subspecies of the 10 or 11 recognised species of baleen whales and 58 individuals representing individuals of 38 species or subspecies of odontocetes . Type sequences in our cytochrome *b* database include 89 sequences, representing all described baleen whale species and 38 odontocete species. Confidence estimates for each species identification were generated using 500 bootstrap simulations for both primary and secondary phylogenetic analyses.

A subset of "test" samples from the JW1999 survey and some previous surveys, plus a set of known-identity reference samples were subjected to molecular probe testing using oligonucleotide ligation assay (OLA) techniques. Two fluorescently-labelled common probes and eight specific identification probes

were synthesized (GenoSys Biotechnologies, The Woodlands, Texas), that targeted species-specific loci within the mitochondrial control region fragments used in this and all our previous sequence-based identification surveys (see Baker et al. 1996b for primer sequences).

Table 2. OLA probes designed for whale product identification.

probe name	5' probe sequence (fluorescently-labelled)/ [target species]	3' probe sequence (all 5'-phosphorylated)	probe size (bp)	ligation product size
TET-54	TET-ATATATTGTACAATAACCRCA		21	
MNOV54	[humpback whale]	GGGCAACAGTACTATGTCCTTT	22	43
BMUS54	[blue whale]	AAGCCACAGTACTATGTCCTTTTTT	25	46
NAMNK-54	[North Atlantic minke whale]	ACACCACAGTACTATGTCCTTTTTTTTT	28	49
6FAM-164	6FAM-ATGTATGTATTTCCMCATAA		20	
MNOV164	[humpback whale]	CCAACTGATAGCACCTTCCAT	21	41
BPHY164	[fin whale]	TTAATAGCGTCTTTCCATGGGTTTT	25	45
BMUS164	[blue whale]	CCAGTTAATCAGTGTTATCCCTTTTTTTTT	30	50
BEDE/ BBOR164	[sei and Bryde's whales]	CTTAATTAATAGTCTTCTCTTGTTTTTTTTTTTT	34	54

OLA probes were diluted to working concentrations of 0.5-0.8 µM with ultra-pure (HPLC quality) water. Ligase chain reaction (LCR) was performed in 20.5 µl total volume using thermostable Taq ligase (New England Biolabs, Beverly, Massachusetts) and the supplied 10X buffer. Per sample, 1.0 µl of labelled probe and species probe were added to 12 µl water and 2 µl 10X buffer, and then 0.5 µl (20 units) of Taq ligase was added. Four µl of PCR product , or purified product passed through a Qiaquick column (Qiagen Inc., Valencia, California) was then added. Thermal cycling conditions for the LCR were: 30"/94°C, 3'00"/50°C, repeated for 25 total cycles, then the ligase was denatured for 10'00"/80°C. Ligation products were detected using an ABI 310 or an ABI 377 automated DNA sequencer, using GeneScan data collection and fragment size analysis software (PE Biosystems, Foster City, California). For sample detection on the ABI 310, 1.5 µl of ligation product was mixed with 14 µl deionized formamide, heated at 94°C for two minutes, then iced briefly before loading into the sample stage for capillary electrophoresis at 15.0 kV for 40 minutes, using POP-6 polymer . For sample detection on the ABI 377, 2 µl of ligation product was mixed with 5 µl blue dextran/deionized formamide loading buffer, heated at 94°C for two minutes, then iced briefly before loadingonto a standard sequencing (4% acrylamide) gel for electrophoresis at 3.0 kV for 60 minutes. Size estimation of ligation products was performed with GeneScan software, using fluorescently labelled size standards prepared from labelled PCR product cut with a variety of standard restriction enzymes to produce fragments of known sizes from 9 to 108 nucleotides in length.

Results and Discussion

Testing OLA Probes for Whale Product Identification

Several OLA probes tested against known-identity reference samples were highly specific and useful for whale product identification, including probes for humpback (MNOV164), fin (BPHY164) and blue whales (BMUS 54 and BMUS164). Each of these produced a clear ligation product signal (Fig. 2), accurately detected the presence of their corresponding species, and never gave a "false positive" (produced a ligation product when the target species was not present). One probe (MNOV54) was not specific, and produced ligation products in tests with both fin and humpback whales (data not shown), fortunately the other humpback probe (MNOV164) is highly specific. Multiplex testing of several probes at once (in a single reaction) were accurate in tests with a number of species (Fig. 3, Table 3). The probe for sei and Bryde's whales (BEDE/BBOR164) was specific, but not sufficiently sensitive (Table 3).

Separate probes for sei, Bryde's, and small form Bryde's whales could be designed at another locus. Specificity could be further enhanced through the use of peptide nucleic acids rather than oligonucleotides for OLA probes, and a wider range of ligation product sizes is possible using pentaethylene oxide tails rather than thymine residues to alter the size of species-specific products (refs. in Table 1).

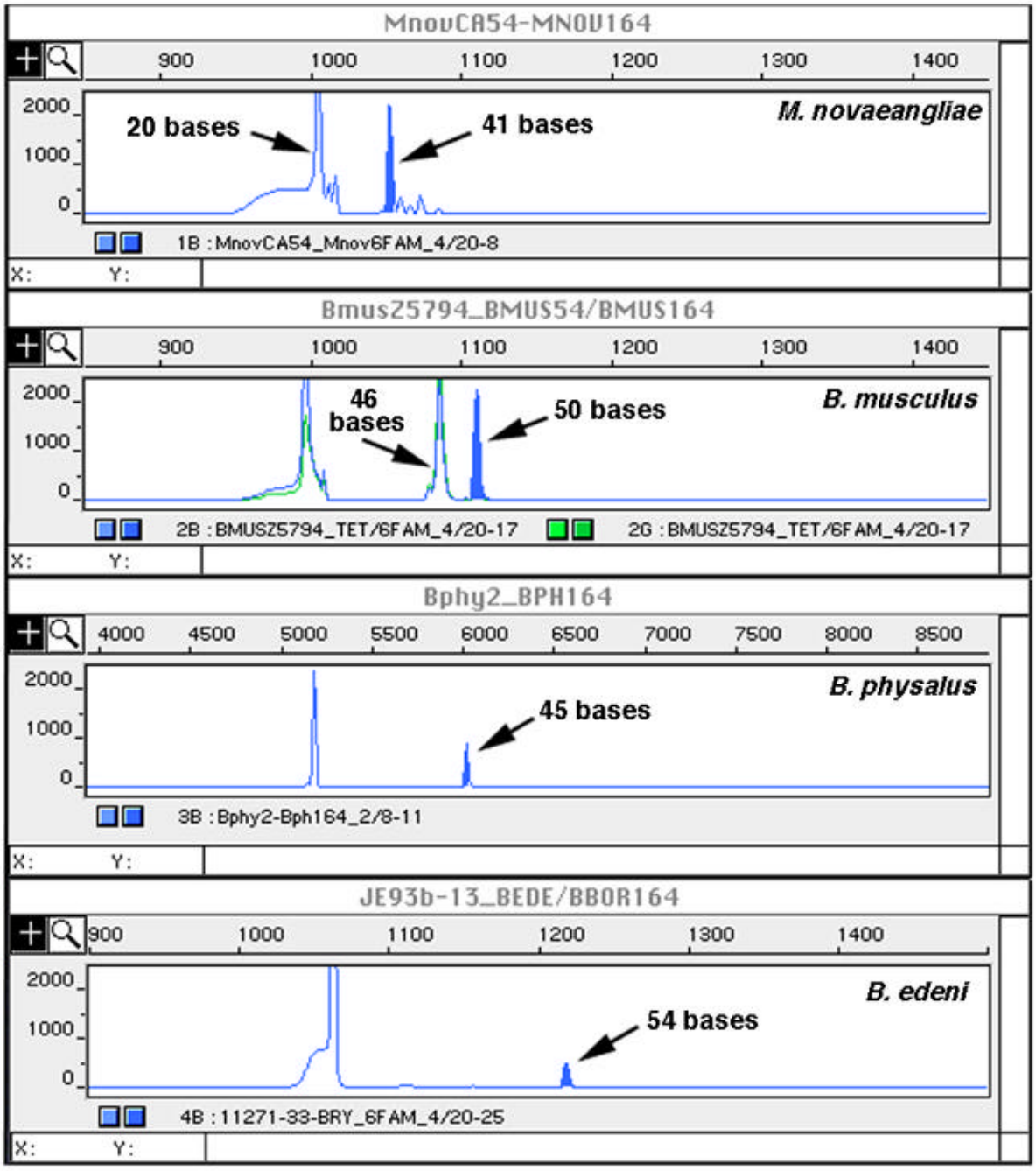

Figure 2. Ligation products from samples of humpback, blue, fin and Bryde's whales tested individually with the four OLA species probes. The left peak in each panel is the unincorporated 5' probe labelled with TET (green, 21 bases) and/or 6FAM (blue, 20 bases). Each probe produces a ligation product (right peak) of different size (or color) when annealed to PCR product from the appropriate species. Two different ligation products (green, 46 bases and blue, 50 bases) are used for identification of blue whale samples.

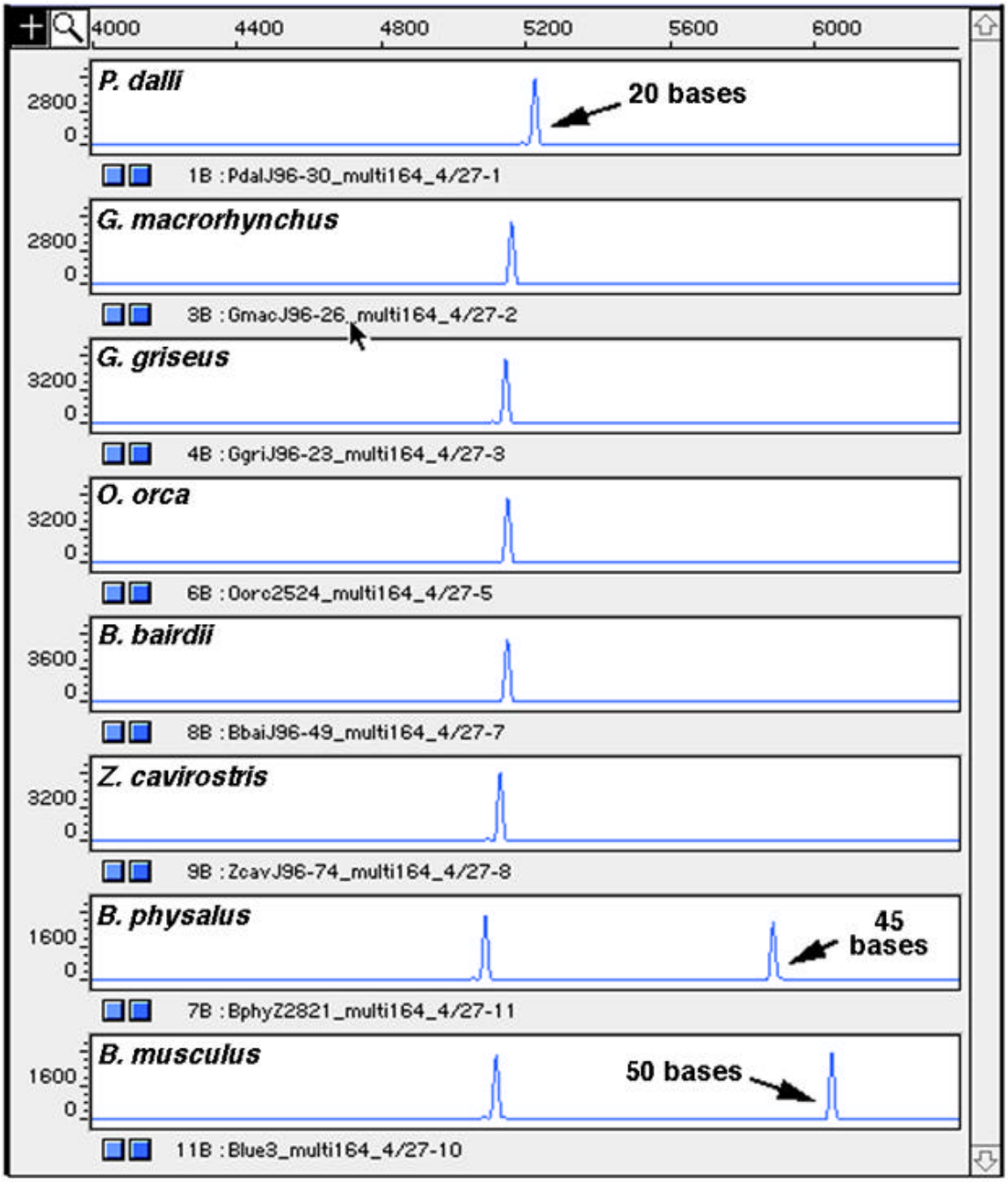

Figure 3. Results from multiplexed OLA analysis. Individual control region PCR products from a variety of species were tested with all four OLA probes (MNOV164, BMUS164, BPHY164, AND BEDE/BBOR164) at once. Ligation products, of the appropriate size, were only formed with products from fin and blue whales, shown in the bottom two panels.

Species Identification of Commercial Market Samples, and the 1999 Japan Survey

Besides testing known-identity reference samples, we also subjected PCR products from commercial market samples to OLA analysis (Table 3). We used products from a new survey of Japanese commercial markets conducted in February 1999, plus products from some earlier surveys for species not encountered in the 1999 survey. Four OLA species probes (MNOV164, BMUS54, BMUS164 and BPHY164) gave excellent results when tested against a variety of commercial market samples: no cases

of false positives (misleading identifications) were observed, and false negatives were observed in only two cases. A low level of false negatives is not a concern, given the ability to obtain control region sequences from samples that did not produce a ligation product from any probe. Supplemental sequencing of all "no ligation product" samples is a conservative approach, and in fact spot-checking of sequences is needed for quality-control of any molecular probe identification system. Although molecular probes could be designed for all cetacean species (and even genetically discreet populations), a more realistic approach might be to identify particular species or populations of interest with molecular probes, and use sequencing to identify the rest.

Of the 130 fresh, frozen, cooked and canned cetacean products collected in 1999, we were able to amplify DNA from and identify 113 to a single species (86.9%), including nine out of fourteen canned products (65%). Two other cans contained mixed products, and seven samples in total included products from more than one cetacean species, but determination of exactly which species awaits cloning of individual control region molecules and sequencing of separate clones. Such mixed products are difficult to sequence without cloning, but multiple species in such mixtures would be quickly identifiable using OLA or other molecular probing techniques, once more species-specific probes are available. A complete list of product types, sources, and species identifications is given in Appendix 1.

Species-specific PCR (and confirmatory sequence analysis from a subset of samples) showed that 56 (49.5%) of the market products were derived from southern minke whales. An additional 29 products (25.7%) were derived from North Pacific minke whales. High-resolution mtDNA analysis of these (see Congdon et al., SC/51/RMP20) showed that 31% were derived from J-stock (Sea of Japan) minke whales. Further discussion of the implications of such a high percentage of J-stock animals being found on the Japanese market is provided by Congdon et al. (SC/51/RMP20).

Five samples collected in 1999 were from protected baleen and sperm whales (4.4%). Two products, from Tokyo and Ayukawa, were derived from two different humpback whales (shown by sequence differences between the two samples). Two products, from different cities in Osaka prefecture, were derived from sperm whales. Sequences from the two sperm whale products were indistinguishable, but because of the low genetic diversity in this species, further genetic testing is needed to determine whether both were derived from the same individual. Another product, steak pieces served sauteéd in a Tokyo restaurant, was derived from a Bryde's whale.

The remaining 1999 samples included seven products derived from Baird's beaked whales , plus an additional sixteen from a variety of dolphin and porpoise species - including the two sperm whale samples, a total of 25 odontocetes (22.1%).

Table 3. Results from individual and multiplexed testing of known identity reference samples and market "test" samples using DNA sequencing and Oligonucleotide Ligation Assay. Species identity of reference samples confirmed by, and of test samples determined by, control region sequence. + = samples correctly identified using OLA probes, - = samples that produced no ligation product with particular species probe, * = samples assayed in a multiplex test using all four species probes at once, false neg. = samples that should have produced a ligation product with a particular species probe, but did not.

Probe/ Sample	Species	OLA result	Specifi -city
MNOV164			
MnovNfld	*M. novaeangliae*	+	correct
MnovCA54	*M. novaeangliae*	+*	correct
Mnov2/9	*M. novaeangliae*	+	correct
JW99-A16	*M. novaeangliae*	-	false neg.
JW99-T36	*M. novaeangliae*	+	correct
Blue3	*B. musculus*	-	correct
BmusZ5794	*B. musculus*	-	correct
BphyZ1862	*B. physalus*	-	correct
BphyZ2821	*B. physalus*	-	correct
Fin6	*B. physalus*	-	correct
JE93b-13	*B. borealis*	-	correct
JW99-T39	*B. edeni*	-	correct
J96-53	*B. a. davidsoni*	-	correct
J96-64	*B. a. davidsoni*	-	correct
J96-80	*B. a. davidsoni*	-	correct
K94-4-4	*B. a. davidsoni*	-	correct
J96-24	*B. a. bonarensis*	-	correct
J96-56	*B. a. bonarensis*	-	correct
J96-30	*P. dalli*	-*	correct
J96-26	*G. macrorhynchus*	-*	correct
J96-23	*G. griseus*	-*	correct
Pcra89-21	*P. crassidens*	-*	correct
Oorc2524f	*O. orca*	-*	correct
J96-75	*B. bairdii*	-*	correct
J96-49	*B. bairdii*	-*	correct
J96-74	*Z. cavirostris*	-*	correct

Probe(s)/ Sample	Species	OLA result	Specifi -city
BMUS54+BMUS164			
Blue2	*B. musculus*	+	correct
Blue3	*B. musculus*	+*	correct
BmusZ5793	*B. musculus*	+	correct
BmusZ5794	*B. musculus*	+	correct
J93b-4	*B. musculus*	+	correct
JS95-019	*B. musculus*	+	correct
Fin6	*B. physalus*	-	correct
BphyZ2821	*B. physalus*	-	correct
BphyZ1862	*B. physalus*	-	correct
Mnov2/9	*M. novaeangliae*	-	correct
MnovCA54	*M. novaeangliae*	-	correct
J96-24	*B. a. bonarensis*	-	correct
J96-56	*B. a. bonarensis*	-	correct
J96-43	*B. a. davidsoni*	-	correct
J96-64	*B. a. davidsoni*	-	correct
K94-4-4	*B. a. davidsoni*	-	correct
Cmar1	*C. marginata*	-	correct
J96-30	*P. dalli*	-*	correct
J96-26	*G. macrorhynchus*	-*	correct
J96-23	*G. griseus*	-*	correct
Pcra89-21	*P. crassidens*	-*	correct
Oorc2524f	*O. orca*	-*	correct
J96-75	*B. bairdii*	-*	correct
J96-49	*B. bairdii*	-*	correct
J96-74	*Z. cavirostris*	-*	correct

Probe/ Sample	Species	OLA result	Specifi -city
BPHY164			
BphyZ2821	*B. physalus*	+*	correct
BphyZ1862	*B. physalus*	+	correct
BphyZ5791	*B. physalus*	+	correct
Fin1	*B. physalus*	-	false neg.
Fin2	*B. physalus*	+	correct
Fin6	*B. physalus*	+	correct
J96-10	*B. physalus*	+	correct
J96-27	*B. physalus*	+	correct
J96-50	*B. a. bonarensis*	-	correct
J96-56	*B. a. bonarensis*	-	correct
JE95-13	*B. a. bonarensis*	-	correct
J96-80	*B. a. davidsoni*	-	correct
K94-4-12	*B. a. davidsoni*	-	correct
MnovNfld	*M. novaeangliae*	-	correct
MnovCA54	*M. novaeangliae*	-	correct
Mnov2/9	*M. novaeangliae*	-	correct
J96-30	*P. dalli*	-*	correct
J96-26	*G. macrorhynchus*	-*	correct
J96-23	*G. griseus*	-*	correct
Pcra89-21	*P. crassidens*	-*	correct
Oorc2524f	*O. orca*	-*	correct
J96-75	*B. bairdii*	-*	correct
J96-49	*B. bairdii*	-*	correct
J96-74	*Z. cavirostris*	-*	correct

Probe(s)/ Sample	Species	OLA result	Specifi -city
BEDE/BBOR164			
JE93b-13	*B. borealis*	+	correct
JW99-T39	*B. edeni*	-	false neg.
K94-4-3	small form Bryde's	-	false neg.
K94-4-15	small form Bryde's	-	false neg.
J96-36	*B. a. bonarensis*	-	correct
J96-50	*B. a. bonarensis*	-	correct
J96-56	*B. a. bonarensi*	-	correct
JE95-13	*B. a. bonarensi*	-	correct
BphyZ2821	*B. physalus*	-	correct
BphyZ1862	*B. physalus*	-	correct

1999 Japan Survey: Coincident Product Identification/Pollutant Analysis

For the first time, the 1999 survey coordinated commercial whale product identification withcoincident pollutant assays, so that pollutant loads associated with commercial food products could be related to species of origin and advertised identity. Of primary concern is the pollutant load being ingested by human consumers of whale products, especially odontocete products which generally contain higher loads of heavy metals and organics. A review of cetacean pollutant loads , regulatory limits and risk assessment guidelines, plus preliminary analysis of a few pollutant types in products from the 1999 survey is provided by Simmonds et al. (SC/51/E13). Of additional concern, from a consumer-protection standpoint, is the selling of mis-labelled products (advertised as derived from mysticetes, while actually derived from odontocetes) and unlabelled products or those advertised only as "whale" ("kujira"), with no actual species designation. In previous surveys, because we wished to concentrate on the availability of protected baleen whales in commercial markets, our sample collectors were instructed never to buy products advertised as derived from dolphins or porpoises. In our 1996 survey, dolphin and porpoise meat being sold as whale meat comprised 27.6% of the sample; with meat from beaked whales odontocetes totalling 34.5% of products sampled (Cipriano and Palumbi 1997, SC/49/NP17).

Some dolphin products (marked "S/D/T dolphins" in Table 4) were derived from Stenella, Delphinus, or Tursiops spp. . These species are relatively closely related and have highly variable control region sequences (A. Dizon, SWFC, personal communication). We also do not have a large sampling of the geographic variability found in these species represented in our reference database, and thus it is often difficult to determine species identity precisely unless the control region sequence almost exactly matches one of the reference sequences in our database. A better sampling of control region sequences from these species is needed.

Sixteen products purchased for the 1999 survey were advertised as derived from odontocetes. Of these, one was actually from a mysticete (A6, a southern minke whale product). Eighteen products which were advertised as derived from one species contained products from another, or a mixture of products from the advertised species plus another (Table 4). Nineteen unidentified or misidentified "whale" products were derived from odontocetes. Eleven products correctly advertised as odontocete and seven of the un- or mis-advertised odontocete-containing products (Table 4) which were analyzed for pollutants contained at least one pollutant type at a level of concern for human consumers (Simmonds et al., SC/51/E13).

Table 4. Mis-advertised cetacean products (advertised as derived from one species while actually containing products from another) and "whale" products whose identity was unadvertised (sold simply as "kujira") which contained products from odontocetes or unusual mysticetes.

Sample Number	Sample Type	advertised identity (if any)	actual identity
JW99-T14	canned red meat	minke	mixed products
JW99-T15	canned red meat		mixed products
JW99-T27	blubber, salted and seasoned		*G. macrorhynchus*
JW99-T36	bacon		*M. novaengliae*
JW99-T41	frozen, salted blubber		*B. bairdii*
JW99-T43	red meat		mixed products
JW99-T44	bacon		*B. bairdii*
JW99-O4	frozen blubber	"from Antarctica"	NP minke
JW99-O5	frozen blubber	"Antarctic minke"	NP minke
JW99-O10	frozen red meat	coastal sei whale	S minke
JW99-O16	shredded bacon	minke	*B. bairdii*
JW99-O19	cooked blubber	sei whale	*P. macrocephalus*
JW99-O20	boiled and dried blubber	minke	*P. macrocephalus*
JW99-S1	frozen sashimi	Antarctic sperm	S minke
JW99-S7	cooked, salted red meat		mixed products
JW99-S8	canned cooked red meat		*P. dalli*
JW99-W6	frozen bacon	pilot whale	*G. griseus*
JW99-W9	bacon, sliced		*T. truncatus*
JW99-W10	cooked liver, intestine	pilot whale	S/D/T dolphin
JW99-W11	frozen bacon		*G. griseus*
JW99-W13	frozen blubber w/ red meat	pilot whale	S/D/T dolphin
JW99-H9	frozen, salted, cooked red meat	minke	*G. melas*
JW99-A3	frozen salted? blubber	minke	mixed products
JW99-A4	frozen salted blubber	minke	*G. macrorhynchus*
JW99-A6	cooked red meat	Baird's from Ayukawa	S minke
JW99-A11	frozen blubber w/ black skin	minke	*G. macrorhynchus*
JW99-A15	frozen blubber w/ no skin	minke	*M. novaengliae*
JW99-A18	frozen salted blubber, black skin	minke	*G. macrorhynchus*

Conclusions

Molecular probe techniques offer a fast, accurate and relatively inexpensive method for identification of whale products to species. They allow commercial scale monitoring of national and international markets by greatly reducing the costs and analytical complexities of current sequence-based approaches. Such techniques, coupled with genetic sampling at point-of-capture for all legally killed whales, could be used as the basis for a comprehensive genetic monitoring program for control of the species sold in commercial markets. It is now widely recognized that resumption of commercial whaling has already been tied to genetic monitoring, e.g.: "If whaling ever resumes again, commercial whaling under IWC control, the monitoring of every whale which is caught will be an integral part of that process" (R. Gambel, BBC interview, Jan. 28 1999). IWC goals are to allow resumption of commercial whaling through re-building of populations to harvestable levels. There is pressure to begin harvest of a population when it and it alone begins to recover, but genetic surveys have consistently shown that legal whaling places threated species and populations at risk. Comprehensive genetic monitoring provides a means for protectionof depleted species and populations, while other populations are legally harvested.

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