Abstract—Procedures for sampling genomic DNA from live billfishes involve manual restraint and tissue excision that can be difficult to carry out and may produce stresses that affect fish survival. We examined the collection of surface mucous as a less invasive alternative method for sourcing genomic DNA by comparing it to autologous muscle tissue samples from Atlantic blue marlin (*Makaira nigricans*), white marlin (*Tetrapturus albidus*), sailfish (*Istiophorus platypterus*), and swordfish (*Xiphias gladius*). Purified DNA from mucous was comparable to muscle and was suitable for conventional polymerase chain reaction, random amplified polymorphic DNA analysis, and mitochondrial and nuclear locus sequencing. The nondestructive and less invasive characteristics of surface mucous collection may promote increased survival of released specimens and may be advantageous for other marine fish genetic studies, particularly those involving large live specimens destined for release.

Surface mucous as a source of genomic DNA from Atlantic billfishes (Istiophoridae) and swordfish (Xiphiidae)

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Understanding genetic variation and identifying evolutionary lineages are important considerations for conservation management of large pelagic fishes (Palumbi, 1994; Ward, 2000). Some methods used for genetic sampling of billfishes (families Istiophoridae and Xiphiidae) require excision of muscle, skin, or fin tissue, and excision requires manual restraint or killing of the animal. Some nondestructive sampling methods, such as fin and scale collection, exist that avoid the need to kill the fish (Yue and Orban, 2001; Wasko et al., 2003; Hoolihan et al., 2004). Tissue excision from very large live specimens is problematic because of personal safety concerns for handlers, and injuries to fish that may reduce survival. Most recreational billfish caught in the United States are released (Prince et al., 2007), whereas possession of billfish by U.S. pelagic longline vessels and sales of Atlantic billfishes have been prohibited since 1988. This regulation eliminates opportunities to obtain DNA samples from landed specimens, warranting a need for alternative methods. To sample genomic DNA with nondestructive techniques and with minimal handling would promote the survival of released individuals and increase the opportunities to conduct genetic studies.

One alternate potential source of genomic DNA is the epidermal cells found in billfish and swordfish surface mucous. Successful extractions of sufficient quantities of genomic DNA from surface mucous of freshwater fishes have been reported for *Salmo trutta fario* (brown trout), *Esox lucius* (northern pike) (Livia et al., 2006) and *Scleropages formosus* (Asian arowana) (Chansue, 2006), providing an experimental model for large pelagic species.

The present study compares DNA extractions from surface mucous and autologous skeletal muscle tissue from billfishes and swordfish to determine the suitability of DNA extracted from surface mucous for random amplified polymorphic DNA (RAPD) and DNA sequencing analyses.

Materials and methods

Surface mucous and autologous skeletal muscle samples were collected

from three individuals from each of the following species: *Makaira nigricans* (blue marlin), *Tetrapturus albidus* (white marlin), *Istiophorus platypterus* (sailfish), and *Xiphias gladius* (swordfish). We compared purified DNA extracted from mucous and muscle using RAPD, mitochondrial DNA (mtDNA) sequencing, and nuclear DNA sequencing analyses.

Sample preparation and DNA purification

Surface mucous samples were collected with a polyurethane sponge as described by Schultz et al. (2006) and immediately processed, or stored at −80°C for later extraction. Each sponge was cut into small pieces, mixed with 1500 μL of phosphate buffered saline, and compressed repeatedly to remove mucous. All fluids were centrifuged through a single QIAamp® (Qiagen Inc., Valencia, CA) spin column. DNA purification was carried out by using Qiagen® buccal swab spin protocol with the following modifications: 1) a final concentration of 1 millimolar (mM) ethylenediaminetetraacetic acid (EDTA) was added to the phosphate-buffered saline extraction buffer; 2) the proteinase K treatment step was eliminated; and 3) an RNase mixture of A and T1 enzymes was used to degrade RNA after the final DNA purification step (Ambion Inc., Foster City, CA). The RNase cocktail enzyme mix was necessary because the Qiagen® spin columns copurify RNA and DNA in parallel when both are present in a sample. Cold ethanol precipitation and inclusion of EDTA in buffers were used to reduce nuclease degradation (Dessauer et al., 1996; Wasko et al., 2003).

For muscle tissue, 25 mg were macerated in 180 μL Buffer ATL (Qiagen Inc., Valencia, CA), and incubated overnight in a 56°C water bath in the presence of 20 μL proteinase K. Purification followed the QIAamp® manufacturer's protocol for tissue. Aqueous samples of nucleic acid (1.5 μL) from mucous and muscle extractions were measured for purity with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific,Wilmington, DE) and showed a range of 1.7 to 2.0 for the DNA and RNA absorbance ratios (260 nm:280 nm).

RAPD analysis

Three different 10-mer oligonucleotide primers (Table 1) of arbitrary sequence (IDT Integrated DNA Technologies, Coralville, IA) were tested against surface mucous DNA and autologous muscle DNA from three individuals of *M. nigricans*, *T. albidus*, *I. platypterus*, and *X. gladius*. PCR reactions for RAPD analysis were performed in a total volume of 25 μL containing 3 μL extracted genomic DNA, 2.5 μL of 10 mM 10-mer primer, 2.5 μL of 2.5 mM deoxynucleotide triphosphate (dNTPs), 0.75 μL of 25 mM $MgCl_2$, 0.20 μL *Taq* DNA polymerase, 2.5 μL 10× buffer, 5 μL 5M betaine (N, N, N-trimethylglycine), 2 μL HotStart-IT® binding protein (USB Corp., Cleveland, OH), and 6.55 μL distilled water. Pre-PCR incubation with the HotStart-IT™ binding protein was executed at 25°C for four hours to prevent mispriming and primer dimerization during amplification (Chou et al., 1992). RAPD PCR was performed in an Eppendorf Mastercycler (Westbury, NY) starting with initial heating for 5 minutes at 94°C, followed by 34 cycles at 94°C for 5 minutes, 42°C for 30 seconds, 72°C for 90 seconds, and a final extension of 72°C for 10 minutes. A negative control (no genomic DNA) was included in each PCR set to verify no reagent contamination. The PCR products were verified by electrophoresing 5 μL in 1.2% agarose gel (ISC BioExpress, Kayville, UT) and TAE buffer (pH 8.5) for 60 min at 100 V (60 mA), stained with ethidium bromide, and visualized through a UV transilluminator.

Table 1

Nucleotide composition of three 10-mer primers used for random amplified polymorphic DNA (RAPD) comparison of DNA extracted from billfish surface mucous and muscle tissue, and percent content of guanine and cytosine (% GC).

Primer no.	Nucleotide composition	% GC
1	5′-GTTGCGGGCT-3′	70
2	5′-CAGCCCGGGT-3′	80
3	5′-AGGCCACCGC-3′	80

Sequencing analysis

Mitochondrial and single-copy nuclear loci were sequenced from three individuals from each of the following species: *M. nigricans*, *T. albidus*, and *I. platypterus*. Insufficient samples of mucous and muscle were available for sequencing *X. gladius*. Mitochondrial NADH dehydrogenase subunit4 (ND4) was amplified by PCR with the primer pair 61F and 1837R and cycling parameters outlined in Shivji et al. (2006). All amplifications of the anonymous single-copy nuclear locus WM13 were performed with the primer pair WM13-F and WM13-R developed by Buonaccorsi et al. (1999). Nuclear PCR reactions were performed in a total reaction volume of 50 μL containing 1 μL of extracted genomic DNA, 10 pmol/μL of each primer, 40 μM dNTPs, 10× PCR buffer, and 1 unit of HotStar *Taq*™ DNA Polymerase (Qiagen Inc., Valencia, CA). The PCR thermal profile consisted of an initial heating at 95°C for 15 minutes to activate the DNA polymerase, followed by 35–40 cycles at 94°C for 1 minute, 1 minute at 50°C, and 1 minute at 72°C, with a 5-minute final extension step at 72°C. Both nuclear and mitochondrial amplifications were performed in a MJ Research PTC-200 thermal cycler (Waltham, MA). A negative control (no genomic DNA) was included in each PCR set to verify that there was no reagent contamination.

All amplified products were purified by using the QIAquick® PCR purification kit (Qiagen Inc., Valencia, CA) and sequenced with an Applied Biosystems 3130

genetic analyzer (Foster City, CA). Forward and reverse sequences were assembled and edited with GeneDoc 2.6.002 (http://www.psc.edu/biomed/genedoc, accessed 1 June 2008). To control for cross-contamination with other billfish or possible parasites or microbes, we compared the mitochondrial and nuclear sequences derived from surface mucous to sequences obtained from autologous muscle tissue. Because autologous muscle tissue was not available for the *T. albidus* sequencing analyses, the mucous sample sequences were compared to homologous locus sequences obtained from reference *T. albidus* tissues available in our laboratory.

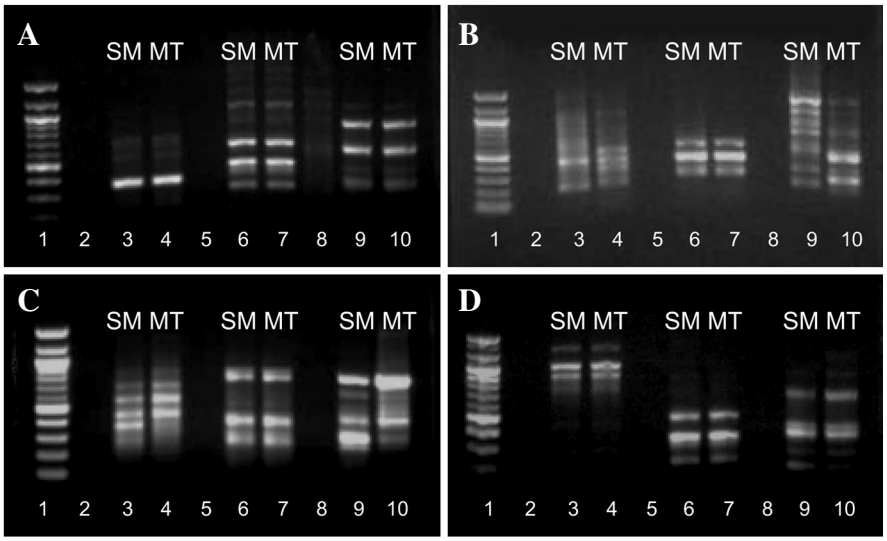

Figure 1

Representative random amplified polymorphic DNA (RAPD) banding patterns from four species: **A**) *Makaira nigricans* (blue marlin), **B**) *Tetrapturus albidus* (white marlin), **C**) *Istiophorus platypterus* (sailfish), and **D**) *Xiphias gladius* (swordfish) for three 10-mer primers (Table 1) tested on each fish for the template DNA from surface mucus (SM), and autologous muscle tissue (MT). Lane contents are as follows: 1, 100-bp ladder; 2, 5, and 8, negative controls (no DNA); 3 and 4, primer no. 1; 6 and 7, primer no. 2; and, 9 and 10, primer no. 3.

Results and conclusions

RAPD analysis

Comparable RAPD amplicons were derived from surface mucous and autologous muscle tissue of *M. nigricans*, *T. albidus*, *I. platypterus*, and *X. gladius* for each of the three 10-mer primers tested. Representative RAPD banding patterns are illustrated in Figure 1. The negative control reactions (minus template DNA) observed with each primer confirmed that the amplified genomic DNA from both sources was not an artifact of the primer concentration. Results indicated that sufficient quantities of genomic DNA are available in surface mucous from all the species tested.

Sequencing analysis

For the three species screened, *M. nigricans*, *T. albidus*, and *I. platypterus*, the quality of nuclear and mitochondrial PCR amplifications were comparable. For both the nuclear WM13 and the mt ND4 locus, sequences derived from a single individual from both sources of genomic DNA (mucous and autologous muscle tissue) were found to be identical when compared with respect to nucleotide base composition (5′–3′ and 3′–5′ directions), thereby demonstrating that the genomic DNA derived from surface mucous was not due to cross-contamination from other billfish or microbes, and in fact originated from the fish sampled. Nuclear and mitochondrial sequences derived from both sources of genomic DNA are available from GenBank under the following accession numbers: *T. albidus*: ND4 (997bp): FJ809995-FJ809997; WM13 (279bp): FJ809988; *M. nigricans*: ND4 (966bp): FJ809991, FJ809992, and FJ809994; WM13 (279bp): FJ809986, FJ809987, and FJ809989; *I. platypterus*: ND4 (1009bp): FJ809990 and FJ809993; WM13 (277bp): FJ809984 and FJ809985.

This study has shown that surface mucous contains sufficient quantities of genomic DNA to carry out RAPD analyses of istiophorid billfishes and swordfish, as well as sequencing applications of istiophorid billfishes. These extractions compare favorably to genomic DNA extractions from surface mucous reported for freshwater species *S. formosus* (Chansue, 2006), *E. lucius,* and *S. trutta fario* (Livia et al., 2006).

The slight differences between some of the mucous and muscle RAPD amplification profiles (Fig. 1) may be a result of contaminant DNA in the mucous (e.g., bacteria, microalgae), or other artifact variations known to occur in RAPD studies (Ellsworth et al., 1993). We found that the pre-PCR incubation of the template DNA master mix with HotStart-IT™ binding protein was crucial for preventing mispriming and primer dimerization that produced these artifact bands (Chou et al., 1992). In addition, the inclusion of betaine (N, N, N-trimethylglycine) improved band visibility by eliminating the smearing attributed to the formation of secondary structure, which is caused by G-C rich regions (Henke et al., 1997). Importantly, for surface mucous RAPD analysis, the RNAase treatment of nucleic acid was a necessary step, whereas the inclusion of proteinase K was not.

The nondestructive and less invasive procedures associated with sampling surface mucous offers many advantages when compared to fin clip and muscle tissue collection, particularly when very large fishes destined for live release are involved. Although the collection of surface mucous with sponges was adequate for the present study, commercially available products such as FTA® cards (Whatman Inc., Florham Park, NJ) may offer advantages. For example, Livia et al. (2006) reported that FTA® cards were a fast and reliable method of collecting, storing, and extracting genomic DNA from *E. lucius* and *S. trutta fario*. FTA® cards can be stored dry at room temperature, thus eliminating the need for laboratory freezers or special shipping considerations. We tested FTA® cards on a limited number of *I. platypterus* and *T. albidus* surface mucous samples (data not shown). Preliminary results were successful for PCR amplification, and further analyses are planned.

We report the first use of surface mucous from marine fishes as an alternative method of DNA sampling. As such, the method offers advantages that warrant consideration when planning genetic studies on other marine species, particularly those where live-release is desirable.

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Literature cited

Buonaccorsi, V. P., K. S. Reece, L. W. Morgan, and J. E. Graves.
1999. Geographic distribution of molecular variance within the blue marlin (*Makaira nigricans*): a hierarchical analysis of allozyme, single-copy nuclear DNA, and mitochondrial DNA markers. Evolution 53:568–579.

Chansue, N.
2006. Rapid isolation of DNA from the mucus of Asian arowana (*Scleropages formosus*, Osteoglossidae). Thai J. Vet. Med. 36:55–59.

Chou, Q., M. Russell, D. E. Birch, J. Raymond, and W. Bloch.
1992. Prevention of pre-PCR mispriming and primer dimerization improves low-copy-number amplifications. Nucleic Acids Res. 20:1717–1723.

Dessauer, H. C., C. J. Cole, and M. S. Hafner.
1996. Collection and storage of tissues. *In* Molecular systematics, 2nd ed. (D. M. Hillis, C. Moritz, and B. K. Mable, eds.), p. 29–47. Sinauer, Sunderland, MA.

Ellsworth, D. L., K. D. Rittenhouse, and R. L. Honeycutt.
1993. Artifactual variation in randomly amplified polymorhic DNA banding patterns. Biotechniques 14:214–217.

Henke, W., K. Herdel, K. Jung, D. Schnorr, and S. A. Loening.
1997. Betaine improves the PCR amplification of GC-rich DNA sequences. Nucleic Acids Res. 25:3957–3958.

Hoolihan, J. P., J. Premanandh, M. A. D'Aloia-Palmieri, and J. A. H. Benzie.
2004. Intraspecific phylogeographic isolation of Arabian Gulf sailfish *Istiophorus platypterus* inferred from mitochondrial DNA. Mar. Biol. 145:465–475.

Livia, L., P. Antonella, L. Hovirag, N. Mauro, and F. Panara.
2006. A nondestructive, rapid, reliable and inexpensive method to sample, store and extract high-quality DNA from fish body mucus and buccal cells. Mol. Ecol. Notes 6:257–260.

Palumbi, S. R.
1994. Genetic divergence, reproductive isolation, and marine speciation. Annu. Rev. Ecol. Systemat. 25:547–572.

Prince, E. D., D. Snodgrass, E. S. Orbesen, J. P. Hoolihan, and J. E. Serafy.
2007. Circle hooks, "J" hooks, and "drop-back" time: a hook performance study of the south Florida recreational live bait fishery for sailfish, *Istiophorus platypterus* (Shaw). Fish. Manag. Ecol. 14:173–182.

Schultz, D. R., N. F. Perez, D. Snodgrass, J. E. Serafy, E. D. Prince, F. Arocha, T. R. Capo, and C. K. Tan.
2006. Enzyme-linked immunosorbent assays and billfish gender: testing muscle tissue and surface mucus in tagging studies. Bull. Mar. Sci. 79:859–864.

Shivji, M. S., J. E. Magnussen, L. R. Beerkircher, G. Hinteregger, D. W. Lee, J. E. Serafy, and E. D. Prince.
2006. Validity, identification, and distribution of the roundscale spearfish, *Tetrapturus georgii* (Teleostei: Istiophoridae): Morphological and molecular evidence. Bull. Mar. Sci. 79:483–491.

Ward, R. D.
2000. Genetics in fisheries management. Hydrobiologia 420:191–201.

Wasko, A. P., C. Martins, C. Oliveira, and F. Foresti.
2003. Non-destructive genetic sampling in fish. An improved method for DNA extraction from fish fins and scales. Hereditas 138:161–165.

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