The process of microsatellite development and profiling involves three primary steps: (i) isolating regions of genomic DNA that contain microsatellite loci; (ii) developing strategies for screening each locus, which requires designing primers for amplification, optimizing reaction conditions, and screening for variation; and (iii) genotyping sampled individuals. The protocols for cloning genomic DNA and isolating microsatellite loci have previously been based on those of Sambrook et al. (1) and Rassman et al. (2). However, recent techniques have been developed that greatly streamline this process and reduce the time and money involved in cloning and isolating microsatellite loci (3). Significant methodological and technical advances have also streamlined the process of genotyping individuals. This revolution began when fluorescent molecules replaced radioisotopes as the primary method of labeling DNA fragments for visualization in sequencing analyses (4). Moreover, fluorescent techniques are safer because they remove the need for radioisotopes.

Although methodological advances have recently occurred in the first and third steps of microsatellite development and profiling, the process of screening markers for polymorphisms has changed little, and this step has become limiting.

To reduce the time and cost and increase the safety involved in screening microsatellites for polymorphisms, several nonradioactive methods have been developed (5–8). However, these techniques still represent additional costs because they require the use of equipment that is unnecessary for other steps in fluorescent-based microsatellite development and profiling. Purchasing fluorescently labeled primers for this step is not economical due to the large cost involved in labeling primers with fluorescent molecules and because a portion of the loci will not be polymorphic. Therefore, a method for screening microsatellite markers for polymorphisms using unlabeled primers and fluorescent detection systems is necessary to reduce the cost and further streamline the process of microsatellite analysis.

Here we describe a method for screening microsatellite markers for polymorphisms using unlabeled primers, an ABI PRISM™ 377 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA), and the DNA stain SYBR® Green I (Cambrex, Rockland, ME, USA).

Right whale (Eubalaena sp.) samples used to develop this technique were collected as previously described (9), and DNA extraction also followed previously described protocols (10). PCR amplification of the dinucleotide microsatellite loci TextVet19, TextVet20 (11), and RW31 (12) was performed on individuals whose genotypes had previously been determined using the method.

Figure 1. Electropherograms showing DNA fragment separation and visualization resolution for microsatellite loci TextVet19, TextVet20, and RW31. (A–C) Electropherograms of individuals who are homozygous; (D–F) heterozygous with a 2-bp difference in allele sizes; and (G–I) heterozygous with a 4-bp difference in allele sizes for each of the tested loci.
Whales with homozygous genotypes representing different allelic spreads for each locus (i.e., homozygotes; 2, 4, 6, and 8 bp differences in allele sizes) were used to test the DNA fragment separation and visualization resolution of the technique. PCR was carried out in 10-μL volumes containing 5 ng template DNA, 1× PCR Buffer [containing Tris-Cl, KCl, (NH₄)₂SO₄, and 1.5 mM MgCl₂], 0.05 U/μL Taq DNA polymerase (Qiagen, Mississauga, ON, Canada), 0.2 mM each dNTP (Amersham Biosciences, Piscataway, NJ, USA), and 0.3 μM each primer. The primers were synthesized by the Institute for Molecular Biology and Biotechnology, McMaster University (Hamilton, ON, Canada). The reaction cycles consisted of 94°C for 5 min; 30 cycles of 94°C for 30 s, 54°C (TextVet19 and RW31) or 60°C (TextVet20) for 30 s, 72°C for 45 s; and 72°C for 10 min. The reactions were carried out on PTC-225 DNA Engine Tetrad ™ Thermal Cyclers (MJ Research, Waltham, MA, USA).

We prepared the PCR products for electrophoresis by making 1:10 and 1:20 dilutions of each and mixing 1 μL of each dilution with 1 μL Orange G loading dye (0.5% Orange G; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), 15% glycerol (Bioshop Canada, Burlington, ON, Canada), and 50 mM EDTA, pH 8.0 (BDH, Toronto, ON, Canada). The samples were loaded onto a 36-cm slab gel, following the protocols recommended for microsatellite analysis in the manual (14), with the following modifications: (i) a nondenaturing 5% polyacrylamide gel was used [5% Long Range ™ acrylamide solution (Applied Biosystems), 2.5% (w/v) glycerol (BDH), 1× TBE, pH 8.3 (0.1 M Tris, 0.1 M boric acid, 0.002 M EDTA, pH 8.0)]; (ii) samples were not heated at 95°C and then placed on ice prior to loading, instead, they remained at room temperature; and (iii) a 1:100,000 dilution of SYBR Green I was mixed with the buffer for both the top and bottom buffer reservoirs. Additionally, 16.7 ng of 100-bp ladder (Invitrogen Canada, Burlington, ON, Canada) were loaded in two lanes for comparison with the samples to obtain the approximate allele sizes. Electrophoresis was performed at 3000 V at 51°C for 1 h.

Whales with homozygous genotypes at each locus had the expected electropherogram pattern of a single peak (Figure 1, A–C). Allelic size differences of 2 bp could not be clearly detected for all loci (Figure 1, D–F); however, alleles differing by 4 bp could be clearly distinguished for all three loci (Figure 1, G–I). Additionally, allelic size differences of over 4 bp could be easily distinguished for all tested loci (data not shown).

We have developed a nonradioactive technique for screening microsatellite markers for polymorphisms using an automated DNA sequencer and SYBR Green I. This technique provides adequate resolution to consistently detect 4 bp differences in allele sizes. If 10 individuals are screened at this stage, an approximate level of allelic diversity can be assessed. This information can then be used to develop primer-labeling strategies for genotyping procedures. If dinucleotide loci are being screened, estimates of allelic diversity will be conservative due to the potential for alleles differing by one repeat unit within an individual to go undetected. It is also possible that variations in dinucleotide loci containing only two alleles, differing by one repeat unit, may go undetected, however, such loci are of limited use in population analyses and are often disregarded in favor of loci with a larger number of alleles. The resolution obtained with this technique, combined with its simplicity and use of fluorescent-based detection systems, will make it a useful method for screening microsatellite markers for polymorphisms prior to the purchase of costly fluorescently labeled primers for genotyping procedures.

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REFERENCES


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