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MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

High-resolution melting analysis (HRMA): a highly sensitive inexpensive genotyping alternative for population studies

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Abstract

High-resolution melting analysis (HRMA) is a highly sensitive closed-tube genotyping method used primarily in clinical studies. As the method is rapid, inexpensive and amenable to high throughput, we decided to investigate its applicability to population studies. Small amplicons and unlabelled probes were used to genotype the nuclear genes, lactate dehydrogenase-A (*ldh*-A), myosin light chain-2 (*mlc*-2), acidic ribosomal phosphoprotein P0 (*ARP*) and calmodulin (*CaM*) in populations of swordfish, *Xiphias gladius*. Results indicate that HRMA is a powerful genotyping tool to study wild populations.

Keywords: genotyping, high-resolution melting analysis, nuclear DNA, population studies, SNP, swordfish

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High-resolution melting analysis (HRMA) is a highly sensitive molecular method for mutation scanning and genotyping confined to date to clinical and diagnostic studies (Reed *et al*. 2007; Vandersteen *et al*. 2007; Lee *et al*. 2008). HRMA detects SNPs and small deletions in a fragment of amplified DNA by comparing fluorescence as a function of temperature. Alleles produce distinct melting curves that can be compared with reference samples. The use of a saturating DNA dye enhances the detection of heteroduplexes (Graham *et al*. 2005) that can be identified through changes in melting curve shape (Palais *et al*. 2005). Variations of HRMA have been developed to enhance resolution. In small amplicon (SA) HRMA, the 3′ ends of a primer set are placed at a very short distance from the informative SNP (Gundry *et al*. 2008). The small size (40–60 bp) of the resulting amplicon enhances the melting temperature (T_m) differences among homozygous genotypes. Alternatively, unlabelled probe (UP) HRMA can be used when multiple informative SNPs are present within a longer stretch of sequence, but also when low GC content or the presence of polymorphisms prevents the placement of SA-HRMA primers. In UP-HRMA, the T_m of the unlabelled probe, and not of the entire amplicon, is used for genotyping (Liew *et al*. 2007; Poulson & Wittwer 2007). The entire procedure from polymerase chain reactions (PCR) to scoring is completed within 15–20 min as a single closed-tube assay. Based on all these attributes, a wide use of this technique would be expected; however, a literature review as of April 2009 revealed no hits of HRMA in wild populations despite the increasing importance of SNPs in this field. Here we use HRMA to genotype alleles in swordfish populations.

Swordfish (n = 121) previously sampled from the North Atlantic, South Atlantic, Mediterranean, Hawaii and Chile (Alvarado Bremer *et al*. 2005, 2006) were used for HRMA. DNA was isolated from a small piece (≈4 μg) of tissue (liver, heart, muscle, or fin clips) using a Proteinase K digestion followed by ETOH precipitation without organic extractions (Grieg 2000). PCR for SA-HRMA

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Table 1 New HRMA primers and PCR profiles used in this study. Amplicon length was minimized to maximize melting temperature differences for genotyping

Locus	HRMA primer name	HRMA primer sequence	Thermocycling profile
CaM (Chow 1998)*	CALMex4-INT-F	5′-TGCACACATTTGATCCTGTGAC-3	94 °C 1 min, followed by 55 cycles at 94 °C 0 s, 60 °C 0 s, 66 °C 10 s
	CALMex5-INT-R	5′-GTAGCCATTTCCGTCCTGGA-3′	
ARP (Takehana *et al*., 2007)*†	Olb03.10-HRM-F	5′-GCTACTCCTGTCTGTCTAAATC-3′	94 °C 1 min, followed by 55 cycles at 94 °C 0 s, 50 °C 0 s, 72 °C 10 s
	Olb03.10-HRM-R	5′-GTCCCTAGCTGCCGGAA-3′	
ldh-A (Grieg 2000)*	ldhA-HRM-SwoF	5′-AGCAAGCCCTGAACTTC-3′	94 °C 1 min, followed by 55 cycles at 94 °C 0 s, 66 °C 10 s
	ldhA-HRM-SwoR2	5′-GCCGAAAGGACAGGGTGAGC-3′	
	ldhA-HRM-Probe	5′-ATTCATC**C**TGTTGATTAGTTTACAAAA CATA**A**TGTACAT/3Phos/-3′	
mlc-2 (Atarhouch *et al*., 2003)*	Mlc2c-F	5′-CTGTCGCACTGGGTGGTCA-3′	94 °C 1 min, followed by 55 cycles at 94 °C 0 s, 55 °C 0 s, 72 °C 10 s
	Mlc2b-R	5′-CCAACACTCACTCCTGAATTGG-3′	
	Mlc2-HRM-Probe	5′-GTAGCGGGAACTT**G**TGCTCA AACACCT/3Phos/-3′	

Polymorphic loci located in the unlabelled probes are underlined and in bold.
*Citations provided for the primers used to obtain DNA sequences and identify SNPs targeted with HRMA primers.
†Locus Olb03.10 is orthologous to locus ARP.

were performed in 10 μl vols in LightCycler capillares (Roche Diagnostics) containing: 0.2 M Trehalose, 250 μg/mL BSA, 1X LCGreen Plus (Idaho Technology), 1X PCR Buffer with 3.0 mM $MgCl_2$ (Idaho Technology), 200 μM of each dNTP, 0.5 U of Taq polymerase (Lucigen), 10 ng DNA template and 0.25 μM of each primer. UP-HRMA required an asymmetric PCR with a reverse primer and an unlabelled 3′-phosphorylated probe at 0.5 μM each, and a forward primer at 0.075 μM, with all other reagents as above. Reaction mixtures were overlaid with mineral oil to prevent evaporative losses ensuring melting profile uniformity. Thermocycling was carried on a RapidCycler II (Idaho Technology) with HRM primers as outlined in Table 1. Prior to melting analysis in a HR-1 instrument (Idaho Technology), samples were heated to 94 °C and cooled rapidly to 40 °C to maximize heteroduplex formation.

Loci calmodulin (*CaM*), acidic ribosomal phosphoprotein P0 (*ARP*) and myosin light chain-2 (*mlc*-2) are bi-allelic. *CaM* and *ARP* were genotyped using SA-HRMA, revealing all three possible genotypes respectively (Fig. 1A–B). Because the regions flanking the informative SNP in *mlc*-2 were not optimal for SA-HRMA primer design, an unlabelled probe producing three distinct melting profiles was designed (Fig. 1C). Locus *ldh*-A also required an unlabelled probe that contained two polymorphic loci, which define three alleles and six genotypes (Fig. 1D). In all instances, the alleles scored with HRMA matched the genotypes from DNA sequences (GenBank AF069912–AF069913, FJ890938–FJ890941, FJ911901–FJ911904).

Population genetic studies targeting SNPs have employed a variety of genotyping methods (reviewed in Kwok 2003), affected by one or more of the following shortcomings associated primarily with post-PCR manipulation: false negatives (e.g. endonuclease inactivity), time consuming with numerous steps and platforms (e.g. gels, enzymatic reactions and cleaning procedures), and or costly (e.g. fluorescently labelled probes). In addition, informative SNPs may not coincide with an endonuclease recognition site, or an allele size polymorphism may not exist within an amplicon. HRMA circumvents most of these limitations. One concern with HRMA is that ionic strength and template concentration could alter the amplicon T_m. This may explain why HRMA has been confined to clinical or diagnostic studies, where uniformity in DNA template quality and quantity is generally higher than the samples collected in the field. Here, however, DNA was isolated from several tissue sources without phenol–chloroform extractions, and regardless of the source, the melting profile for each allele was unambiguous and repeatable (Fig. 1). While we relied on the HR-1 instrument for melting analyses, the sensitivity and effectiveness of other platforms have been evaluated (Herrmann *et al.* 2007). The high throughput, low cost and high resolution to discriminate alleles makes HRMA a desirable genotyping tool for studying wild populations.

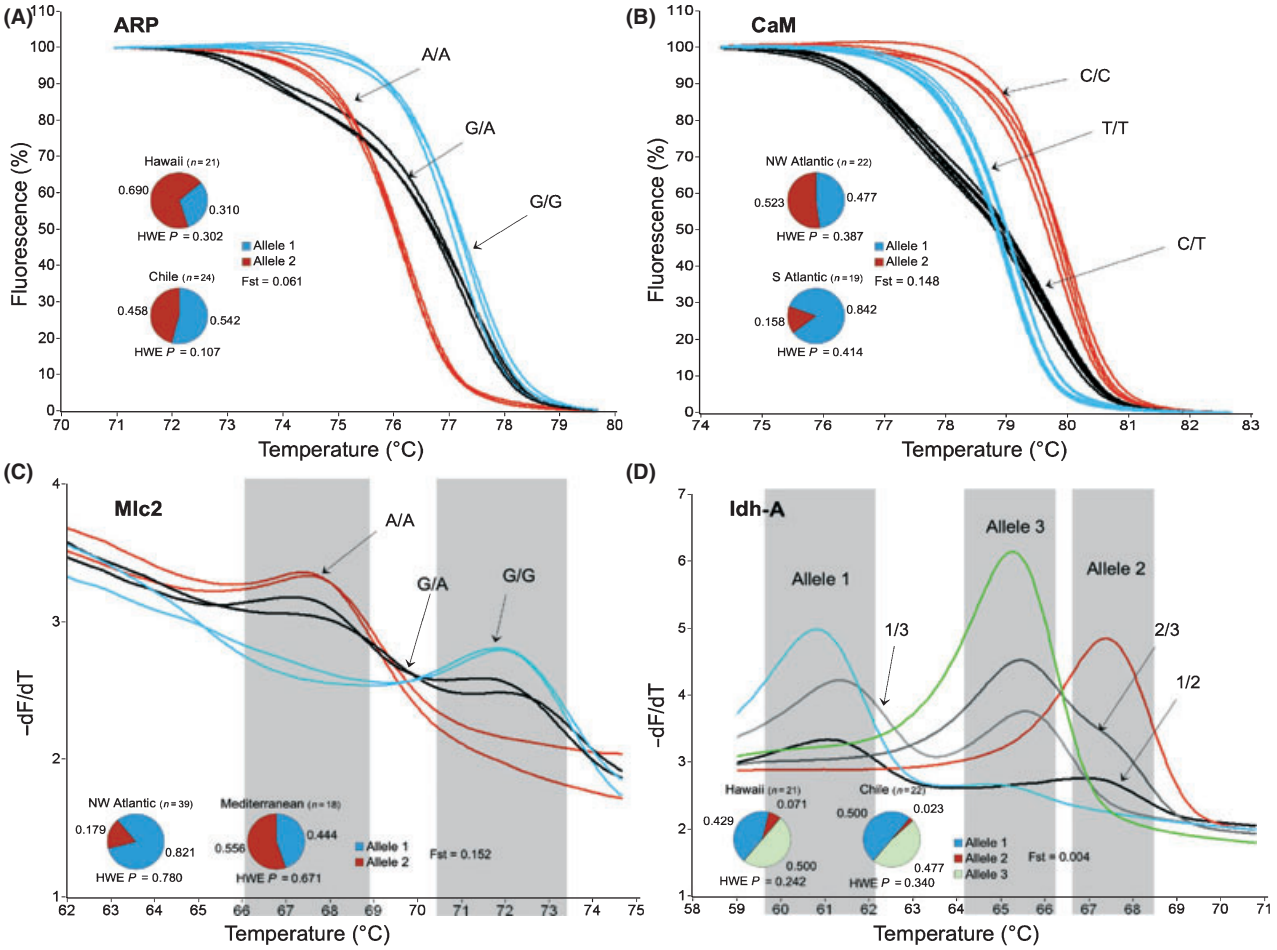

Fig. 1 Genotyping swordfish populations using HRMA. Each graph (A–D) depicts melting curves from multiple individuals for the corresponding locus. Pie charts (insets) summarize the allele frequencies for different samples and the respective pairwise F_{ST} value. All samples are in HWE ($P > 0.05$). In the normalized curves of *ARP* (A) and *CaM* (B) using SA-HRMA, homozygous genotypes are distinguished by $T_{m,}$ whereas heterozygous individuals by melting curve shape. In UP-HRMA (C, D), negative derivative plots of fluorescence with respect to temperature (−dF/dT) were obtained from the melting curves of the probes for *mlc-2* and *ldh*-A (Table 1). The *mlc-2* unlabelled probe targets an A/G SNP. The *ldh*-A unlabelled probe targets two polymorphic loci (T/C/A and A/C) that define the three most common alleles described in Alvarado Bremer *et al*. (2006) for Pacific swordfish. Homozygous individuals have one peak and heterozygous individuals two peaks (e.g. genotype 1/3) within the corresponding melting points (shaded regions). Details of sampling locations are given in Alvarado Bremer *et al*. (2005, 2006).

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