Using the DCode™ System to Identify DNA Sequence Variation for Studies of Population Structure in Marine Organisms

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Introduction

DNA polymorphisms are useful tools for ecological and evolutionary studies of both terrestrial and marine organisms, with applications ranging from species identification to delineation of population structure to monitoring genetic change in wild or domesticated populations. Denaturing gradient gel electrophoresis (DGGE) using the DCode universal mutation detection system provides a convenient means of identifying genes with useful levels of polymorphism, and subsequently screening populations for variation at selected loci.

Unlike biomedical model organisms, most species of interest in ecological or evolutionary studies are not genetically well characterized. Genes of interest are amplified by the polymerase chain reaction (PCR) using 'universal primers' targeting highly conserved regions (Palumbi 1991). Mitochondrial genes are popular candidates because of their typically rapid evolution, easy amplification, and sensitivity to reduced effective population size (Avise 1994). For DGGE, the haploid state of mitochondrial DNA (mtDNA) offers two advantages. First, except for heteroplasmic individuals, the electrophoretic pattern of homoduplex DNA is simple. Secondly, the deliberate construction of heteroduplex molecules by pooling homoduplex PCR products often yields unique heteroduplex electrophoresis patterns, enabling identification of different haplotypes that cannot be separated on the basis of homoduplex mobility. We use DGGE to locate and score both nuclear and mtDNA polymorphisms in a variety of marine finfish and invertebrates, and describe a few examples here.

Methods

Genomic DNA was prepared from 1–3 mg tissue snips of gill or adductor muscle from eastern oysters (*Crassostrea virginica*) stored in 70–95% ethanol and from frozen or ethanol-preserved tissue samples from the tautog (*Tautoga onitis*), a commercially and recreationally important Atlantic finfish, using a commercial guanidinium isothiocyanate DNA extraction kit (PureGene, Gentra). For the oyster, a ~400 bp fragment of the mitochondrial 16S RNA subunit was amplified using a GC-clamped version of the universal primer 16SAR (Kocher et al. 1989) and an oyster-specific primer 16SOB (Banks et al. 1993). Although most authors recommend a GC clamp of 35–40 nt, we obtained satisfactory results with a 15 nt clamp. For the tautog, a ≈380 bp fragment of the mitochondrial cytochrome b oxidase (*cyt b*) gene was amplified using the universal primers CB2-H and CB1-L3, with a 15 nt GC clamp attached to the 5' end of CB2-H. In addition, universal primers LDHA6F1 and LDHA6R (J. Quattro, University of South Carolina, personal communication), with a 15 nt GC clamp attached to LDHA6F1, were used to amplify muscle-type lactate dehydrogenase (LDH) intron six. Oligonucleotides were prepared by commercial manufacturers (Genosys, Life Technologies) without additional cartridge or HPLC purification.

Amplification of both products was performed using two thermal cyclers (PerkinElmer 480 and MJ Research PTC-100) with comparable results. Standard 50 µl amplification reactions used 1.25 U of *Taq* polymerase (Promega, Life Technologies) with supplied buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 10 pmol of each primer, and 1–3 µl of template DNA (concentration not quantitated). For all amplifications, hot-start PCR was initiated by addition of polymerase and primers following an initial 2 min denaturation at 94°C. For the 16S product, 30–35 cycles (45 sec at 94°C, 1 min at 48°C, 1 min at 72°C) were followed by a 7 min final extension at 72°C. For the *cyt b* fragment, 35 cycles (1 min at 95°C, 1 min at 50°C, 1 min at 72°C) were followed by a 5 min final extension at 72°C. For the LDH intron, 35 cycles (1 min at 95°C, 1 min at 52°C, 1 min at 72°C) were followed by a 2 min final extension at 72°C.

Heteroduplex DNA was formed by pairwise pooling of 10 µl aliquots of unpurified PCR products in a linked series (product 1 + 2, 2 + 3, 3 + 4,... k + 1). Pooled aliquots were placed in 0.6 ml PCR tubes with a silicone oil (Aldrich) overlay. Several protocols were used to form heteroduplex DNA, including 1) heating the template mix to 95°C for 10 min, snap-cooling at –20°C, and gradual warming to room temperature, and 2) using a thermal cycler to heat the template mix to 95°C for 10 min, followed by controlled cooling to 65°C at a rate of 1°C/min and subsequent uncontrolled acclimation to room temperature.

Because no sequence data were available for the two tautog PCR products, perpendicular gradient denaturing gels were run to determine their melting profiles empirically. The resulting profiles (not shown) suggested a parallel gradient range of 40–60% denaturant for the *cyt b* product, and a range of 20–40% for the LDH intron. For the oyster product, we had preliminary sequence data for the entire fragment except for a short stretch immediately downstream from the forward (GC-clamped) primer. For this region, we substituted the homologous sequence from *Drosophila yakuba* inferred from a manual alignment of oyster and fly 16S sequences, in order to construct a theoretical melting profile of the PCR product (Figure 1). The theoretical profile suggests a gradient range of 20–40% denaturant.

After heteroduplex formation, pooled PCR products were loaded onto 6% acrylamide gels and run at constant temperature (60°C) for 4–5 hr, followed by staining in ethidium bromide and visualization with a standard UV transilluminator.

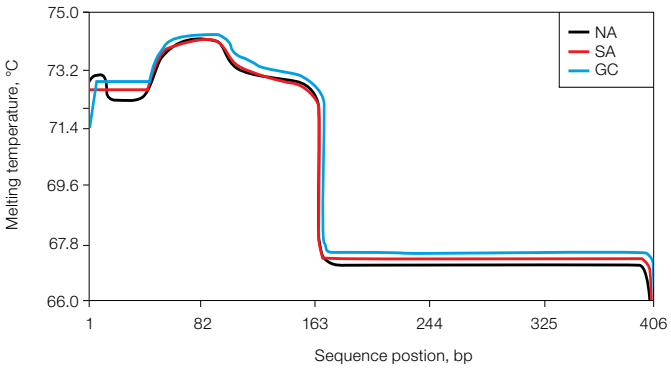

Fig. 1. MeltMap™ software analysis of a 402 bp fragment of oyster 16S mtDNA illustrating three common haplotypes. Haplotypes differ from one another at two variable nucleotides only (positions 332 and 340), containing TA, GA, and GG, respectively. NA, North Atlantic; SA, South Atlantic; GC, Gulf Coast.

Results

For the tautog, no previous genetic data were available, so the amount of genetic variation to be expected in the amplified fragments was unknown. Seventy-two individuals collected from the geographical range of the species (Rhode Island to Virginia) were examined. The LDH intron appeared to be virtually invariant, with only a single variant encountered among the 144 alleles screened. The *cyt b* fragment was likewise nearly monomorphic; 70 individuals possessed the common haplotype and two individuals were unique variants (singletons).

As an indirect test of the possibility that sequence variation in these fragments was going undetected with our protocols, we examined *cyt b* products from spot (*Leiostomus xanthurus*), a fish species shown by restriction fragment length polymorphism (RFLP) analysis to have high mtDNA sequence diversity (T. Lankford, University of Delaware, personal communication). Even without optimizing DGGE protocols, we detected 4–6 different haplotypes among the 8 individuals examined, suggesting that the low diversity observed in the tautog was not due to poor resolution by DGGE.

For the oyster 16S product, more than 250 individuals were examined using heteroduplex DGGE. Initial screening showed that each geographical region tended to have a single common haplotype (frequency >90%) plus a number of rare variants. To identify new or questionable haplotypes with more certainty, a standard heteroduplex panel of 5–6 known variants was used. If an individual in question did not show banding patterns identical to that of a previously characterized variant for all heteroduplex combinations, it was classified as a new haplotype. For the total data set, 3 extremely common haplotypes (Gulf Coast, South Atlantic, North Atlantic), 3 moderately common variants (1 from Long Island Sound, 2 from Prince Edward Island), and 8 rare variants were found (Figure 2). All were further characterized by direct sequencing.

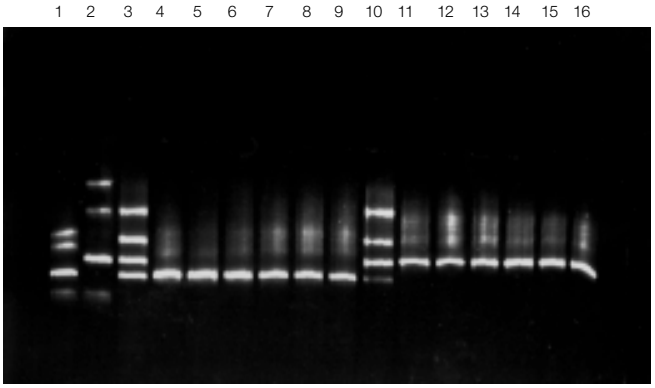

Fig. 2. Parallel DGGE gel of oyster 16S mtDNA PCR products, illustrating pairs of samples combined and heated after PCR to induce heteroduplex formation. Lane 1, GC+SA; lane 2, GC+NA; lane 3, SA+NA; lanes 4–9, SA+SA combinations; lane 10, SA+NA; lanes 11–16, NA+NA combinations. In lanes 1 and 2, the amount of GC DNA is low, resulting in faint bands for the GC homoduplex molecule.

Discussion

DGGE analysis of PCR products amplified using universal primers may be coupled with heteroduplex analysis to yield a powerful and versatile tool for both the detection of genetic polymorphisms and subsequent haplotype identification. This approach, applicable to temperature gradient gel electrophoresis (TGGE) (Campbell et al. 1995) as well as the related constant denaturant gel electrophoresis (CDGE), is gaining popularity in population genetics and phylogenetic studies as an alternative to direct sequencing of population samples. Although identification of haplotypes by denaturing gel electrophoresis does not yield the same type of information that DNA sequencing does, it has two particular advantages: 1) it allows the investigator to identify amplified regions with desirable levels of polymorphism, which may vary depending on the purpose of the study; 2) large population samples typically collapse into a much smaller set of haplotypes, which may be sequenced, avoiding repetitive sequencing of common haplotypes.

The use of an outgroup reference standard as a heteroduplex generator greatly improves detection of allelic variants, many of which will have homoduplex molecules with similar electrophoretic mobility. For the oyster 16S product, several common haplotypes were available to use as reference standards. In a similar study of population structure in Atlantic horseshoe crabs, we are using two different Gulf Coast haplotypes as standards for detecting sequence variants in Atlantic populations (Figure 3). In the gel shown, both homoduplex and heteroduplex bands are visible as doublets, which may be an artifact of amplification with *Taq* polymerase under certain PCR conditions (Zhu et al. 1997). We have not observed these doublets in other DGGE analyses.

The approach taken may vary with the amount of previous sequence information available to the investigator. When sequence data are unavailable, as was the case for the two tautog loci described above, perpendicular DGGE of the PCR product is necessary to define the appropriate gradient for parallel electrophoresis. When sequence data are available, it is possible to proceed directly to parallel DGGE, although empirical 'fine-tuning' of the gradient range and run time is still essential.

For PCR products larger than ≈0.5 kb, the extremely high sensitivity of gradient gel electrophoresis is diminished, and it becomes difficult to obtain very large fragments with ideal melting properties. One strategy is to amplify the larger product, with one or both primers GC clamped, and digest it with a restriction enzyme that yields several smaller fragments, which are then run on a denaturing gel. Although the melting profiles of the individual fragments may not be ideal (particularly for fragments without a GC clamp), many polymorphisms will still be detected. This 'fall-back' option is useful in cases where the size of the PCR product is not known in advance, as in the case of some introns.

Population biologists searching for intraspecific polymorphisms are drawn to introns, which often show a greater degree of site and size variation than coding regions. Introns may be amplified using conserved primers targeted to flanking exon sequence, a strategy termed exon-primed intron-crossing (EPIC) amplification (Palumbi 1996). When the size of a homologous intron varies among species, one may analyze the smaller products by direct gradient gel electrophoresis and use a RFLP/DGGE approach for the larger ones.

This two-pronged approach may be used in developing polymorphic markers when available universal primers are inadequate. Using available cDNA sequence data, one may design primers to amplify a moderate-sized (100–400 bp) product with a suitable melting profile for DGGE. If the resulting PCR product is larger than expected due to the presence of an intron, it can still be screened for polymorphisms with the RFLP/DGGE approach. Both population genetics surveys and genetic mapping require numerous polymorphic DNA markers. Denaturing gel electrophoresis, in its various forms, offers a flexible and powerful means of identifying polymorphisms, as well as screening large population samples for identified variants.

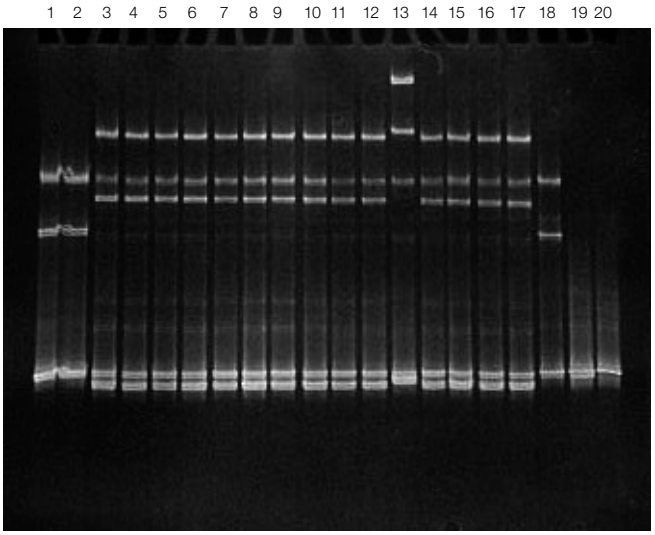

Fig. 3. Parallel DGGE gel of a 312 bp fragment of the mitochondrial cytochrome oxidase subunit 1 gene from the horseshoe crab, *Limulus polyphemus*. Each lane represents a different crab from the Atlantic coast combined with a Gulf Coast reference standard, except for the first two lanes, which represent other Gulf Coast individuals. The 10–25% gradient gel was run for 4 hr at 130 V. Fixed sequence differences between Gulf Coast and Atlantic haplotypes result in heteroduplex formation for each Gulf-Atlantic combination. Of the three Atlantic haplotypes revealed by the heteroduplex bands, one (lanes 3, 19, and 20) has a homoduplex band indistinguishable from the Gulf Coast homoduplex under these DGGE conditions.

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