

PERMANENT GENETIC RESOURCES NOTE

General-use polymerase chain reaction primers for amplification and direct sequencing of enolase, a single-copy nuclear gene, from different animal phyla

RYAN P. KELLY and STEPHEN R. PALUMBI

Stanford University, Hopkins Marine Station, 120 Oceanview Blvd., Pacific Grove, CA 93950, USA

Abstract

In contrast to mitochondrial DNA, remarkably few general-use primer sets are available for single-copy nuclear genes across animal phyla. Here, we present a primer set that yields a c. 364-bp coding fragment of the metabolic gene enolase, which includes an intron in some taxa. In species where introns are absent or have few insertions/deletions, the amplified fragment can be sequenced directly for phylogenetic or population analysis. Between-species variation in the coding region occurs widely at third codon positions, even between closely related taxa, making the fragment useful for species-level systematics. In low gene-flow species, the primers may also be of use for population genetics, as intraspecific polymorphisms occur at several silent positions in the taxa examined.

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The dearth of general-use (i.e. quasi-'universal') primer sets for the amplification of single-copy nuclear DNA (scnDNA) loci, combined with the availability and ease of use of mitochondrial (mtDNA) gene regions, has led to a large disparity in the number of publications using each data type. Nuclear primers that amplify single fragments from different taxa have generally focused on ribosomal DNA (e.g. 18S and 28S, Whiting *et al.* 1997) or histones (e.g. H3 or H4 *et al.* 1998; Pineau *et al.* 2005) that are present in many copies that evolve in concert within a species (Avisé 2004). Large-scale systematics work has yielded highly degenerate primers that target single-copy nuclear genes but these often amplify multiple fragments from genomic DNA, requiring reverse transcription–polymerase chain reaction (RT–PCR) or cloning before sequencing (e.g. Peterson *et al.* 2004). Other systematics efforts have resulted in phylum-specific scnDNA primers (Regier & Schultz 1997; Schultz, Regier 2000; Regier 2007).

Nuclear DNA offers several benefits for systematics or population genetic studies work: diploidy allows heterozygosity to be measured to infer selection and demographic processes (Hartl 2000; Bazin *et al.* 2006), recombination creates independent gene regions whereas the entire

mitochondrion is linked (Ballard & Whitlock 2004), and a wide variety of mutation rates is observed in the nuclear genome (Avisé 2004). Finally, nuclear DNA represents orders of magnitude more genetic information than does mtDNA (Ballard, Whitlock 2004). Unfortunately, many nuclear genes are multicopy or part of larger gene families, making it difficult to amplify single PCR products without multiple rounds of cloning and developing taxon-specific primers (Graur & Li 2000). In addition, the presence of multiple gene copies may make gene homology among individuals or taxa unclear (Palumbi & Baker 1996; Zhang & Hewitt 2003).

Here, we present a primer set that reliably amplifies and sequences a single fragment from the enolase (2-phospho-D-glycerate hydrolyase) gene, which functions in glycolysis (Berg *et al.* 2002), from species in at least five metazoan phyla. This region is intronless in many species and, where this is the case, amplicons can be sequenced directly and read cleanly. In species where introns are present, introns may be sufficiently short or uniform in length that clean reads from direct sequencing are nevertheless possible. The resulting fragment is less variable than mtDNA fragments such as cytochrome *c* oxidase I (COI) but more variable than 18S rDNA in the taxa examined, making it useful for population genetics in low gene-flow species or else for species-level systematics work.

Correspondence: Ryan P. Kelly, Fax: (831) 375 0793; E-mail: rpk@stanford.edu

Alignments were created from sequences publicly available in GenBank (Table 1), and degenerate primers designed at conserved regions spanning from position 481–910, inclusive (forward primers Enol F1, F2, and reverse Enol R4; Table 1). These positions are relative to the *Loligo pealei* and *Drosophila melanogaster* start codons for alpha enolase (GenBank S80961 and X17034, respectively). Another degenerate reverse primer (Enol R5; designed with the help of E. Jacobs-Palmer; Table 1) was designed internal to Enol R4, yielding a smaller (209-bp) fragment. This reverse primer was used to generate sequence for the barnacle *Balanus glandula*. For ease of sequencing, M13 tails (forward and reverse) were added to the 5' end of all of the below primers, following Regier (2007).

PCRs were carried out on genomic DNA extracted using QIAGEN DNeasy (QIAGEN Corp.) or Nucleospin Tissue (Macherey-Nagel Corp.) kits and kept frozen (–20 °C) in water. Amplification was achieved in 12.5- μ L reactions using *AmpliTaq* DNA Polymerase (Applied Biosystems Corp.) with additional MgCl₂ (1.25 μ L of 25 mM), bovine serum albumin (1.25 μ L of 10 mg/mL solution), a final concentration of 10 nM of dNTPs, and 5 pmol of primer. Other polymerases successfully amplified the fragment as well. Thermocycling followed a basic protocol of 95 °C initial denaturation for 1 min, then 35–40 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and then a final 72 °C elongation period of 3 min. Amplicons were cleaned by adding 0.5 μ L each of Shrimp Alkaline Phosphatase and Exonuclease (USB, Inc.) to 5 μ L of PCR product and incubating at 65 °C for 30 min and 80 °C for 15 min, in order to digest single-stranded DNA and unincorporated dNTPs.

Cycle sequencing of amplified products was carried out using 0.25–.5 μ L BigDye (Applied Biosystems Inc.), 5 \times sequencing buffer, 2.5 pmol primer, 5 μ L, and 1–2 μ L PCR product per reaction. These were cycled using the following protocol: 30 cycles of 95 °C for 15 s, 50 °C for 15 s and 72 °C for 4 min. Sequences were cleaned using Sephadex (GE Healthcare, Inc.), eluted in water, and read on an ABI PRISM 3700 sequence analyser.

To date, the primer set has been used to generate sequence data by direct sequencing for various species across five phyla (Table 2). In addition, they have reliably amplified in a sixth phylum, although multiple identically sized fragments generated in the annelid species *Nereis* sp. made accurate reading of the fragments impossible.

Intraspecific variability at the enolase locus appears to be generally low: within individuals of four *Pagurus* spp. (*P. samuelis*, *P. granosimanus*, *P. hirsutiussculus* and *P. venturenensis*) for example, enolase has a mean number of 0.93 pairwise differences among genotypes across 364 bp (nucleotide diversity = 0.0025), whereas the same species average nearly an order of magnitude more variability in 658 bp from the COI mtDNA region (mean pairwise distance = 8.7; nucleotide diversity = 0.013; R.P.K. and S.R.P., unpublished).

Table 1 Novel primers for amplifying a fragment of the enolase gene, with the GenBank sequences used to design them. Positions refer to the complete enolase sequence of *Drosophila melanogaster*

Taxon (Phylum) (GenBank Accession no.)	5' Position 481–515	5' Position 724–740	5' Position 880–910
<i>Loligo pealei</i> (Mollusca) (S80961)	AACAATTTGGCCATGCAGGAGTTTATGATCCTTCC	ATTGGCATGGATGTTGC	ATTGAGGATCCCTTTGACCAAGATGACTGGG
<i>Drosophila melanogaster</i> (Arthropoda) (X17034)	AACAAGCTGGCCATGCAGGAGTTTATGATCCTTCC	ATCGGCATGGACGTGGC	ATTGAGGATCCCTTTGACCAAGATGACTGGG
<i>Limulus polyphemus</i> (Arthropoda) (AF258665)	AACAAGCTTAGCTATGCAGGAGTTTATGATCCTTCC	ATTGGCATGGATGTTGC	ATTGAAAGATCCATTTGACCAAGATGACTGGG
<i>Alligator mississippiensis</i> (Chordata) (AF072586)	AACAAGCTGGCCATGCAGGAGTTTATGATCCTTCC	ATTGGCATGGATGTTGC	ATTGAAAGATCCCTTTGACCAAGATGACTGGG
<i>Biomphalaria glabrata</i> (Mollusca) (DN837236)	?	ATTGGTATGGATGTTGC	?
Enol F1	ATGCAGGAGTTTATGATCCTKCC		
Enol F2	AACAAGCTGGCCATGCAGGAG		
Enol R4			ATTGARGATCCWTTTGGACCARGAYSACTGGG
Enol R5		ATTGGNATGGATGTINGC	

Substantially more variation at the enolase locus is observed among species (mean pairwise distance = 5.83; nucleotide diversity = 0.016 for the same four species), demonstrating that the locus is variable enough to distinguish among even closely related taxa. By contrast, a 550-bp fragment of the 18S rDNA locus is invariant among these and other hermit crab species. Despite its low intraspecific variability, the enolase fragment was also able to identify population genetic structure in at least one highly structured species, *P. hirsuti-sculus*, which yielded an F_{ST} of 0.44 between populations in Cape Blanco, Oregon, and Sitka, Alaska. These results are consistent with those from the COI mtDNA fragment.

Based on these results, we believe the enolase primer set will be broadly useful for species-level systematics work or, in species with a high degree of structure, population-level inquiry. If direct sequence reads are not possible in a given species, these primers are an easy starting point for cloning amplified PCR product and designing primers specific to the relevant project. We hope to see more such primers enter the literature in the future in order to encourage the use of scnDNA for species- and population-level analysis.

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