

MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

Intron-spanning primers for the amplification of the nuclear ANT gene in decapod crustaceans

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Abstract

We describe polymerase chain reaction primers that amplify the low-copy nuclear adenine nucleotide transporter gene in decapod crustaceans. These were tested on 35 species from 14 decapod families, and a single polymerase chain reaction product amplified in 32 species. Of 49 sequences generated, only two did not contain an intron, and the longest intron identified was more than 834 nucleotides in length. The amplified fragment is likely to be useful at various taxonomic levels. While the intron is suitable for phylogeographical/population genetic studies and to identify cryptic speciation, the second exon region is sufficiently long to provide signal at both the phylogeographical and phylogenetic levels.

Keywords: ATP/ADP translocase, cryptic species, EPIC primers, nuclear DNA, phylogeography, phylogenetics

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The last 10 years have seen an escalation of surveys using multilocus DNA data in phylogenetic, phylogeographical and population genetic studies (Beheregaray 2008). A large number of universal primers are available for certain groups, such as mammals (e.g. Lyons *et al.* 1997) and teleosts (e.g. Li *et al.* 2007), but crustaceans have been neglected. With the exception of primers amplifying regions containing microsatellites, few primers have been developed that work reliably for crustaceans, and most researchers working at the phylogeographical or population genetic level have exclusively used mitochondrial DNA sequences (Beheregaray 2008). Of the few studies on crustaceans that have employed nuclear sequence data, most have either used elongation factor 1 α (EF1 α) or the internal transcribed spacers (ITS1 and ITS2). A major problem with both these markers is that multiple versions are often present in the genome of a single individual (France *et al.* 1999; Harris & Crandall 2000).

Here, we describe exon-priming, intron-crossing (EPIC) primers developed to amplify the adenine nucleotide transporter (ANT) gene (also known as ATP/ADP translocase): DecapANT-F (forward primer):

5'-CCTCTTGAYTTCGCKCGAAC-3'

DecapANT-R (reverse primer):

5'-TCATCATGCGCCTACGCAC-3'

We consider this marker to be a useful alternative to EF1 α and ITS, because the number of copies present in the genome tends to be low (Jarman *et al.* 2002). The primers were designed based on a consensus sequence obtained from the prawns *Pacifastacus leniusculus* (DQ874397) and *Marsupenaeus japonicus* (EF077712) using the program Oligo version 7.0. The exon regions to which they anneal are highly conserved in arthropods, as indicated by comparison with *Drosophila subobscura* (AF025799): primer annealing regions of the forward and reverse primers differ from the corresponding regions in *D. subobscura* by 20% and 11%, respectively. Both primers were designed in such a way that their 3' ends anneal to the most conserved portions of the target sequence. For that reason, they should be suitable to amplify the ANT gene in a wide range of decapod species, and possibly also some other crustacean orders.

We tested the primers in 35 decapod species from 14 families, and a single polymerase chain reaction (PCR) product amplified in most of them (Table 1). PCRs contained 1 μ L of template DNA (~150 ng), 3 μ L of reaction buffer (Promega), 3.6 μ L of 25 mM MgCl₂ (i.e. 3 mM MgCl₂), 6 μ L of dNTP mixture containing 125 mM of each dNTP, 1.2 μ L of each primer (5 mM dilutions), 1 U of *Taq* DNA polymerase (Promega) and water to a final volume of 30 μ L. The PCR profile consisted of an initial denaturation step (94 °C for 3 min) followed by 35 cycles of 94 °C for 30 s, 50–55 °C for 45 s and 72 °C for 45 s, and a final extension step (72 °C for 7 min). PCR products

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Table 1 Characteristics of the ANT sequences of various decapod crustaceans

Higher taxon	Family	Species	Intron length (in bp)	GenBank Accessions		
Achelata	Palinuridae	<i>Jasus verreauxi</i>	> 131	FJ432007		
	Scyllaridae	<i>Ibacus peronii</i>	> 199	FJ432008		
		<i>Thenus orientalis</i>	> 113	FJ432009		
Anomura	Coenobitidae	<i>Coenobita variabilis</i>	No product	—		
	Galatheididae	<i>Munida ios</i>	No product	—		
Astacidea	Parastacidae	<i>Cherax destructor</i>	No product	—		
Brachyura	Grapsidae	<i>Paragrapsus laevis</i>	> 207	FJ432010-18		
		<i>Amarinus paralacustris</i>	No intron	FJ432020, FJ432021		
		<i>Elamena producta</i> *	> 358	FJ432022		
		<i>Halicarcinus cooki</i> *	314	FJ432023		
		<i>Halicarcinus innominatus</i> *	> 149	FJ432024		
		<i>Halicarcinus ovatus</i>	> 123	FJ432025		
		<i>Halicarcinus varius</i> *	294	FJ432026, FJ432027		
		<i>Hymenosoma depressum</i> *	> 39	FJ432028		
		<i>Hymenosoma geometricum</i>	596	FJ432029		
		<i>Hymenosoma hodgkini</i>	> 834	FJ432030, FJ432031		
		<i>Hymenosoma orbiculare</i>	> 287	FJ432032		
		<i>Hymenosoma sp. 1</i>	503	FJ432033, FJ432034		
		<i>Hymenosoma sp. 2</i>	322	FJ432035, FJ432036		
		<i>Hymenosoma sp. 3</i>	> 38	FJ432037		
		<i>Neohymenicus pubescens</i> *	> 82	FJ432038		
		<i>Neorhynchoplax bovis</i>	> 242	FJ432039		
		Mictyridae	<i>Mictyris longicarpus</i>	> 751	FJ432040, FJ432041	
			Pilumnidae	<i>Pilumnopeus serratifrons</i>	> 379	FJ432019
				Portunidae	<i>Portunus pelagicus</i>	> 379
<i>Scylla serrata</i>	> 789	FJ432043				
Caridea	Palaemonidae	<i>Macrobrachium sp.</i>	341	FJ432044, FJ432045		
Dendrobranchiata	Penaeidae	<i>Metapenaeus bennettiae</i>	> 630	FJ432046		
		<i>Metapenaeus macleayi</i>	> 53	FJ432047		
		<i>Penaeus longistylus</i>	> 134	FJ432048		
		<i>Penaeus merguensis</i>	> 140	FJ432049		
Eryonoidea	Polychelidae	<i>Pentacheles laevis</i> †	> 149	FJ432050		
		<i>Polycheles enthrix</i> †	> 163	FJ432051		
		<i>Polycheles sculptus</i> †	> 175	FJ432052		
Thalassinoidea	Callianassidae	<i>Biffarius sp.</i>	> 387	FJ432053-55		

Samples not collected by the first author were provided by *Colin McLay (University of Canterbury, New Zealand) and †Stephen Keable (Australian Museum, Sydney). In some cases, the complete length of the introns could not be determined, either because the 3' end of the first exon region was not identifiable in the trace files, or because the individuals sequenced were heterozygotes whose two copies of the introns differed in length. Minimum intron lengths are therefore indicated in most cases.

were purified using the UltraClean™15 DNA Purification Kit (MO BIO Laboratories, Inc.), sequenced in both directions using BigDye terminator version 3.1 (Applied Biosystems) and run on a 3130xl Genetic Analyser (Applied Biosystems) according to the manufacturer's instructions.

Introns were found in all but one species (Table 1). The primer annealing site of the forward primer is located close to the intron, and most trace files therefore did not contain readable exon sequences at their 5' ends. The second exon region, on the other hand, was readily recognizable in all sequences. In most cases, the introns of different species were too divergent to be alignable, even among members of the same families. We explored their variation in three

species pairs in which alignment was possible: (i) *Halicarcinus cooki* vs. *H. varius*, (ii) *Hymenosoma geometricum* vs. *Hymenosoma sp. 1*, and (iii) *Ibacus peronii* vs. *Thenus orientalis*. Intron sequences of these species pairs differed by 5%, 7% and 24%, respectively. This amount of differentiation is similar to that of the mitochondrial COI gene, which was 5%, 15% and 22%, respectively. As the species status of the two *Hymenosoma* species was only recently discovered using COI sequences and morphological data (Edkins *et al.* 2007), these results indicate that the intron may be useful to identify cryptic speciation. We also found genetic variation within a single species, namely among two representatives of the crab *Hymenosoma hodgkini* that had been collected at the same

locality, whose intron sequences differed by 1% (nine nucleotide differences) and also contained two large indels that could be coded as additional characters. The amplified portion of the second exon is sufficiently long (281 bp) to be useful to study decapods at higher taxonomic levels. A phylogeny reconstructed using sequences of crabs from the family Hymenosomatidae was approximately as resolved as one constructed from sequences of the mitochondrial 12S rRNA (Teske *et al.* submitted).

In summary, the portion of the ANT gene that amplifies using the primers presented here provides signal at various taxonomic levels. Therefore, these primers could be useful for phylogenetic, phylogeographical and, in cases where long introns are present, population genetic studies of decapod crustaceans.

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