

TECHNICAL NOTE

Group-specific primers for amplifying DNA sequences that identify Amphipoda, Cephalopoda, Echinodermata, Gastropoda, Isopoda, Ostracoda and Thoracica

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Abstract

We describe seven group-specific primer pairs that amplify small sections of ribosomal RNA genes suitable for identification of animal groups of major importance as prey items in marine ecosystems. These primer sets allow the isolation of DNA from the target animal groups from mixed pools of DNA, where DNA-based identification using universal primers is unlikely to succeed. The primers are designed for identifying prey in animal diets, but could be used in any situation where these animal groups are to be identified by their DNA.

Keywords: diet, diversity, identification, mixed, taxonomy

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DNA sequences can be very useful tools for making taxonomic identifications of biological materials. For macroscopic life, universal polymerase chain reaction (PCR) primers are generally applied to a pure source of tissue to amplify the DNA region that can be compared to sequences of known provenance. This approach has gained recent widespread acceptance and is being implemented with 'DNA barcoding' projects (Schander & Willassen 2005). However, approaches based on universal PCR primers will often not work when the PCR has to be applied to a mixed source of DNA, as in studies of microbial communities (Harmsen *et al.* 1997) or animal diets (Jarman *et al.* 2004; Deagle *et al.* 2005). Universal primers may fail to amplify all target sequences in these situations because early round of the PCR is dominated by the more common sequences and rarer sequences fail to amplify. Even if the target sequence is amplified, it then needs to be isolated from the pool of all amplified sequences. This generally involves cloning the PCR product and sequencing a number of clones proportional to the diversity of sequences in the library. Group-specific primers allow the diversity of sequences in the product of a PCR to be reduced to more manageable levels (Jarman *et al.* 2004). The primer sets described here were designed for identifying prey items in

the pool of DNA that can be purified from the gut contents or faeces of predators.

Primer sets were designed by making multiple sequence alignments of nuclear and mitochondrial rDNA regions because they often have appropriate characteristics for producing group-specific PCR primers (Jarman *et al.* 2004). Sequences from the target group and from closely related groups were obtained from GenBank and aligned with CLUSTAL_X software (Thompson *et al.* 1997). PCR primers were then designed with assistance from AMPLICON software (Jarman 2004). Promising looking primers were checked against all sequences in GenBank for the target group to ensure that they all contained good binding sites using a search for short, nearly exact matches (McGinnis & Madden 2004). The primers were compared against all sequences in GenBank to check that nontarget species did not contain effective primer binding sites.

Optimal annealing temperatures for each primer set were determined empirically by temperature gradient PCR on an MJ Research PTC200 with Chromo4 real time PCR system on several representative species from the target group. The components of these 20 µL PCRs were 1 × PCR buffer, 2.5 mM MgCl₂, 100 µg/mL bovine serum albumin, 2 mM of each dNTP, 1 µM of each primer, 1 U Taq DNA polymerase (Sigma), 1 × SyBr Green (Molecular Probes) and c. 20 ng template DNA. PCR thermal cycling conditions were: 2 min at 95 °C; 35 cycles of 5 s at 95 °C,

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Table 1 Group specific primers for amplifying rRNA genes from seven groups of marine animals

Target taxon	Primer names	Primer sequences 5'–3'	Annealing temperature	Product size	Taxonomic resolution
Amphipoda	AmphNSSf1	CTGCGGTAAAGGCTCGTAGTTGAA	51 °C	Range = 204–375 \bar{x} = 310.0, s^2 = 44.37, n = 15	Species/genus
	AmphNSSr1	ACTGCTTTTRAGCACTCTGATTTAC			
Cephalopoda	CephMLSf1	TGCGGTATTWTAACGTACT	52 °C	Range = 212–244 \bar{x} = 235.3, s^2 = 10.73, n = 18	Genus
	CephMLSr1	TTATTCCTTRATCACCC			
Echinodermata	EchinNSSf1	GCGTGCCTTTTATTAGGA	51 °C	Range = 157–163 \bar{x} = 159.3, s^2 = 1.36, n = 18	Species/genus
	EchinNSSr1	CGACCATGRTARGCGCATAACG			
Gastropoda	GastNLSf1	GCGGYAACGCAACGAAGT	52 °C	Range = 188–198 \bar{x} = 192.7, s^2 = 3.43, n = 10	Genus
	GastNLSr1	CGAAAWTMACACCGTCTCCG			
Isopoda	IsopodNSSf1	TCATGATTYATGGGATGT	51 °C	Range = 201–278 \bar{x} = 242.8, s^2 = 20.41, n = 15	Species/genus
	IsopodNSSr1	AAGACCTCAGCGCTCGGC			
Ostracoda	OstracodMSSf1	GTGACAAGAAGACCTARGAG	46 °C	Range = 247–252 \bar{x} = 249.2, s^2 = 1.40, n = 11	Species
	OstracodMSSr1	AATCCAACATCGAGGTCA			
Thoracica	ThorMSSf1	TTTAGAGGAACCTGCC	53 °C	Range = 134–139 \bar{x} = 136.5, s^2 = 1.18, n = 17	Genus/family
	ThorMSSr1	AGCTGCACCTTTAYTTGAAG			

The primer names are based on the target taxon, the rDNA gene being amplified (N for nuclear rDNAs, M for mitochondrial rDNAs, LS for large subunit, SS for small subunit, and f for the forward primer and r for the reverse primer). The optimal annealing temperature was determined by temperature gradient PCR on pure DNA from the target group. The size range in bp of the expected PCR products from n representatives of the target taxon in GenBank and the mean (\bar{x}) and its standard deviation (s^2) are given.

30 s at annealing temperature, 30 s at 72 °C; and finally 10 min at 72 °C. Approximate levels of taxonomic informativeness were estimated by creating a neighbour-joining-tree from the aligned sequences to determine whether there was enough information in the region to separate different taxonomic levels. Details of the primer sets are given in Table 1.

Once an appropriate annealing temperature was determined, single-temperature PCR was carried out on a range of target group species that were reasonably representative of the phylogenetic diversity of the target group. Nontarget species closely related to the target group were also tested to check the group-specificity of the primers and to check that the primers did not amplify the DNA of predator groups of interest to us such as mammals, birds, fish and crayfish. The DNAs were also tested with universal primers ('NSF1419/20' ATAACAGGTCTGTGATGCCC, 'NSR1642/16' GACGGGCGGTGTGTRC) for an $c.$ 200–240 bp region of nuclear SSU rDNA gene to ensure that the DNA extract could act as a PCR template. PCR products were separated on 1.5% Tris–borate–EDTA, 0.5 × GelStar (Cambrex) gels run at 5 V/cm and visualized by fluorescence > 500 nm of GelStar-associated DNA excited by 400 nm transillumination. This checked that products of a similar size to that predicted from alignment of sequences from GenBank were being amplified. The results of these tests are given in Table 2.

The primers amplified DNA from a selection of the target group species that was as phylogenetically diverse as we could arrange. The primers did not amplify from a

selection of nontarget species, including several that prey on the targets of the primer sets. This suggests that they will be useful for isolating DNA of the target group from mixed pools of DNA, making them suitable for prey species identification in dietary samples. We have successfully applied two of these primer sets to identification of echinoderm and gastropod prey species of the southern rock lobster *Jasus edwardsii*; and two more sets to detecting amphipod and ostracod prey of the myctophid fish *Krefflichthys andersoni*.

The primers could also be useful for identification of larval stages of these groups, many of which are unidentifiable by morphological analysis. The DNA regions targeted are short in all cases, so the primer sets are useful for ancient DNA studies, where DNA is degraded into short fragments. The primers would be especially useful in situations where the ancient DNA is from a mixed source, such as in studies of organisms preserved in marine sediments.

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Table 2 Success or failure of eight PCR primer pairs in amplifying DNA from a range of animals

Phylum	Class	Order	Family	Species	Amph- NSS	Ceph- MLS	Echin- NSS	Gastr- NLS	Isopod- NSS	Ostracod- MSS	Thor- MSS	Universal
Crustacea	Branchiopoda	Anostraca	Artemiidae	<i>Artemia salina</i>	-	-	-	-	-	-	-	+
Crustacea	Cirripedia	<u>Thoracica</u>	Balanidae	<i>Elminius modestus</i>	-	-	-	-	-	-	+	+
Crustacea	Cirripedia	<u>Thoracica</u>	Cthamalidae	<i>Cthamalus antematus</i>	-	nt	-	nt	-	-	+	+
Crustacea	Cirripedia	<u>Thoracica</u>	Cthamalidae	<i>Chamaesipho tasmanica</i>	-	nt	-	nt	-	-	+	+
Crustacea	Copepoda	Calanoida	Euchaetidae	<i>Euchaeta antarctica</i>	-	-	-	-	-	-	-	+
Crustacea	Malacostraca	Decapoda	Palinuridae	<i>Jasus edwardsii</i>	-	nt	-	-	-	nt	-	+
Crustacea	Malacostraca	Euphausiacea	Euphausiidae	<i>Euphausia superba</i>	-	-	-	-	-	-	-	+
Crustacea	Malacostraca	<u>Amphipoda</u>	Hyperiididae	<i>Themisto gaudichaudii</i>	+	-	-	-	-	-	-	+
Crustacea	Malacostraca	<u>Amphipoda</u>	Caprellidae	<i>Caprella</i> sp.	+	-	-	-	-	-	-	+
Crustacea	Malacostraca	<u>Amphipoda</u>	Gammaridae	Unidentified sp. 1	+	-	-	-	-	-	-	+
Crustacea	Malacostraca	<u>Amphipoda</u>	Gammaridae	Unidentified sp. 2	+	-	nt	nt	-	-	nt	+
Crustacea	Malacostraca	<u>Amphipoda</u>	Gammaridae	Unidentified sp. 3	+	-	nt	nt	-	-	nt	+
Crustacea	Malacostraca	Cumacea	Bodotriidae	<i>Cyclapsis caprella</i>	-	-	-	-	-	-	-	+
Crustacea	Malacostraca	Tanaidacea	Paratanaidae	<i>Paratanais</i> sp.	-	-	-	-	-	-	-	+
Crustacea	Malacostraca	Mysidacea	Mysidae	<i>Tenagomysis australis</i>	-	-	-	-	-	-	-	+
Crustacea	Malacostraca	<u>Isopoda</u>	Ligidae	<i>Ligia australis</i>	-	-	-	-	+	-	-	+
Crustacea	Malacostraca	<u>Isopoda</u>	Phraeroicidae	<i>Colubotelson thomsoni</i>	-	-	-	-	+	-	-	+
Crustacea	Malacostraca	<u>Isopoda</u>	Armadillidiidae	<i>Armadillidium vulgare</i>	-	-	-	-	+	-	-	+
Crustacea	Malacostraca	<u>Isopoda</u>	Anthuridae	Unidentified sp.	-	-	-	-	+	-	-	+
Crustacea	<u>Ostracoda</u>	Halocyprida	Cypridae	<i>Gigantocypris muelleri</i>	-	-	-	-	-	+	-	+
Crustacea	<u>Ostracoda</u>	Halocyprida	Cypridae	Unidentified sp.	-	-	-	-	-	+	-	+
Mollusca	<u>Cephalopoda</u>	Teuthida	Loliginidae	<i>Lolius noctiluca</i>	nt	+	-	-	-	-	-	+
Mollusca	<u>Cephalopoda</u>	Teuthida	Onychoteuthidae	<i>Moroteuthis ingens</i>	-	+	-	-	-	-	-	+
Mollusca	<u>Cephalopoda</u>	Teuthida	Omnastrepidae	<i>Nototodarus gouldii</i>	nt	+	-	-	-	-	-	+
Mollusca	<u>Cephalopoda</u>	Octopoda	Incirrata	<i>Octopus</i> sp.	nt	+	-	-	-	-	-	+
Mollusca	Polyplocophora	Neoloricata	Chitonidae	<i>Chiton pelliserpentis</i>	-	-	-	-	-	-	-	+
Mollusca	<u>Gastropoda</u>	Stylomatophora	unidentified	Unidentified slug	-	-	-	+	-	-	-	+
Mollusca	<u>Gastropoda</u>	Archaeogastropoda	Haliotidae	<i>Haliotis rubra</i>	-	-	-	+	-	-	-	+
Mollusca	<u>Gastropoda</u>	Archaeogastropoda	Turbinidae	<i>Turbo</i> sp.	-	-	-	+	-	-	-	+
Mollusca	<u>Gastropoda</u>	Archaeogastropoda	Turbinidae	<i>Astralium</i> sp.	-	-	-	+	-	nt	-	+
Mollusca	Gastropoda	Pulmonata	Siphonariidae	<i>Siphonaria tasmanica</i>	-	-	-	+	-	nt	-	+
<u>Echinodermata</u>	Asteroidea	Forcipulatida	Asteriidae	<i>Asterias amurensis</i>	-	-	+	-	-	-	-	+
<u>Echinodermata</u>	Asteroidea	Spinulosida	Asterinidae	<i>Patriella parvivivipara</i>	-	-	+	-	-	-	-	+
<u>Echinodermata</u>	Echinoidea	Echinoidea	Echinometridae	<i>Heliocidaris erythrogramma</i>	-	-	+	-	-	-	-	+
<u>Echinodermata</u>	Echinoidea	Diadematoida	Diadematidae	<i>Centrostephanus rogersii</i>	-	-	+	-	nt	nt	nt	+
<u>Echinodermata</u>	Holothuroidea	Aspidochirotida	Stichopodidae	<i>Stichopus nollis</i>	nt	-	+	-	nt	nt	nt	+
<u>Echinodermata</u>	Crinoidea	Articulata	Comasteridae	<i>Cenolia trichoptra</i>	nt	-	+	-	nt	nt	nt	+
Chordata	Aves	Sphenisciformes	Spheniscidae	<i>Aptenodytes patagonica</i>	-	-	-	-	-	nt	nt	+
Chordata	Mammalia	Carnivora	Otariidae	<i>Arctocephalus gazella</i>	-	-	-	-	-	nt	nt	+
Chordata	Mammalia	Cetacea	Physeteridae	<i>Physeter macrocephalus</i>	-	-	-	+	-	nt	nt	+
Chordata	Actinopterygii	Perciformes	Nototheniidae	<i>Dissostichus eleginoides</i>	-	-	-	-	-	-	-	+
Chordata	Actinopterygii	Myctophiformes	Myctophidae	<i>Kreffichthys andersoni</i>	-	-	-	-	-	-	-	+
Cnidaria	Anthozoa	Actinaria	Actiniidae	<i>Actina tenebrosa</i>	-	-	-	-	-	-	-	+

A taxonomy of the tested DNAs is given with groups underlined that correspond to the seven targets of the group-specific primer sets in Table 1. A universal primer set was also used as a positive control for DNA quality. Results of these tests are shown as positive (+), negative (-), or untested (nt).

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