Sodmergen K, Kawano S, Tano S, Kuroiwa T (1991) Degradation of chloroplast DNA in second leaves of rice (*Oryza sativa*) before leaf yellowing. *Protoplasma*, **160**, 89–98.

Zilberstein A, Koch T, Altschuler Y, Lers A, Zamir A (1987) Characterization of DNase (s) activity in tobacco leaf extracts. *Plant Science*, **52**, 57–65.

An ultrasensitive method for detection of single crab larvae (*Sesarma reticulatum*) by PCR amplification of a highly repetitive DNA sequence

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For studies of planktonic organisms, even the basic question of whether a particular species is represented in a plankton sample may be difficult to answer. Traditional methods of morphological identification by microscopy are labour intensive and require species-specific diagnostic characters (Silberman & Walsh 1992; Fuhrman *et al.* 1994; Medeiros-Bergen *et al.* 1995). These characters may be absent, highly variable, or obscured by specimen damage during collection (Smith 1977; Medeiros-Bergen *et al.* 1995). Studies of planktonic communities would thus benefit from methods of identification that do not depend on morphology. Here we report a novel approach for the detection of larval *Sesarma reticulatum* (marsh crab) that is based on the presence of a characteristic repetitive sequence element. This sequence element is $\approx$ 600 bp in length, represented by about 5×10^5 copies per haploid genome, and bears no significant similarity to sequences in the GenBank database (release 108). Because of its high copy number, it can be detected in samples that contain less DNA from *S. reticulatum* than is present in a single cell (Rheinsmith *et al.* 1974).

A small genomic library for *S. reticulatum* was created in the plasmid vector pUC18. A large percentage of the sequences in this library exhibited similar restriction site maps and cross-hybridized on Southern blots (methods described in Neigel *et al.* 1991). Partial sequence analysis of several of these plasmids confirmed that they contained overlapping portions of a repetitive sequence, which we designated Sr16.

Using the computer program NAR (Rychlik & Rhoads 1989), we designed a pair of PCR primers to amplify a 294-bp region of Sr16, hereafter designated as Sr16-PCR. Primers were synthesized by BioSynthesis, Inc. (Lewisville, Texas) and are: SR16F-F1, 5'-ATCGAAGAATAAGAGGCGACT-3'; SR16F-R1, 5'-ACGTAATTAACCGAGCATTGA-3'.

Amplification conditions were optimized for these primers, and used for all subsequent experiments. The optimized amplification profile was 35 cycles of 1 min at 94 °C, 1 min at 50 °C, ramp to 72 °C for 2 min, and 1.5 min at 72 °C. Each 25 μL reaction included 10 pM of each primer and final concentrations of 2 mM of each dNTP; 2.5 mM $MgCl_2$; 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin; 1.25 U *Taq* polymerase (Boehringer Mannheim); and template DNA (varying concentrations). Reaction mixtures were covered with a layer of sterile mineral oil to reduce evaporation.

Genomic DNA was extracted from muscle tissue of adult crabs by phenol/chloroform extractions and from larval crustaceans with 'protocol A for larval extraction' and 'protocol B – alcoholic tissues' (Palumbi *et al.* 1991). For extractions of individual larval *S. reticulatum*, 25 μL of naupliar larval *Artemia* sp. (Ocean Star International, Inc.) was added as a 'carrier' to provide a visible quantity of material. Each PCR experiment consisted of three replicates and a negative control that lacked template DNA. Amplification products were electrophoresed through 2% agarose/Tris-borate EDTA gels alongside a DNA size standard and then visualized by staining with 25 μg/mL ethidium bromide and UV transillumination (Maniatis *et al.* 1982). A reaction was scored as positive if a product of the same mobility as Sr16-PCR was observed.

PCR theoretically can detect a single DNA molecule; in combination with this extreme sensitivity the high copy number of Sr16 provided the basis for an ultrasensitive method for detection of genomic DNA from *S. reticulatum* (SrDNA). Sr16-PCR was consistently amplified from quantities of SrDNA that spanned five orders of magnitude (Table 1), including quantities below the haploid genome size of 4.1 pg measured for the closely related species *Armases cinereum* (see Rheinsmith *et al.* 1974).

Amplifications of Sr16-PCR were also successful when SrDNA was mixed with DNA from other species. In mixtures with DNA from larval *Artemia* sp., it was possible to amplify Sr16-PCR from as little as 1 pg of SrDNA, although the concentration of DNA from *Artemia* was 10 000-fold higher (Table 1). This sensitivity provided consistent detection of DNA from single larval *S. reticulatum* that were coextracted with 25 μL of *Artemia* (about 500 nauplii). Amplifications were also successful in the presence of DNA extracted from a

Table 1 Detection of genomic DNA from *Sesarma reticulatum* mixed with DNA from *Artemia*. Numbers in parentheses are the mass ratios of DNA from *S. reticulatum* to DNA from *Artemia*

	Artemia	
Sesarma	1 ng	10 ng
10 ng	+ (10:1)	+ (1:1)
1 ng	+ (1:1)	+ (1:10)
0.01 ng	NA	+ (1:1000)
0.001 ng	NA	+ (1:10 000)

+ indicates successful amplification in all three replicates; NA indicates amplification was not attempted.

brachyuran crab (*Menippe*) and a sample of freshwater plankton (Cypress Lake, LA). Sr16-PCR was not amplified (at detectable levels) in controls with DNA from these other species alone.

For accurate identification, specificity of the PCR amplification as well as sensitivity is essential. Species with sequences similar to Sr16 could generate 'false positives' if PCR products similar in size to Sr16-PCR were amplified. However, although many other species could potentially occur with *S. reticulatum* in plankton samples, similar sequences would most probably occur in related species. We therefore tested six other species from the family Grapsidae, including species closely related to *S. reticulatum*. None generated false positives (Table 2).

Sensitive detection of SrDNA was not dependent on the method of sample preservation. Amplifications were successful with larvae frozen immediately after collection or preserved in either glycerol or 95% ethanol. Prior to extraction, glycerol was removed by four rinses with TE (20 mM Tris, pH 7.5, 100 mM EDTA).

Repetitive sequences such as Sr16 are seldom used as taxonomic characters because they are subject to unusual modes of evolution (Arnheim 1983), and homologous sequences are often restricted to closely related species (Felger & Hunt 1993). However, the variability of repetitive sequences could make them ideal species tags. They are easier to detect than low-copy sequences and, if characteristic of a particular species, their presence alone is sufficient to identify that species. Much greater effort and expense is required to characterize nuclear or mitochondrial sequences when used for species identification. We expect that the approach described here can be applied to other species and will be useful for quantification as well as detection of planktonic organisms.

Table 2 Species of grapsid crabs tested for false-positive amplification of a PCR with primers for Sr16-PCR

Species	Product	Relationship
Sesarma reticulatum (+ control)	+	Self
Sesarma cuacaoense	–	Congener
Sesarma fossarum	–	Congener
Armases cinereum	–	Same subfamily
Cyclograpsus integer	–	Same subfamily
Hemigrapsus oregonensis	–	Different subfamily
Pachygrapsus transversus	–	Different subfamily

+ indicates that a product of the expected size was amplified;
– indicates that no discrete product was amplified.

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References

Arnheim N (1983) Concerted evolution of multigene families. In: *Evolution of Genes and Proteins* (eds Nei M, Koehn RK), pp. 38–61. Sinauer Associates, Sunderland, MA.

Felger I, Hunt JA (1993) Detection and characterization of transposable elements. *Methods in Enzymology*, **224**, 322–334.

Fuhrman JA, Lee SH, Masuchi Y, Davis AA, Wilcox RM (1994) Characterization of marine prokaryotic communities via DNA and RNA. *Microbiology and Ecology*, **28**, 133–145.

Maniatis T, Gritsch EF, Sambrook J (1982) *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.

Medeiros-Bergen DE, Olsen RR, Conroy JA, Kocher TD (1995) Distribution of holothurian larvae determined with species-specific genetic probes. *Limnology and Oceanography*, **40**, 1225–1235.

Neigel JE, Felder DL, Chlan CA, La Porte R (1991) Cloning and screening of DNA probes for genetic studies in stone crabs (Decapoda: Xanthidae: *Menippe*). *Journal of Crustacean Biology*, **11**, 496–505.

Palumbi SR, Martin A, Romano S, McMillan WO, Stice L, Grabowski G (1991) *The Simple Fool's Guide to PCR*. University of Hawaii, Honolulu.

Rheinsmith EL, Hinegardner R, Bachman K (1974) Nuclear DNA amounts in Crustacea. *Comparative Biochemical Physiology*, **48B**, 343–348.

Rychlik W, Rhoads RE (1989) A computer program for choosing optimal oligonucleotides for filter hybidization, sequencing and *in vitro* amplification of DNA. *Nucleic Acids Research*, **17**, 8543–8551.

Silberman JD, Walsh PJ (1992) Species identification of spiny lobster phyllosome larvae via ribosomal DNA analysis. *Molecular Marine Biology and Biotechnology*, **1**, 195–205.

Smith DL (1977) *Marine Coastal Plankton and Marine Invertebrate Larvae*. Kendal/Hunt Publishing, Dubuque, Iowa.