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Species identification of bivalve larvae using random amplified polymorphic DNA (RAPD): differentiation between *Cerastoderma edule* and *C. lamarcki*

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The polymerase chain reaction (PCR) was used to produce species-specific DNA markers (RAPDs) from two sibling cockle species and five other co-occurring intertidal bivalves. Amplification reactions with one single primer readily distinguished larvae and adults of *Cerastoderma edule* from larvae and adults of *C. lamarcki*, and from adults of *Mya arenaria*, *Macoma balthica*, *Scrobicularia plana*, *Venerupis pulastra* and *Mytilus edulis*. Random amplified polymorphic DNA (RAPD) is suggested as a simple and quick method to determine species identity in taxa that are difficult to identify on the basis of morphological characters alone, such as marine bivalve larvae.

Many benthic invertebrates disperse via a planktonic larval phase and the resultant variability in recruitment is potentially important for the dynamics and structure of benthic populations and communities (Caley et al., 1996). Ecological studies of early life stages in marine benthic organisms have, however, hitherto been hampered by the inability to identify larvae and juveniles to species level (Levin, 1990). Their small size, usually less than 500 μm, makes it difficult or impossible to discriminate between closely related taxa based on morphological characters, and a variety of molecular techniques have been developed instead. The polymerase chain reaction (PCR) for DNA amplification, which requires only nanogram amounts of total genomic DNA, has been used in several ways for the species identification of larvae. For example, specific genes have been amplified, sequenced and species-specific probes generated to identify individual larvae (Medeiros-Bergen et al., 1995; Heath et al., 1996). Alternatively, amplification products have been digested with restriction enzymes (Silberman & Walsh, 1992). Both these techniques require previous knowledge about the genome of interest, and the development of specific primers can be time consuming and costly. In this study the PCR based method random amplified polymorphic DNA (RAPD) was used to distinguish between larvae for two congeneric bivalve species. Random amplified polymorphic DNA has been applied to various questions in ecology (reviews in Burton, 1996; Grosberg et al., 1996), including species identification (Wilkerson et al., 1993; Coffroth & Mulawka, 1995).

The bivalves *Cerastoderma edule* (Linnaeus, 1785) and *Cerastoderma lamarcki* (Reeve, 1845) are two morphologically similar species which are common in soft sediment, intertidal habitats along the European coast (Lindegarth et al., 1995). Both species have external fertilization and a planktotrophic larval stage which lasts for 2–3 weeks. High rates of dispersal have been inferred from allozyme studies (Hummel et al., 1994). *Cerastoderma edule* is often associated with sandy sediments, whereas *C. lamarcki* is mostly found in more sheltered locations with silty sediments. However, the two species are sometimes found in sympatric populations (Brock, 1979; C.A., personal observations). The mechanisms behind the separation in habitats are not clear. To investigate the relative importance of recruitment processes such as larval supply, habitat selection and juvenile mortality it is critical to identify larvae and early juveniles of the two species in routine samples. This is presently not possible using morphological characters.

Here, a simple technique to distinguish between the larvae of the two species *Cerastoderma edule* and *C. lamarcki*, using both adults and artificially reared larvae of each of the two species is described. Ten-mer random oligonucleotide primers (Operon Technologies, USA) were screened for diagnostic banding patterns using PCR procedures. Thirty-one primers were tested in the search for a single primer that yielded consistent, clear interspecific variation without showing any intraspecific variation. To reduce the risk of sampling from one genetically distinct subpopulation, the adults used in the initial screening were sampled from two localities in Sweden, Lysekil and Strömstad, 80 km apart. When one diagnostic primer was found it was necessary to show that the banding patterns produced by *C. edule* or *C. lamarcki* was not also produced by other species. To investigate this possibility we sampled five adult individuals from five other common co-existing intertidal species: *Mya arenaria*, *Macoma baltica*, *Scrobicularia plana*, *Venerupis pullastra* and *Mytilus edulis*. Ideally, there should be absolute correspondence of the RAPD banding patterns between larvae and adults of a certain species. It is, however, possible that variability in for example DNA concentration may produce different banding patterns between adults and larvae. To test this possibility and to develop a protocol for extraction of total DNA from larvae we analysed laboratory reared larvae from both *C. edule* and *C. lamarcki*. Adult cockles were collected in early June 1995, determined by morphology to species (cf. Brock, 1978) and induced to spawn (cf. André et al., 1993). For each species at least three different pairs of males and females were crossed. During larval development, between 12 and 28 d after fertilization, and at sizes ranging from 200 to 320 μm, the cultures were harvested repeatedly. The larvae were then starved for 12–24 h to void their stomachs prior to storage.

Larvae were stored in Tris-Borate buffer (TBE) pH 8.0 and individually frozen at −74°C. Initial trials with larvae

Table 1. *Primer screening for diagnostic banding patterns in* Cerastoderma edule *and* C. lamarcki *using random 10-mer Operon primers. Thirty-one primers were tested with 5–10 individuals from each species. Primer OP-B16 were selected for further analyses.*

	Cerastoderma edule		*Cerastoderma lamarcki*	
Primer (OP-#)	PCR products	Diagnostic markers	PCR products	Diagnostic markers
A01	s	n	s	y
A02	v	y	s	y
A06	s	n	s	y
A12	s	y	v	n
A13	s	n	s	y
A14	s	y	w	n
A15	s	y	v	n
B02	s	y	s	y
B03	s	y	s	y
B05	s	y	s	y
B08	s	n	s	n
B11	s	n	s	n
B12	s	y	s	y
B13	w	n	s	y
B15	w	n	s	y
B16*	**s**	**y**	**s**	**y**
B18	s	n	s	n
B20	s	y	s	y
D02	s	n	s	y
D03	s	y	s	y
D07	w	n	w	n
D08	s	n	s	y
D09	w	n	w	n
D12	w	n	w	n
D14	w	n	w	n
D15	w	n	s	y
D16	v	n	w	y
D17	w	n	w	n
D18	s	y	s	y
D19	s	n	s	n
D20	s	y	s	y

s, strong; w, weak; v, variable; y, yes; n, no; *, primer sequence (5′3′)=TTTGCCCGGA.

preserved in 95% ethanol yielded variable results, with only ~50% of the individuals showing consistent bands. DNA from single larvae was extracted in separate 0.2 ml tubes containing 10 μl Chelex 100 (Sigma Chemical Co.) and 1 μl of 2.0 mg ml^{-1} Proteinase K. After 2 h at 56°C the tubes were heated at 94°C for 5 min. The lysates were then vortexed and spun at 8000 rpm for 2 min before the supernatant (~9.5 μl) were transferred to new tubes. The resultant DNA concentration from individual larval extractions was often <1 ng μl^{-1}. In adults, DNA was extracted from ~1 mg of muscle tissue from either fresh individuals, individuals preserved in 95% ethanol or adults individually frozen at −74°C. The tissue was placed in 1.5 ml tubes with 50 μl Chelex 100 and 1 μl of 2.0 mg ml^{-1} Proteinase K. The tissue was incubated at 56°C for 2 h, with another addition of 1 μl Proteinase K after 1 h. Finally, to denature Proteinase K the tubes were heated at 94°C for 5 min. The lysate was then vortexed and spun at 8000 rpm for 2 min before the supernatant was transferred to new tubes. Prior to amplification the extracted DNA was diluted to 10 ng μl^{-1}. The PCR reactions for both adults and larvae were carried out in reaction volumes of 25 μl. Each reaction cocktail contained: 4.0 μl 25 mM $MgCl_2$; 2.0 μl 10×Stoffel buffer; 10.4 μl 2 mM (0.5 mM each) GeneAmp® dNTPs (Perkin Elmer); 0.5 μl (5×10^{-3} mg μl^{-1}) bovine serum albumin; 5.0 μl 6.0 μM 10-mer primer; 0.2 μl (2.0 units) AmpliTaq®DNA polymerase (Stoffel fragment, Perkin Elmer); and 3 μl template DNA (larvae) or 2 μl template DNA+1 μl H_2O (adults). The PCR was run on a PTC-100™ thermal controller with initial 2 min at 94°C followed by 40 cycles of 94°C (1 min), 36°C (1 min) and 72°C (2 min). DNA fragments were separated on 1.5% agarose gels (MetaPhor™ FMC Bioproducts, Denmark) containing 0.10–0.15 μl of 0.5 μg ml^{-1} ethidium bromide solution per millilitre of gel; the gels were run in 1×TBE at 3.3 V cm^{-1}. The gels were documented with a 35 mm camera using black & white film and yellow filter (Wratten no. 2 and no. 22). The photographs were then digitized and analysed with NCSA Gelreader v. 2.0.

Most primers yielded amplification products for both species and some of these primers produced diagnostic bands (Table 1). In several cases the number of bands produced was so high that the diagnostic bands could not be identified without ambiguity. One primer, OP-B16, however, gave simple diagnostic banding patterns for both species (Figure 1A). In *C. edule* B16 yielded one strong band of 815 and one of 986 bp (Figure 1A). Occasionally, other small fragments (200–600 bp) appeared, but *C. edule* was unambiguously identified by these two larger markers. *Cerastoderma lamarcki* is characterized by a single fragment of 280 bp (Figure 1A). The banding patterns exhibited by reared larvae (Figure 1B) were similar to those produced by adults although estimations of fragment length varied slightly among gels. Though it is here safe to conclude that the diagnostic bands produced by adults are homologous to the bands produced by larvae it is important to note that in work with the identification of field-collected larvae or juveniles it is advisable to use known samples (adults) as references. This pattern has now been reproduced in amplifications of over 200 independent individuals, using four different PCR-machines in three different laboratories (C.A., unpublished data).

To be unambiguously used as markers, diagnostic bands must be present in all individuals of the taxon under consideration, but not in any individuals from other taxa. In bivalves, larvae are morphologically very similar, also among species that are distantly related (Lutz et al., 1982). It would therefore be desirable to screen every potential recruiting bivalve species in recruitment studies. This is not always possible since some species may at times be rare and difficult to obtain, and since it is impossible to anticipate which species may settle in an area and subsequently die before growing to a size where morphological identification is possible. Here we analysed the five bivalve species most commonly co-occurring with *C. edule* and *C. lamarcki* on the west coast of Sweden (C.A., personal observations), and none of these produced the banding patterns diagnostic for *C. edule* and *C. lamarcki* (Figure 1C).

The RAPD technique proved efficient to distinguish between larvae of *C. edule* and *C. lamarcki*. This opens new prospects for observational and experimental studies of the early life history of these two species, like for example recruitment processes. In this study we employed a very simple extraction protocol, using only Chelex and Proteinase. With extraction run in the afternoon, PCR overnight and electrophoreses the following morning, the analyses were completed in 24 h. For individual larvae, the total amount of extracted DNA was sufficient for four separate amplifications. Here, we used only one primer for the identification of larvae, and the remaining DNA could be used to run replicates or multiple primers.

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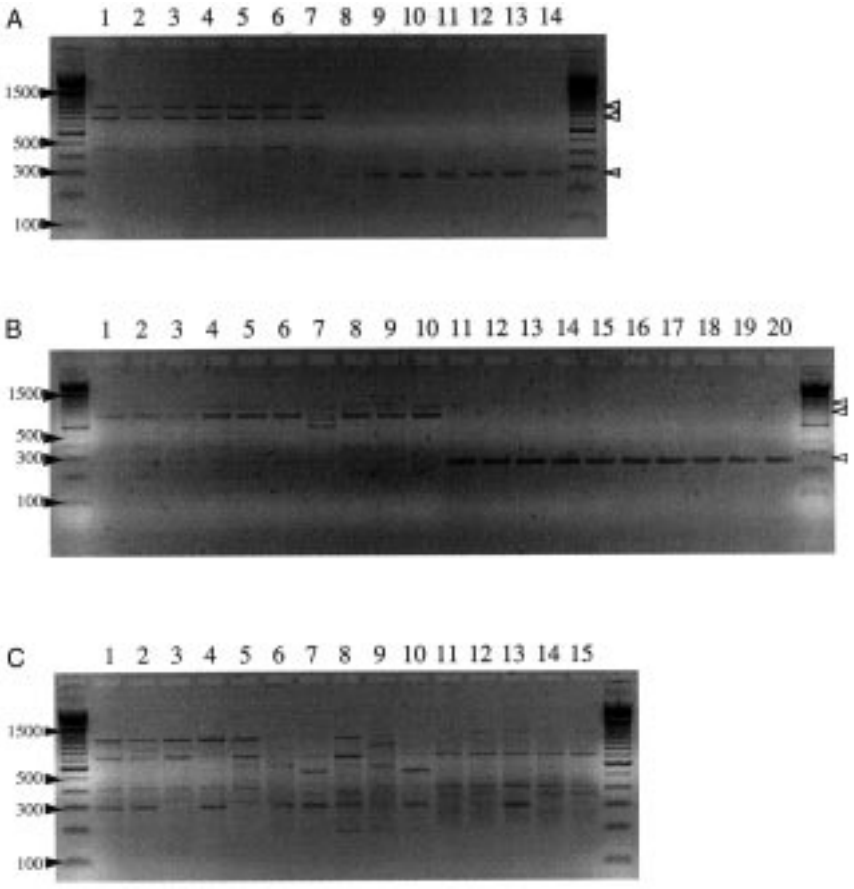

Figure 1. Random amplified polymorphic DNA markers generated with the primer OPB-16. Lanes flanking the RAPD-markers are size markers with 100 bp difference between consecutive bands. (A) Lanes 1–7 contain DNA from seven adult individuals of *Cerastoderma edule* and lanes 8–14 seven adult *C. lamarcki*. Arrows on the right hand side denote the two diagnostic bands of 815 ±8.9 bp (mean±$CI_{0.95}$) and 986 ±10.2 bp in *C. edule*, and the single band 280 ±0.44 bp in *C. lamarcki*. (B) Lanes 1–10 contain DNA from ten larvae of *Cerastoderma edule*, and lanes 11–20 ten larvae of *C. lamarcki*. Note that the two diagnostic bands of 773 ±3.8 bp (mean ±$CI_{0.95}$) and 969 ±11.1 bp in *C. edule*, and the single band of 264 ±1.4 bp in *C. lamarcki*, are similar to those produced by adults (A). (C) Lane 1–5 *Mya arenaria*, lane 6–10 *Macoma balthica* and lane 11–15 *Scrobicularia plana*. All these three species exhibited several bands, but none that were consistent with the diagnostic markers for *C. edule* and *C. lamarcki*. Only one individual of *Venerupis pullastra* yielded amplification products (423 and 367 bp), and in *Mytilus edulis* one individual showed bands of 250 and 325 bp, and one individual produced a single 250 bp fragment only.

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