

A Combined Approach of DNA Probe and RFLP for Family and Species Identification of Larval Stages of Commercially Important Aquatic Species: A Study on the Surfclam *Spisula solidissima*

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This paper deals briefly with a technique developed for the identification of the early stages of veligers of the surfclam *Spisula solidissima* from the larval stages of other common bivalve species using a combination of DNA probe and restriction fragment length polymorphism (RFLP) analysis. An oligonucleotide sequence designed from the 18S ribosomal RNA gene (nucleotide position 259-276) provided a sensitive probe for the Family Mactridae, to which *S. solidissima* belongs. DNA of unknown larvae picked from the plankton samples was extracted using Chelex-100. The 600-bp fragment of the 18S rRNA gene was amplified by polymerase chain reaction (PCR). PCR products and controls were tested with the mactrid-specific probe by dot-blot hybridization. The probe hybridized only with controls and larvae of *S. solidissima* and *Mulinia lateralis* (The only other member of Family Mactridae present in the study site). Higher percentage of larval amplifications were detected by dot-blot hybridization than by agarose gel electrophoresis indicating that colorimetric detection with a probe is more sensitive than ethidium bromide in the visualization of DNA. Restriction enzymes *Bts*UI and *Ava*I having different restriction sites were used in RFLP analysis to separate the larvae of *S. solidissima* from those of *M. lateralis*. It is suggested that a similar approach in fishery biology may be very effective and quick in identification of eggs and larval stages of commercially important species of shell and finfishes in studies pertaining to population dynamics, reproductive behaviour, larval recruitment, resource evaluation and management.

Problem

Identification of eggs and larval stages (particularly early larval stages) of finfish and shellfish (molluscs and crustaceans) using conventional methods invariably poses problem in studying the spawning behaviour and larval recruitment/settlement pattern which are important in understanding the population dynamics and resource potential of economically important species. Therefore, it is necessary to develop an effective and sensitive technique to identify the eggs and larval stages of the target species distributed in the planktonic realm. Development of species specific DNA probe is the most reliable solution to this problem.

An attempt was made in this direction by developing a family-specific DNA probe for distinguishing the members of the family Mactridae (To which the commercially important surfclam *S. solidissima* belongs) from members of other bivalve families co-existing in the plankton population (Bell, Grassle and Achuthankutty, 1997). To differentiate *S. solidissima* from *M. lateralis* (The only other member of the mactrid family occurring in the study site), RFLP analysis was done by using restriction enzymes which have different restriction sites for the two species. Thus the combined approach of DNA probe and RFLP analysis was found to be a reliable tool for identifying the mactrid family and the two species belonging to this family from members of other families occurring in the plankton samples.

Approach

Development of a family-specific DNA probe can be used in many geographical regions since species distribution may vary between regions.

18S ribosomal RNA (18S r RNA) gene is fairly conserved for bivalve families.

Complete sequence alignment of 18S rRNA gene for different families and species of bivalves are available.

Highly variable region between nucleotide position 209 and 294 could be used for species identification of bivalves.

Single nucleotide restriction sites may be ideal for species specificity within the family.

A combination of oligonucleotide probe hybridization with DNA amplified by PCR from individual larvae and subsequent digestion with restriction enzymes, tested positive for probe may provide accurate identification of family and species, respectively.

Probe Development

The variable region in nucleotide sequence of 18S rRNA was examined for different bivalve families. An insert unique to Family Mactridae (nucleotide position 259-276) was chosen as the probe region. An 18-mer probe (5'-AACGGCGGCCCGGCCCAA-3') complementary to the synonymous strand was designed to hybridize to the insert region. A probe (digoxigenin tailed) unique to this region distinguished larvae belonging to the Family Mactridae.

Probe and Larval Controls

A 600 bp fragment of 18S rRNA gene from 5' end (including the variable region) extracted from adult mantle tissue of *S. solidissima* was amplified along with those of *M. lateralis* and three known species of bivalves belonging to other families. The primers used were forward (Primer A: 5'-CTGGTTGAT-

CCTGCCAGT-3') and reverse (536R: 5'-G A/T ATTACCGCGGC G/T GCTG-3')- In 100 µl reactions, the reagent concentrations used were as follows. Each reaction contained, 1-2 µl of template DNA, 10 µl of 10x PCR buffer (Boehringer Mannheim, Germany), 0.2 mM dNTP, 0.2 (M of each primer, 40 µg of bovine serum albumin (BSA) and 1.0 U. *Taq* polymerase (Boehringer Mannheim, Germany). A Perkin-Elmer (Irvine, California) DNA thermocycler was used to cycle the temperature for the PCR with the following parameters.

An initial denaturation at 95° C for 5 m was followed by 5 amplification cycles (denaturation at 95° C for 40 s, annealing at 40° C for 1 m, extension at 72° C for 1 m). This was followed by 35 amplification cycles (where denaturation and extension parameters were as above, but annealing was done at 50° C for 1 m) and a final extension at 72° C for 7 m. The presence of PCR products was checked by electrophoresis on 2% agarose gel containing ethidium bromide.

The PCR products were denatured by heating at 95° C for 2 m. On to a posi-

tively charged nylon membrane (Boehringer Mannheim, Germany), 1 µl of the heated PCR product was applied and the DNA was crosslinked to the membrane by UV light (1200 mV). The Genius Non-radioactive Nucleic Acid Labeling and Detection System was used for labeling and detection of the probe. The probe was detected with antidigoxigenin conjugated to alkaline phosphatase and nitroblue tetrazolium salt-X-phosphate combination. The probe was found to hybridize only with DNA of the two species of the mactrids viz. *S. solidissima* and *M.*

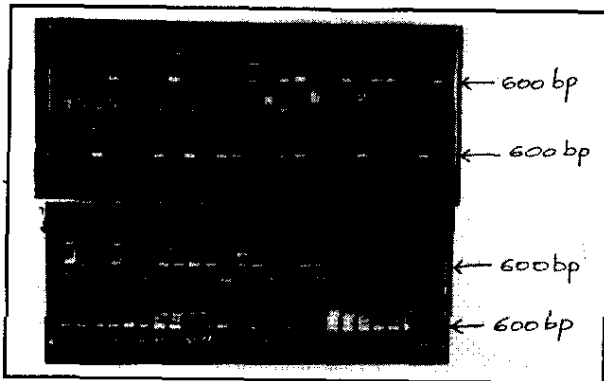


Fig. 1 PCR product of first 600 bp of the 18S rRNA of unknown bivalve larvae on agarose gel. Positive controls of 5 known bivalve species are in lanes 7-11 on the right half of bottom row of the bottom gel. Lanes 7 and 8 are PCR amplified DNA templates from *S. solidissima* and *M. lateralis*, respectively. Negative control (without DNA) is in lane 12.

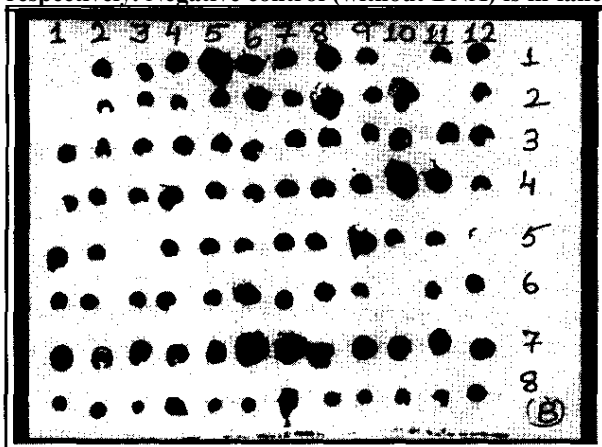


Fig 2B. Dot-blot hybridization of all bivalve larvae and positive controls of all bivalves (Row 8, lanes 8-12) with universal bivalve DNA probe.

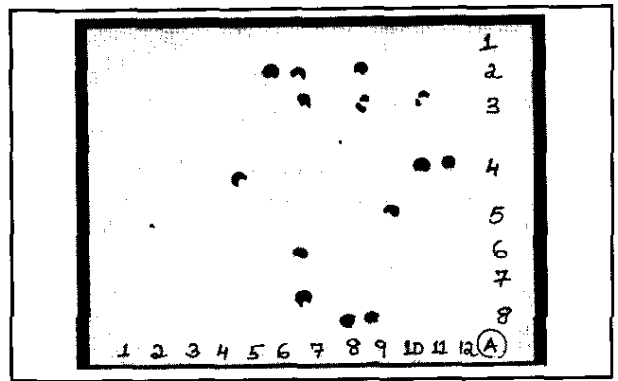


Fig. 2A Dot-blot hybridization of PCR product of 12 unknown bivalve larvae and positive controls of *S. solidissima* and *M. lateralis* with mactrid-specific DNA probe. Row 8, lanes 8 and 9 are positive controls.

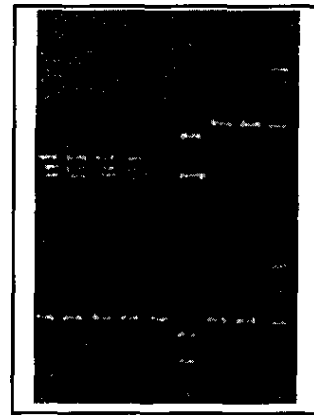


Fig. 3. RFLP of first 600 bp of unknown lane positive to mactrid-specific probe digested with *Btsul* and *Aval*. Lanes 1-4 of row 1 are DNA from *S. solidissima* digested with *Btsul* and lane 6 with that of *M. lateralis*. Lanes 1-5 of row 2 are DNA from *S. solidissima* digested with *Aval* and lane 7 with that of *M. lateralis*. Lanes 7 and 8 of row 1 and 2 are undigested DNA controls of *S. solidissima* and *M. lateralis*. Lane 9 of both rows contains the 100 bp ladder.

lateralis and not with bivalves belonging to other families.

The probe was also tested on the 600 bp fragment of 18S rRNA gene amplified from the veliger stage larvae of *S. solidissima* reared in the laboratory. It was found that DNA extracted from the larvae as small as 80 µm in shell length was sufficient to be amplified by PCR (Bell, Grassle and Achuthankutty, 1997). PCR amplified larval DNA behaved in the same manner as that derived from the adult.

Unknown Bivalve Larvae from Plankton Samples

Active bivalve larvae were picked from plankton samples under a stereozoom binocular microscope and stored individually in eppendorf tubes at -80° C. DNA from 90 larvae was individually extracted using Chelex-100. The 600 bp fragment of 18S rRNA gene was amplified by PCR from the unknown larvae and 5 bivalve controls, viz., *S. solidissima*, *M. lateralis*, *Pitar morrhuanus*, *Telina agilis* and *Placopecten magellanicus*. PCR product of 57 larvae and five controls were shown to be positive by agarose gel electrophoresis (Fig. 1).

Chelex Extraction Protocol

The protocol used for extraction of DNA from individual larva is given below.

Dissolve 0.5 g of 5% Chelex-100 in 10 ml of double distilled water.

Keep eppendorf tubes containing frozen larvae on heating block at 95° C for 4 m.

Grind larva well individually in the tube using sterilized plastic pestle.

Add 20 µl of 5% Chelex-100 Resin (Bio-Rad, Hercules, California) in sterile water in each tube.

Heat the tubes for 10 m at 100° C.

Transfer supernatant to new tube.

Protocol for PCR Amplification of Larval DNA

The 600 bp fragment containing the

variable region was amplified by PCR in a V-bottom microtiter plate, using the forward (Primer A) and reverse (536 R) primers. In a 50 µl reaction, 5 µl of Chelex supernatant from individual larva was used as template DNA. Each reaction contained five µl 10x PCR buffer, 0.2 mM dNTP, 0.2 µM of each primer, 20 µg BSA and 0.5 U Taq polymerase.

A Stratagene Robocycler 96 (La Jolla, California) was used to cycle the temperature for PCR. The first 5 cycles were performed with the following parameters: denaturation at 95° C for 1 m, annealing at 40° C for 1.5 m, extension at 72° C for 2 m. The next 35 cycles were done at 95° C for 1 m, 50° C for 1.5 m, 72° C for 2 m and was followed by a final extension at 72° C for 7 m. The presence of PCR products was confirmed by electrophoresis on a 2% agarose gel containing ethidium bromide.

Probe Hybridization

PCR products from the larvae and controls were tested with mactrid-specific probe in dot-blot hybridization. The probe hybridized with 12 PCR products from larvae and controls of only *S. solidissima* and *M. lateralis* and not with other bivalve controls and negative control (Fig. 2A).

The membrane was later exposed to the universal 18S rRNA bivalve probe to detect the presence of PCR products from all bivalve larvae belonging to other families. The universal probe was designed with the template DNA from *S. solidissima*. The 216-b probe was amplified from a conserved region of the gene between nucleotide positions 410 and 626, using primers 300F (5'-AGGGTTCGATTCCGGAG-3') and 536R and labeled with biotin. The universal probe detected 84 out of the 90 larval samples (as against 57 in agarose gel electrophoresis) and all bivalve controls (Fig. 2B), indicating that colorimetric detection with a probe is more sensitive than ethidium bromide in the visualization of DNA (Schriefer, Sacchi, Wirtz and Azad, 1991).

RFLP Analysis

M. lateralis larvae is the only other

species belonging to the mactrid family that was present in small numbers in the plankton samples collected from the study site along with *S. solidissima*. To differentiate the larval stages between the two species, RFLP analysis was carried out taking advantage of a single base pair difference (CGAG for *M. lateralis* and CGCG for *S. solidissima*). The PCR products of larval samples that were tested positive for mactrid family were subjected to restriction digests with *Bst*UI and *Ava*I. Since *Bst*UI has a common restriction site for both species and one specific restriction site for *S. solidissima*, the DNA from *S. solidissima* was cut twice whereas that from *M. lateralis* was cut only once. On the other hand, *Ava*I has one restriction site for DNA from *M. lateralis* and no restriction site for DNA from *S. solidissima*. Therefore the former was cut once and the latter was uncut (Fig. 3).

Advantages of Combination of DNA Probe and RFLP Analysis

Reliable tool for identification of eggs and larvae which are difficult and time consuming to identify by conventional methods.

Oligonucleotide probe is sensitive for family identification and restriction enzymes distinguish species within the family.

Combination of DNA probe and RFLP allows multiple species identification in a sample.

Availability of three different colorimetric labels (digoxigenin, biotin and fluorescein) allows detection of 3 probes on a single membrane.

DNA probe is quick, sensitive and facilitates more detection than agarose gel electrophoresis.

In case of a single species dominated family (when other species within the family are rare), RFLP analysis can be avoided.

Probe solution is sensitive for three or more uses.

Application

The combined approach of a DNA

probe for family identification and the restriction digest for species recognition within the family may have several applications in fishery biology particularly in differentiating eggs and early larval stages, spawning behaviour, population dynamics, resource evaluation and management.

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References

BELL, J.L., GRASSLE, J.P. and ACHUTHANKUTTY, C.T., 1997. Application of a molecular probe to species identification of bivalve larvae in

nearshore plankton samples. ASLO 97, Aquatic Sciences Meeting, Current and emerging issues in aquatic science. *The American Society of Limnology and Oceanography*, Santa Fe, New Mexico, p. 95.

SCHRIEFER, M.E., SACCI, J.B. JR., WIRTZ, R.A. and AZAD, A.F., 1991. Detection of polymerase chain reaction-amplified malarial DNA in infected blood and individual mosquitoes. *Exp. Parasitol.*, 13 n-3\6.