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Single-step species identification of bivalve larvae using multiplex polymerase chain reaction

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Abstract One of the biggest obstacles to studying recruitment variation in marine bivalves is the need to collect and process large numbers of plankton samples. Larval bivalves are notoriously difficult, if not impossible, to identify to species using morphological criteria alone. Remote time-series collections could satisfy the sampling challenge, but efficient identification techniques must be developed to obtain species-specific data. Thus, we have developed a multiplex polymerase chain reaction (PCR) identification assay in which a single reaction is capable of accurate and efficient discrimination of five target bivalve species based on the size of cytochrome oxidase I products. The assay was tested with cultured and field-sampled larvae as well as adult genomic DNAs. Using a single whole larva as template, multiplex PCR reactions were capable of discriminating among the commercially important bivalves: *Mercenaria mercenaria*, *Argopecten irradians*, *Mulinia lateralis*, *Spisula solidissima* and *Mya arenaria*. Overall accuracy was 92%, including very few false positives. The efficiency of this assay stems from its ability to discriminate multiple target species with a single molecular step that ultimately can be automated to process large numbers of larvae.

Introduction

Understanding the causes of variation in recruitment of benthic marine invertebrates requires measurements of larval dispersal patterns within and among populations. Yet, quantifying dispersal pathways can be difficult and laborious because of (1) rapid advection and turbulent diffusion of larvae in the water column, and (2) lengthy planktonic periods in many temperate species. Moreover, identification of the geographic source of settling larvae is often equivocal using morphological, genetic or isotopic tracers. Therefore, delineating the larval dispersal "cloud" and its movements for a given species requires long-term, remote, time-series sampling within the potential larval transport region, coupled with an efficient and accurate means of identifying and quantifying the sampled organisms.

Several remote, time-series, larval samplers have been developed for use in very shallow water, and one instrument was designed for long-term (months) deployments at water depths ≤1500 m (Doherty and Butman 1990; Butman 1994). For some taxa, however, morphological identification to species of especially the smallest larvae collected in long-term deployments has been problematic because of limited diagnostic characters (Garland and Butman 1996; Garland 2000). In an initial effort to alleviate this problem for coastal bivalves, this paper describes the development of a rapid molecular assay for the definitive identification of five western North Atlantic species (see Table 1).

The planktonic larvae of many bivalve species are nearly impossible to identify morphologically, using a light microscope, during the early straight-hinge stage of veliger development when total length is approx. 90 to 120 μm (Loosanoff et al. 1966; Chanley and Andrews 1971; Le Pennec 1980). Later-stage larvae, while relatively easier to identify, may not be as abundant in plankton samples (Chanley and Andrews 1971) and still require considerable expertise and effort for reliable identifications. Currently, the most reliable morphological identifications are based on the structure of the larval shell-hinge teeth, which must be observed using scanning electron microscopy (e.g. Lutz and Hidu 1979; Lutz and Jablonski 1979; Lutz et al. 1982). These morphological methods of identification are impractical,

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Table 1 Bivalve species targeted for identification in this study, name abbreviations used in Fig. 1, and collection localities for adult bivalves in Massachusetts, USA

Species	Abbreviation	Locality	Latitude; Longtitude
Mercenaria mercenaria	*Merc. merc.*	Waquoit Bay	41°34′27″N; 70°30′59″W
Argopecten irradians	*A. irrad.*	Great Harbor	41°29′00″N; 70°40′00″W
Mulinia lateralis	*Mul. later.*	Waquoit Bay	41°34′27″N; 70°30′59″W
Spisula solidissima	*S. solidis.*	Cape Cod Bay	41°50′00″N; 70°20′00″W
Mya arenaria	None	Little Buttermilk Bay	41°42′00″N; 70°40′00″W

however, in a study of larval dispersal where thousands of identifications are required.

Several molecular methods for identifying bivalve species are particularly attractive because of their potential for automation (Powers et al. 1988, 1990). Immunological techniques are appealing because they are typically applied to whole organisms and thus, conceivably, samples could be screened with minimal handling of the larvae (Yentsch et al. 1988; Ward 1990). From a practical and monetary perspective, polyclonal antibodies are preferable over monoclonal antibodies because the latter requires the culture and assay of large numbers of isolated cell lines. However, polyclonal antibodies have been wrought with high cross-reactivity and lack of specificity (Feller et al. 1979; Feller and Gallagher 1982), such that the highest taxonomic resolution for a larval invertebrate thus far is at the familial level (Demers et al. 1993). Monoclonal antibodies have been more successful in terms of species-specificity (Miller et al. 1991; Hanna et al. 1994) but the identification protocol can be tedious. Miller et al., for example, distinguished between three barnacle species by applying antibodies in two steps and making binary comparisons.

DNA sequence variation has also been used to differentiate species by several means. Larval identifications have been made by hybridizing a species-specific DNA probe with larval DNA or with a larval polymerase chain reaction (PCR) product bound to a membrane (Banks et al. 1993; Bell and Grassle 1998). Conserved "universal" primers have also been used in PCR to amplify a locus containing diagnostic variation, followed by digestion of the amplification products with informative restriction enzymes and visualization by electrophoresis (Banks et al. 1993; McKay et al. 1997; Bell and Grassle 1998; Toro 1998; Lindstrom 1999; Spatz et al. 1999). Genetic identification of species has been accomplished without the hybridization or digestion step by combining several species-specific primers in a multiplex PCR using template DNA from a single specimen. The PCR primers were designed to amplify a different-sized product depending on the species used for template. To date, the multiplex approach has been used to identify one or two species at a time, using template DNA previously extracted from larval tissue (Banks et al. 1993; Romstad et al. 1997; Rocha-Olivares 1998). Not all of these molecular assay procedures can be automated.

To increase the efficiency of molecular larval identifications, we have developed a multiplexed PCR reaction that will amplify a species-specific sized fragment of the mitochondial cytochrome oxidase I (COI) gene from a single whole larva from any of the five target species (see Table 1). As a positive control a portion of the nuclear ribosomal 18S RNA gene is also amplified in each reaction. Electrophoresis and staining with ethidium bromide allows visualization of the PCR products. This fast and inexpensive assay had high accuracy when tested on cultured and field-collected larvae. Also, the small number of steps in this assay will facilitate its automation in the future.

Materials and methods

Assay approach

Conventional PCRs have two opposing oligonucleotides priming the polymerase enzyme. In specific PCR, one or both of the primers is complementary to a section of DNA that is unique to the target species. Primer specificity is mostly conferred by the last few nucleotides at the 3′ end of the oligonucleotide, so even a single unique nucleotide can be used to direct species-specific PCR (Bottema et al. 1993). However, additional nucleotide or insertion/deletion differences between the target sequence and potential nontarget templates may reduce the likelihood of unintended amplification or false positives. The fact that marine bivalves are highly variable at COI (A. Frese personal communication) led to the design of "species"-specific primers at this locus. Primers are referred to here as species-specific not because they have been tested on all potential congeners, but because congeners of the target species that might be present in New England waters are expected to be rare based on adult distributions (Abbot 1974). The primers may, in fact, be species-specific outside this geographic region, but further study is needed to verify this possibility.

Two elements are critical in a PCR assay to identify larvae. First, reactions that produce no species-specific product because of to an absence of target DNAs must be distinguished from failure of the overall enzymatic reaction. This was accomplished by including a positive control amplification in every multiplex reaction. Positive control primers were designed from highly conserved portions of the nuclear 18S ribosomal RNA gene because it was impossible to design "universal" nondegenerate COI primers that would amplify reliably from all bivalve species tested (data not shown). The region of 18S sequence amplified by these primers contains variation that is diagnostic for some bivalve families and genera (Bell and Grassle 1998). Thus, sequencing the 18S positive control product yielded an additional, sequence-based means for identifying wild larvae that were used in assay tests.

Second, a PCR assay should be efficient, both in terms of minimizing manipulations of larvae or amplification products and in terms of maximizing the number of species that are potentially identified by a single reaction. Here, by initiating the PCR with a boiling step, sufficient template DNA for amplification was liberated from single whole larvae without the need for a separate DNA extraction. Also, by designing primers to amplify species-specific products that differed in size for each species, post-PCR manipulations such as restriction digestion were obviated. Finally, multiple species-specific primer pairs have been multiplexed in a single reaction, along with the positive control primers, so that a species-

specific product and an 18S positive control are amplified from any one of five target species (Table 1). In non-target species only an 18S product is amplified.

Primer design

Cytochrome oxidase I sequences were aligned and compared among 17 species of marine bivalves (A. Frese personal communication). For each of the five target species, oligonucleotide primer pairs were designed for PCR using OLIGO (National Biosciences, Inc.) based on four criteria (see Fig. 1): (1) two out of four nucleotides at the 3′ end of the primers must be unique to one target species in the alignment, (2) the 3′ terminal nucleotide of each primer must correspond to a first or second codon position, (3) melting temperature, Tm = 45 to 50 °C, (4) the expected PCR product size is different for each species. Primer lengths ranged from 18 to 22 nucleotides (Table 2). Primers designed to amplify a 430 base pair (bp) portion of the nuclear 18S ribosomal RNA gene were used to amplify a positive control product (Table 2).

Adult bivalve samples were collected in the Cape Cod region of Massachusetts and from near Panacea, Florida (see Tables 1 and 4). Massachusetts samples were preserved in 70% ethanol for later DNA extraction, whereas fresh tissue was used for DNA extractions from Florida specimens. Genomic DNA was prepared by homogenizing adult gill tissue in liquid nitrogen and purifying the DNA in a Wizard Plus Miniprep column (Promega).

Each primer pair was tested in PCR amplifications alone and in combination with other primer pairs using adult genomic DNA. Acceptable COI primer pairs amplified a single COI product of the expected size from the species for which they were designed, and produced no product from other species. Primer pairs for COI in the five target species plus the 18S primer pair were initially combined in an equimolar mixture for multiplex PCR reactions, and amplification conditions were optimized as per Henegariu et al. (1997). Optimum reaction conditions included 0.15 units of Qiagen *Taq* polymerase, 0.7x Qiagen PCR buffer, 2.2 mM $MgCl_2$, 200 μM of each dNTP and each COI primer at 0.4 μM in 12.5 μl total volumes. Different ratios of 18S to COI primers were tested by increasing the concentration of each 18S primer to 0.8 and 1.2 μM per reaction. With genomic DNA as template, PCR was performed on a PTC-100 thermocycler (MJ Research) using a 2 min soak at 95 °C, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s. Ramping rate from 50° to 72 °C was 0.5° per second. Negative control reactions, containing no template, were performed in every batch of reactions to test for contaminating template. Results were analyzed only if the negative control was blank.

Cultured larvae were obtained from Mook Sea Farms, Damariscotta, Maine (*Spisula solidissima*), Beale's Island Hatchery, Beale's Island, Maine (*Mya arenaria*), Marthas Vineyard Shellfish Group, Marthas Vineyard, Massachusetts (*Mercenaria mercenaria*, *Crassostrea virginica* and *Argopecten irradians*), or cultured at the Woods Hole Oceanographic Institution from broodstock provided by the Virginia Institute of Marine Science, Wachapreague, Virginia (*Mulinia lateralis*). Cultured larvae were preserved in 80% ethanol and stored at 4 °C. Field samples of larvae were collected by towing a 100 μm plankton net from a boat at Isle of Shoals, New Hampshire (43°02′00″N; 70°38′00″W) on 10 May and 3 July 1999, and by suspending a 64 μm net in the tidal current at Iselin dock, Woods Hole, Massachusetts (41°29′00″N; 70°39′50″W) on 16 June 1999. Field samples were preserved in 70% ethanol and manually sorted to concentrate and separate mollusk veligers. Ethanol-preserved larvae were rehydrated in water for 1 to 15 min before pipeting each individual into a separate 200 μl tube along with approx. 1.0 μl of water. Tubes were visually inspected to confirm the presence of a single larva. PCR reaction mixtures were then added directly to tubes containing whole larvae. PCR of larvae was identical to that using genomic DNA except that the initial denaturing step was lengthened to 15 min to activate a hot-start enzyme (Qiagen Hotstar) and liberate DNA from the larva. PCR products were separated in 2 to 2.5% agarose gels and visualized with ethidium bromide (EtBr).

Template for 18S sequencing was generated by diluting a trace amount of the primary multiplex reaction product in 200 μl water and using 0.5 μl as template in a 25 μl secondary PCR containing only the 18S primers. Five μl of the secondary product was visualized on a EtBr-stained gel and 5 μl was treated to remove remaining primers by mixing with 0.25 units of shrimp alkaline phosphatase (SAP), 2.5 units of exonuclease I and 0.5 μl SAP dilution buffer (Amersham). Reactions were incubated at 37 °C for 30 min, then 80 °C for 15 min. Two μl (approx. 50 ng) of this template was used for cycle sequencing of each DNA strand with BigDye chemistry (Applied Biosystems) and visualized on an automated ABI 377 sequencer. After comparing the two 18S strands for a particular sample to check for sequencing errors, the sequence was compared to the GenBank data base using gapped BLAST 2.0 (Altschul et al. 1997).

Criteria for determining accuracy

The correctness or accuracy of molecular identifications is measured by the frequency of false negative and false positive results. A reaction containing a target species template that produces an 18S product but no visible COI product is a false negative. A false positive can result from either a target or non-target template if the size of an amplified product mimics that expected from a different target species. A reaction that produces no visible COI or 18S product is "blank" and uninformative. Blank reactions can result from overall enzymatic failure or by inadvertently running a reaction without template. Cultured larvae provide template of known species origin whereas the species identification of field samples is initially unknown. For field samples, sequencing of the 18S positive control product subsequently provides a positive identification to the level of order, family or genus, depending on the availability of reference sequences in GenBank and diagnostic characters in 18S.

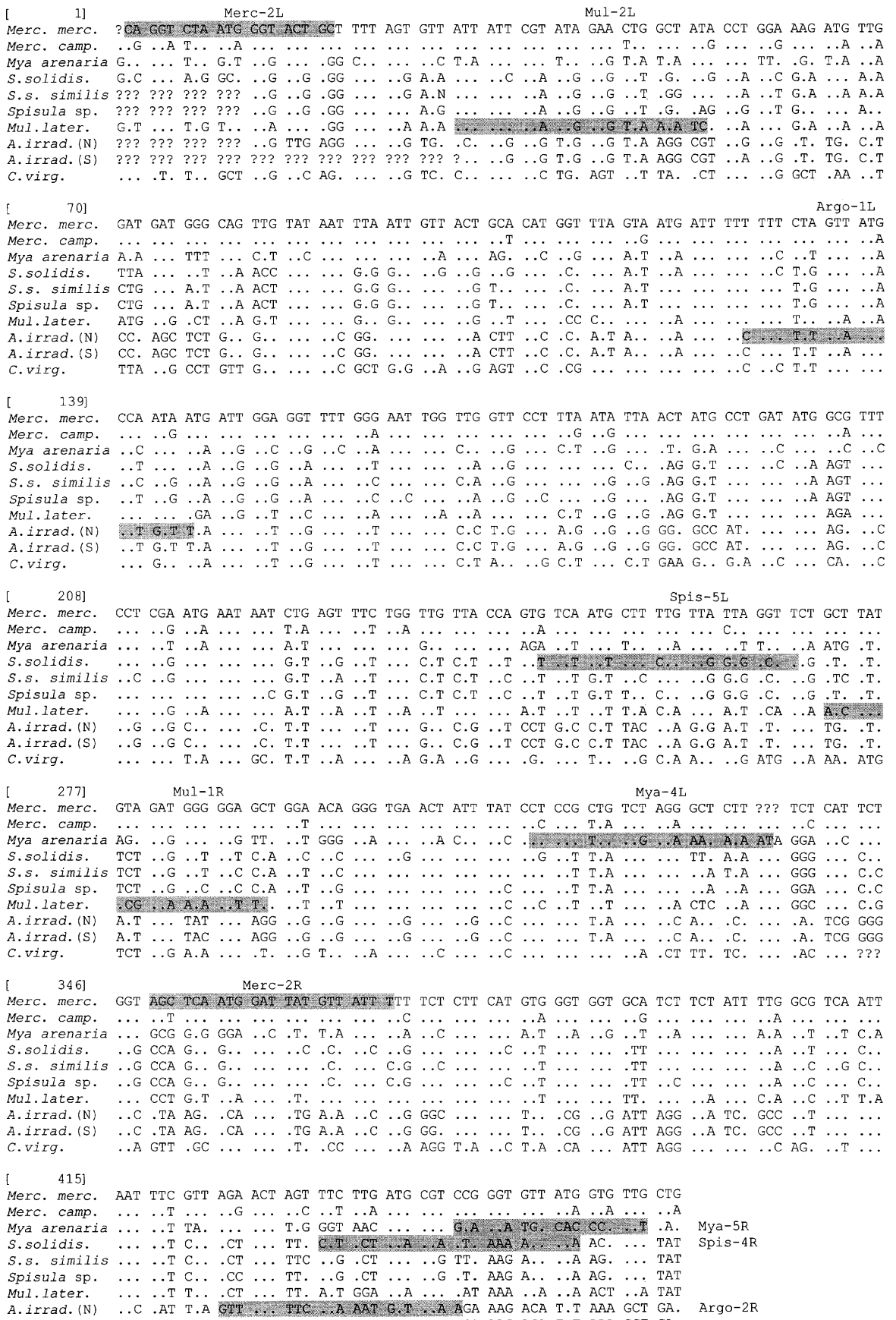
Results

COI sequence comparisons

After COI primers had been designed, DNA sequence comparisons were made between geographically distant populations in two target species, *Spisula solidissima* and *Argopecten irradians*, and also between *Mercenaria* congeners to examine the potential for primer specificity in COI amplifications (Fig. 1). The uncorrected COI sequence difference was 10.2% between *M. mercenaria* and *M. campechiensis*, 11.9% between *S. solidissima* and *S. solidissima similis* (Say, 1822) and 0.7% between *A. irradians* from Massachusetts and the Gulf of Mexico.

Testing identifications made with multiplex PCR

In most tests involving one of the five target species, the multiplexed primers resulted in a two-banded pattern after electrophoresis; a smaller species-specific sized band and a larger 18S band that varied in size from 410 to 440 base pairs (bp) depending on the species, presumably due to insertions and deletions at that locus (Fig. 2a). Multiplex reactions containing an equimolar mixture of all 12 primers did not always result in the co-amplification of 18S with a COI product (data not shown). To increase consistent amplification of the 18S positive control, 2:1 and 3:1 molar ratios of 18S to COI primers were tested. The overall proportion of correct identifications out of 248 multiplex larval PCRs was 94% with the 3:1 multiplex


[1] Merc-2L Mul-2L
Merc. merc. ?CA GGT CTA ATG GGT ACT GCT TTT AGT GTT ATT ATT CGT ATA GAA CTG GCT ATA CCT GGA AAG ATG TTG
Merc. camp. ..G ..A T.. ..A ... ... T.. ... ..G ... ..G ... ..A ..A
Mya arenaria G.. ... T.. G.T ..G ... .GG C.. ... ..C T.A ... ... T.. ..G T.A T.A ... ... TT. .G. T.A ..A
S.solidis. G.C ... A.G GC. ..G ..G .GG ... ..G A.A ... ..C ..A ..G ..G ..T .G. ..G ..A ..C G.A ... A.A
S.s. similis ??? ??? ??? ??? ..G ..G .GG ... ..G A.N ... .A ..G ..G ..T .GG ... ..A ..T G.A ..A A.A
Spisula sp. ??? ??? ??? ??? ..G ..G .GG ... ... A.G ... .A ..G ..G ..T .G. .AG ..G ..T G.. ... A..
Mul.later. G.T ... T.G T.. ..A ... .GG ... ..A A.A ... .A ..G ..G T.A A.A TC. ..A ... G.A ..A ..A
A.irrad.(N) ??? ??? ??? ??? ..G TTG AGG ... ..G TG. .C. ..G ..G T.G ..G T.A AGG CGT ..G ..G .T. TG. C.T
A.irrad.(S) ??? ??? ??? ??? ??? ??? ??? ??? ??? ??? ?.. ..G ..G T.G ..G T.A AGG CGT ..A ..G .T. TG. C.T
C.virg. ... .T. T.. GCT ..G ..C AG. ... ..G TC. C.. ... ..C TG. AGT ..T TA. .CT ... ..G GCT .AA ..T
[70] Argo-1L
Merc. merc. GAT GAT GGG CAG TTG TAT AAT TTA ATT GTT ACT GCA CAT GGT TTA GTA ATG ATT TTT TTT CTA GTT ATG
Merc. camp. ... ...T ... ...G ... ...A
Mya arenaria A.A ... TTT ... C.T ..C ... ...A ... AG. ..C ..G ... A.T ..A ... .C ..T ... ..A
S.solidis. TTA ... ..T ..A ACC ... ... G.G G.. ..G ..G ... .C. ... A.T ..A ... .C T.G ... ..A
S.s. similis CTG ... A.T ..A ACT ... ... G.G G.. ... ..G T.. ... .C. ... A.T ... ... T.G ... ..A
Spisula sp. CTG ... A.T ..A ACT ... ... G.G G.. ... ..G T.. ... .C. ... A.T ... ... T.G ... ..A
Mul.later. ATG ..G .CT ..A G.T ... ... G.. G.. ... ..G ..T ... .CC C.. ... ..A ... ... T.. ..A ..A
A.irrad.(N) CC. AGC TCT G.. G.. ... ..C GG. ... .A CTT ..C .C. A.T A.. ..A ... ..C ... T.T ..A ...
A.irrad.(S) CC. AGC TCT G.. G.. ... ..C GG. ... .A CTT ..C .C. A.T A.. ..A ... ..C ... T.T ..A ...
C.virg. TTA ..G CCT GTT G.. ... ..C GCT G.G ..A ..G AGT ..C .CG ... ...C ..C T.T ... ...
[139]
Merc. merc. CCA ATA ATG ATT GGA GGT TTT GGG AAT TGG TTG GTT CCT TTA ATA TTA ACT ATG CCT GAT ATG GCG TTT
Merc. camp. ... ..G ... ...A ... ...G ..G ... ...A ...
Mya arenaria ..C ... ..A ..G ..C ..G ..C ..A ... ... C.. ..G ... C.T ..G ... .T. G.A ... ..C ... ..C ..C
S.solidis. ..T ... ..A ..G ..G ..A ... ..T ... .A ..G ... ... C.. .AG G.T ... ..C ..A AGT ...
S.s. similis ..C ..G ..A ..G ..G ..A ... ..C ... ... C.A ..G ... .G ..G .AG G.T ... .A AGT ...
Spisula sp. ..T ..G ..A ..G ..G ..A ... ..C ..C ... ..A ..G ..C ... ..G ... .AG G.T ... .A AGT ...
Mul.later. ... ... .GA ..G ..T ..C ... ..A ... ..A ..A ... ... C.T ..G ..G .AG G.T ... ... AGA ...
A.irrad.(N) ..T G.T T.A ... ..T ..G ... ..T ... ... C.C T.G ... A.G ..G ..G GG. GCC AT. ... ... AG. ..C
A.irrad.(S) ..T G.T T.A ... ..T ..G ... ..T ... ... C.C T.G ... A.G ..G ..G GG. GCC AT. ... ... AG. ..C
C.virg. ... G.. ..A ... ..T ..G ... ..T ... ... C.T A.. ..G C.T ... C.T GAA G.. G.A ..C ... CA. ..C
[208] Spis-5L
Merc. merc. CCT CGA ATG AAT AAT CTG AGT TTC TGG TTG TTA CCA GTG TCA ATG CTT TTG TTA TTA GGT TCT GCT TAT
Merc. camp. ... ..G ..A ... ... T.A ... ..T ..A ... ...A ... ... C.. ... ...
Mya arenaria ... ..T ..A ... ... A.T ... ... G.. ... ... AGA ..T ... T.. ..A ... ..T T.. ..A ATG .T.
S.solidis. ... ..G ... ... G.T ..G ..T ... C.T C.T ..T ..T ..T ... C.. ..G G.G .C. ..G .T. .T.
S.s. similis ..C ..G ... ... G.T ..A ..T ... C.T C.T ..C ..T ..T G.T ..C ... ..G G.G .C. ..G .TC .T.
Spisula sp. ... ...C G.T ..G ..T ... C.T C.T ..C ..T ..T G.T T.. C.. ..G G.G .C. ..G .T. .T.
Mul.later. ... ..G ..A ... ... A.T ..A ..T ..A ..T ... ... A.T ..T ..T T.A C.A ... A.T .CA ..A A.C ...
A.irrad.(N) ..G ..G C.. ... .C. T.T ... ..T ... G.. C.G ..T CCT G.C C.T TAC ..A G.G A.T .T. ... TG. .T.
A.irrad.(S) ..G ..G C.. ... .C. T.T ... ..T ... G.. C.G ..T CCT G.C C.T TAC ..A G.G A.T .T. ... TG. .T.
C.virg. ... ... T.A ... GC. T.T ..A ... ..A G.A ..G ... .G. ... T.. ..G C.A A.. ..G ATG ..A AA. ATG
[277] Mul-1R Mya-4L
Merc. merc. GTA GAT GGG GGA GCT GGA ACA GGG TGA ACT ATT TAT CCT CCG CTG TCT AGG GCT CTT ??? TCT CAT TCT
Merc. camp. ... ...T ... ...C ... T.A ... ..A ... ...C ... ...
Mya arenaria AG. ..G ... ..G TT. ..T GGG ..A ... ..A C.. ..C ..... T.. ..G ..A AA. A.A ATA GGA ..C ...
S.solidis. TCT ..G ..T ..T C.A ..C ..C ... ..G ... ...G ..T T.A ... ... TT. A.A ... GGG ... C..
S.s. similis TCT ..G ..T ..C C.A ..T ..C ... ...T T.A ... .A T.A ... GGG ... C.C
Spisula sp. TCT ..G ..C ..C C.A ..T ..G ... ...C ... ..T T.A ... .A ..A ... GGA ... C.C
Mul.later. .CG ..A A.A ..T T.. ..T ..T ... ...C ..C ..T ..T ... ..A CTC ..A ... GGC ... C.G
A.irrad.(N) A.T ... TAT ... AGG ..G ..G ... ..G ... ..G ..C ... ... T.A ... ..C A.. .C. ... .A. TCG GGG
A.irrad.(S) A.T ... TAC ... AGG ..G ..G ... ..G ... ..G ..C ... ... T.A ... ..C A.. .C. ... .A. TCG GGG
C.virg. TCT ..G A.A ... .T. ..G T.. ..A ... ..C ... ..C ... ...A .CT TT. TC. ... .AC ... ???
[346] Merc-2R
Merc. merc. GGT AGC TCA ATG GAT TAT GTT ATT TTT TCT CTT CAT GTG GGT GGT GCA TCT TCT ATT TTG GCG TCA ATT
Merc. camp. ... ..T ... ...C ... ...A ... .G ... ...A ... ...
Mya arenaria ... GCG G.G GGA ..C .T. T.A ... ..A ..C ... ... A.T ..A ..G ..T ..A ... ... A.A ..T ..T C.A
S.solidis. ..G CCA G.. G.. ... ..C .C. ..C ..G ... .C ..T ... ... .TT ... ...A ..T ... C..
S.s. similis ..G CCA G.. G.. ... ... .C. ... C.G ..C ... .T ... ... .TT ... ...A ..C ..G C..
Spisula sp. ..G CCA G.. G.. ... ... .C. ... C.G ... .C ..T ... ... .TT ..C ... .A ..C ... C..
Mul.later. ... CCT G.T ..A ... .T. ... ...T ... ... TT. ... ..A ... C.A ..C ..T T.A
A.irrad.(N) ..C .TA AG. .CA ... .TG A.A ..C ..G GGC ... ... T.. .CG ..G ATT AGG ..A TC. GCC ..T ... ...
A.irrad.(S) ..C .TA AG. .CA ... .TG A.A ..C ..G GG. ... ... T.. .CG ..G ATT AGG ..A TC. GCC ..T ... ...
C.virg. ..A GTT .GC ... ... .T. .CC ... ..A AGG T.A ..C T.A .CA ... ATT AGG ... .C AG. ..T ...
[415]
Merc. merc. AAT TTC GTT AGA ACT AGT TTC TTG ATG CGT CCG GGT GTT ATG GTG TTG CTG
Merc. camp. ... ..T ... ..G ... ..C ..T ..A ... ...A ..A ... ..A
Mya arenaria ... ..T TA. ... ... T.G GGT AAC ... ... G.A ..A TG. CAC CC. ..T .A. Mya-5R
S.solidis. ... ..T C.. .CT ... TT. C.T .CT ..A ..A .T. AAA A.. ..A AC. ... TAT Spis-4R
S.s. similis ... ..T C.. .CT ... TTC ..G .CT ... ..G TT. AAG A.. ..A AG. ... TAT
Spisula sp. ... ..T C.. .CC ... TT. ..G .CT ... ..G .T. AAG A.. ..A AG. ... TAT
Mul.later. ... ..T T.. .CT ... TT. A.T GGA ..A ... .AT AAA ..A ..A ACT ..A TAT
A.irrad.(N) ..C .AT T.A GTT ... TTC ..A AAT G.T ..A AGA AAG ACA T.T AAA GCT GA. Argo-2R
A.irrad.(S) ..C .AT T.A GTT ... TTC ..A AAT G.T ..A AGA AAG ACA T.T AAA GCT GA.
C.virg. ... ..T A.A GT. ..C .T. AGA AAC ... ..A T.T .T. .GG GGT CAC A.A T.A

◀
Fig. 1 Alignment of COI sequences from 10 bivalve specimens, including 5 target species, their close relatives, and *Crassostrea virginica* (*C. virg.*) [*Names abbreviated* as in Table 1, except *Merc. camp.* = *Mercenaria campechiensis*; *dots* show identity with top sequence; *spaces* separate amino acid codons; COI primer annealing positions are *highlighted in gray* and labeled with primer names (Table 2) either above or to right of sequence; *question marks and dashes* denote unknown bases and insertion/deletions, respectively]

mixture and 90% with the 2:1 mixture (Table 3). No false positive results were observed using the 3:1 mixture in 78 trials with larvae of known or 18S-confirmed species identifications. Two false positives were observed out of 61 trials using the 2:1 mixture, for a total rate of 1.4% false positives from larvae. *Spisula solidissima* was falsely indicated by a field-collected *Ensis directus* and a veneroid larva (Table 3).

In all tests of the multiplex assay using cultured larvae, the COI primers consistently amplified the expected size product from *Mercenaria mercenaria*, *Spisula solidissima* and *Mya arenaria* (Table 3). *Mulinia lateralis* and *Argopecten irradians* showed slight and moderate false negative frequencies, respectively. None of the five target species generated a product size expected from a different species, i.e. a false positive result. The 18S product amplified reliably in all target species except in *Mya arenaria*, where it was typically faint (Fig. 2a).

Genomic DNA was used to test the multiplex assay on Gulf of Mexico populations of *Argopecten irradians* and *Spisula solidissima similis*. Two target-species congeners were also tested, *Mercenaria campechiensis* (Abbott 1974) from the Gulf of Mexico and *Spisula* spp. (A. Frese personal communication) from Vineyard Sound, Massachusetts (41°33′07″N; 70°32′49″W). Multiplex PCR of *M. campechiensis* and *A. irradians* from the Gulf of Mexico showed product sizes expected for *M. mercenaria* and *A. irradians*, respectively. In contrast, neither the *S. solidissima similis* or *Spisula* spp. specimens generated a diagnostic *S. solidissima* product (Table 4). Comparison of the COI sequences of these species at PCR priming sites shows sequence differences that presumably disrupt COI amplification in *S. solidissima similis* and *Spisula* spp., and sequence similarities that usually allow COI amplification in *M. campechiensis* and Gulf of Mexico *A. irradians* (Fig. 1).

Multiplex PCR performed with adult genomic DNA from eight non-target species, four of them confamilial with target species, showed no false positive results (Table 4). An exception occurred with three additional species, *Crassostrea virginica*, *Gemma gemma* and *Pitar morrhuana*, each of which showed a faint *Spisula*-sized COI product in some trials. However, false positive results were not observed in multiplex reactions with single cultured *C. virginica* larvae (Table 3).

Table 2 Oligonucleotide primers used in multiplex PCR reactions to identify single larvae

Primer	Sequence (5′ to 3′)
Merc-2L	CAGGTCTAATGGGTACTGC
Merc-1R	AAATAACATAATCCATTGAGCT
Argo-1L	CTTTTTTGTAATGCCTGTTT
Argo-2R	TTCGAACATTTAAGAAAGTAAC
Mul-2L	TTATTCGAATGGAGTTAACATC
Mul-1R	GAACCTCTTTCCGCATAGGT
Spis-5L	TATTCTTCTGTTGGTGGCTT
Spis-4R	TATAATTTTCAGTCGTATAGAAAG
Mya-4L	CTCCGTTGTCGAGAAATATAAT
Mya-5R	AAACGGGTGACATCCTGC
18S-Biv-3L	TGGCTCATTAAATCAGTTAT
18S-Biv-1	AAGAGTCCCGTATTGTTATT

Fig. 2 Fragment profiles produced with multiplex PCR on 5 target species (**a**) (full specific names in Table 1) and on 21 individual larvae collected at Woods Hole (**b**) (*Unlabeled lanes* show molecular size standards with some fragment sizes defined on right) 18S-positive control, although variable in size, is always slightly >400 bp whereas the COI species-specific fragments are all <400 bp. Among the larval samples in **b**, Lane 5 shows pattern diagnostic for *Mercenaria mercenaria*, Lane 18 has *Argopecten irradians* pattern, and *Spisula solidissima* diagnostic profile was seen in Lanes 10, 17 and 19–21. Faint COI products of expected size for *S. solidissima* were also observed in Lanes 1 and 14 of gels, but are difficult to see in the reproduction. Both of these faint products are false positive results from non-*Spisula* taxa (Table 3)

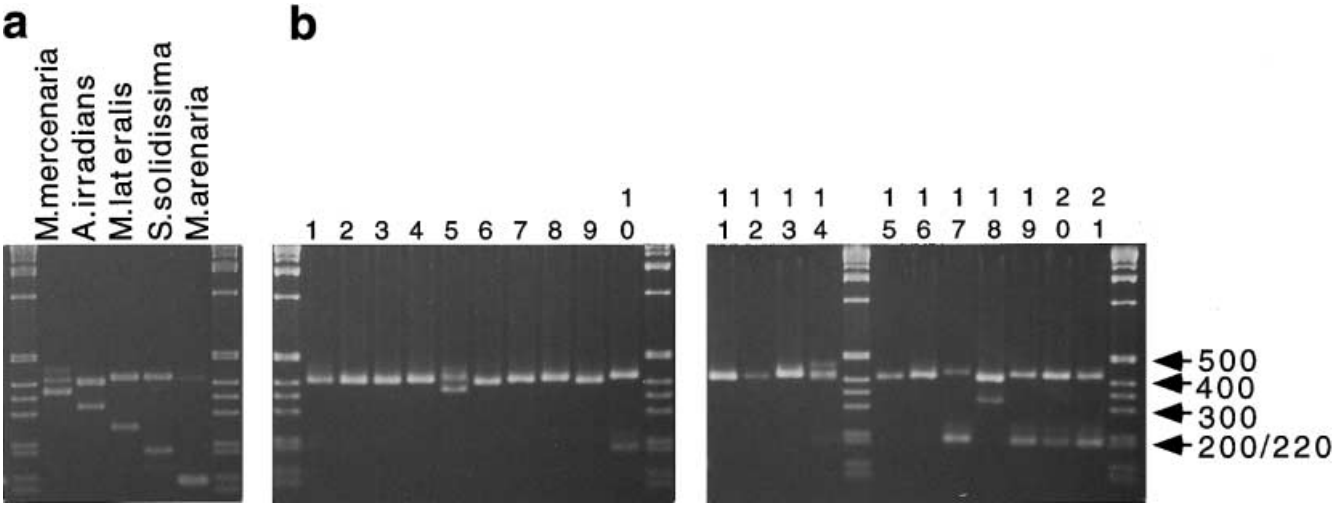


Table 3 Accuracy of multiplex PCR assay for identification of five target bivalve species using two different molar ratios, 3:1 and 2:1, of ribosomal 18S and COI primer pairs (see "Results"). Subset of PCR results using wild larvae were followed up with sequencing of 18S product. False negative (*neg.*) and false positive (*pos.*) results are defined in "Materials and methods"; they sum with the number of correct identifications to sample size [*No. PCR* for cultured larvae, No. sequenced (*No. seq.*) for wild-caught larvae] in that row (*WHOI* Woods Hole Oceanographic Institute, Massachusetts; *IOS* Isle of shoals, New Hampshire) Blank PCR results still produced amplifiable 18S product in some cases

Sample	No. PCR	PCR identification	No. seq.	18S sequence identification	False neg.	False pos.	Correct
3:1 Primer mix							
Cultured	7	*Mercenaria mercenaria*			0	0	7
	6	*Argopecten irradians*			3	0	3
	6	*Mulinia lateralis*			0	0	6
	6	*Spisula solidissima*			0	0	6
	7	*Mya arenaria*			0	0	7
Wild WHOI	3	*Spisula solidissima*	3	*Spisula* spp.	0	0	3
	3	*Argopecten irradians*	3	*Argopecten* spp.	0	0	3
	9	Nontarget	1	*Spisula* spp.	1	0	0
			5	*Ensis directus*	0	0	5
			2	Mytilidae	0	0	12
			1	Veneroida	0	0	1
	1	Blank	1	Mytilidae	–	–	–
IOS 5/99	24	Nontarget	1	*Mya arenaria*	1	0	0
			15	Veneroida	0	0	15
IOS 7/99	31	Nontarget	15	Mytilidae	0	0	15
	3	Blank	3	Mytilidae	–	–	–
Totals	106		50		5	0	73
Proportions					6.3		93.6
2:1 Primer mix							
Cultured	8	*Mercenaria mercenaria*			0	0	8
	8	*Argopecten irradians*			3	0	5
	8	*Mulinia lateralis*			1	0	7
	8	*Spisula solidissima*			0	0	8
	8	*Mya arenaria*			0	0	8
Wild WHOI	3	*Mercenaria mercenaria*	1	*Mercenaria mercenaria*	0	0	1
	2	*Argopecten irradians*	1	*Argopecten irradians*	0	0	1
	21	*Spisula solidissima*	5	*Spisula solidissima*	0	0	5
	1	*Spisula solidissima*	1	*Ensis directus*	0	1	0
	1	*Spisula solidissima*	1	Veneroida	0	1	0
	74	Nontarget	3	*Ensis directus*	0	0	3
			4	Veneroida	0	0	4
			2	Mytilidae	0	0	2
			1	Unionidae	0	0	1
			1	Pterioida	0	0	1
			1	Corbulidae	0	0	1
	1	Blank			–	–	–
Totals	142		21		4	2	55
Proportions					6.6	3.3	90.2

Larval samples from three plankton tows were screened in order to apply and further test the multiplex PCR assay. A subset of the identifications made from multiplex PCR profiles (Table 3, Fig. 2b) were subsequently confirmed by sequencing the 18S product amplified in each multiplex reaction. A total of 175 wild larvae were assayed and five (3%) gave blank PCR results, with no visible 18S or COI product. A secondary amplification of (invisible) 18S from four of the blank reactions generated products whose sequence corresponded to Mytilidae species in GenBank, indicating that blank reactions occur at low frequency for reasons other than the absence of template. Additional mytilid specimens (with identical 18S sequences) and at least six other non-target bivalve taxa were represented among the 71 larvae for which 18S was sequenced (Table 3).

The Woods Hole sample contained the greatest bivalve diversity, including three target species, *Mercenaria mercenaria*, *Argopecten irradians* and *Spisula solidissima*. Based on 18S sequences, 1 *M. mercenaria*, 4 *A. irradians* and 5 *S. solidissima* were accurately identified from their PCR profiles (e.g. Fig. 2b). Two false positive results were obtained, both involving a very faint *Spisula*-sized product amplified from a non-target taxon (Fig. 2b, Lanes 1 and 14). In the Isle of Shoals samples only a single larva proved to be from a target species, and this *Mya arenaria* individual gave a false negative PCR result (Table 3).

Discussion

Accurate and efficient (rapid, low-cost) identification of bivalve larvae to species in a large number of samples is

Table 4 Results of multiplex PCR with adult genomic DNA from nontarget species or distant populations (*S* southern; *FL* Florida). Nontarget species were expected to show 18S only, but sometimes had a non-diagnostic fragment in addition to 18S that was not an expected size for COI in any of target species. Diagnostic fragment refers to species-specific-sized COI product, with identified target species listed by genus only

Species	Locality	Latitude; Longitude	Amplification products			
			No. of reactions attempted	18S only	18S + non-diagnostic fragment	18S + diagnostic fragment
Mercenaria campechiensis	Panacea, FL	30°00′00″N; 84°20′00″W	2	1	0	1 *Mercenaria*
Pitar morrhuana	Massachusetts	41°34′27″N; 70°30′59″W	6	3	0	3 *Spisula*
Gemma gemma	Massachusetts	41°34′20″N; 70°30′50″W	5	1	1	3 *Spisula*
Argopecten irradians (S)	Panacea, FL	30°00′00″N; 84°20′00″W	2	0	0	2 *Argopecten*
Placopecten magellanicus	Massachusetts	41°00′00″N; 70°00′00″W	2	2	0	0
Rangia cuneata	Panacea, FL	30°00′00″N; 84°20′00″W	2	2	0	0
Spisula solidissima similis	Panacea, FL	30°00′00″N; 84°20′00″W	2	2	0	0
Spisula sp.	Massachusetts	41°33′07″N; 70°32′49″W	2	2	0	0
Macoma tenta	Massachusetts	41°30′40″N; 70°42′20″W	2	2	0	0
Tellina agilis	Massachusetts	41°32′18″N; 70°39′53″W	2	2	0	0
Crytopleura costata	Massachusetts	41°31′40″N; 70°43′20″W	2	1	1	0
Crassostrea virginica	Massachusetts	41°35′40″N; 70°39′50″W	2	1	0	1 *Spisula*
Mytilus edulis	Massachusetts	41°29′00″N; 70°39′50″W	2	2	0	0

required to determine the relationship between larval dispersal, settlement and recruitment. Larval identifications are particularly challenging with marine bivalves because there is a high diversity of species represented in the plankton (e.g. as many as 20 common species in New England waters alone; Abbott 1974), and this diversity increases the potential for mis-identification. In the molecular approach to larval identification reported here, the design of primers to amplify a species-specific sized product from individual larvae in multiplex PCR eliminated the need for any DNA extractions or restriction digestions. Efficiency was increased over previous multiplex assays by increasing the number of target species that can be positively identified from PCR with a single larva. Application of this method to identify larvae of five common and commercially important New England bivalve species showed 92% overall accuracy with only 1.4% false positives. This level of efficiency and accuracy can facilitate screening of the large numbers of samples needed to describe bulk larval movements. Moreover, both accuracy and efficiency of this assay potentially can be improved.

Accuracy of PCR identifications will partly depend on whether there is intraspecific polymorphism that can obstruct amplification in some samples, producing a false negative result. Additionally, closely related species, not initially available during primer design, may have enough sequence similarity to a target species to allow amplification, producing a false positive result. Thus, molecular identification assays must meet the unavoidable challenge of designing primers to discriminate among sequence differences at the species level while retaining insensitivity to polymorphism within the target species.

Diagnostic sequence variation was sufficiently abundant in bivalve COI for both primers of each target species to contain diagnostic nucleotides (Fig. 1, and A. Frese unpublished data). Therefore, discrimination of the target species by PCR was achieved with specificity of priming by two oligonucleotides to produce COI amplification. This provided higher specificity than if one conserved primer (complementary to all target species) was multiplexed with numerous species-specific opposing primers (Rocha-Olivares 1998). Although the specificity achieved here did not always distinguish closely related species (e.g. *Mercenaria campechiensis*, Table 4), addition of those species to the COI sequence alignment will make it possible to design even more accurate species-specific primers (compare *Mercenaria* spp. sequences in Fig. 1).

Distant populations of *Argopecten irradians* had 0.009% sequence divergence at COI, but this intraspecific polymorphism was not at primer sites and therefore did not disrupt COI amplification (Fig. 1, Table 4). The three *Spisula* taxa examined all had the same 12 nucleotides at the 3′ end of the Spis-5L primer site, but differed at the first and third 3′ nucleotides of the Spis-4R primer site (Fig. 1). These differences promoted discrimination of the three taxa so that even the southern subspecies, *S. solidissima similis*, was not confused with *S. solidissima* based on PCR results (Table 4).

More thorough sampling among populations of the target species is needed to test for intraspecific polymorphism that could generate false negative results, but several considerations suggest that any such effect would be small. First, COI seems to have low levels of polymorphism in marine bivalve species, as evidenced by *Argopecten irradians* (see above) and a sample of four *Mercenaria mercenaria* individuals with $<0.01\%$ sequence divergence (A. Frese personal communication). Second, rates of nucleotide change vary among sites within COI, and this predictable pattern facilitates the design of primers less sensitive to polymorphism. Because selection acts to reduce amino acid variation in COI, third codon positions typically have the highest

rate of evolution in coding sequence (compare the two *Argopecten irradians* sequences or the *Mercenaria*. congeners in Fig. 1). In PCR, specificity of primer annealing is primarily determined by complementarity at the 3′ end of the primer, with the terminal nucleotide most critical (Newton et al. 1989; Bottema et al. 1993). For these reasons, most COI primers used here had the first and second 3′ nucleotides complementary to first and second codon positions in COI, and the third position from the primer 3′ end corresponded to the so called "wobble" site that is most likely to be polymorphic. Two exceptions are primers with the penultimate 3′ position complementary to the wobble site (Argo-lL and Spis-5L, Fig. 1). Thus, primers used in this study minimize the likelihood that intraspecific polymorphism would influence PCR amplification.

Future improvement of accuracy in this multiplex bivalve assay could entail decreasing the frequency of blank reactions, false negatives or false positives. However, the 3% frequency of blank reactions observed here is relatively low (compared, e.g., with 9% in Bell and Grassle 1998), a surprising result given that DNA was crudely liberated from larvae during PCR in our procedure. The 6% frequency of false negatives here is typical of previously reported PCR identification assays (e.g. as high as 5% in Rocha-Olivares 1998). Decreasing the ratio of 18S to COI primers from 3:1 to 2:1 did not have the desired effect of reducing the frequency of false negatives. However, this ratio could be modified for individual COI primer pairs to improve amplification in those species – *Argopecten irradians* and *Mulinia lateralis* – that show the highest frequency of false negatives (Henegariu et al. 1997).

Some false positive results are due to incomplete information about species differences at the time of primer design (e.g. *Mercenaria* spp., see above), and these errors can be reduced as more comparative sequence information becomes available. However, false positive signals can also result from spurious amplification of a non-homologous product that is similar in size to that expected from a target species. As the number of primers is increased in a multiplex reaction there is an increasing probability that among genomic DNA templates from different species, two primers in the mix will find non-specific annealing sites capable of priming PCR. This may explain the false positive result in *Crassostrea virginica* because the COI sequence in this species is very unlikely to support amplification by the Spis-5L and Spis-4R primers (Fig. 1), and null results from the negative controls indicate a lack of generalized contamination. Given the low-resolution agarose electrophoresis used in this study, it is possible that the *Spisula solidissima*-sized products amplified in *C. virginica*, *Gemma gemma* and *Pitar morrhuana* (Table 4) were not actually COI at all, but were close enough in size to the expected *S. solidissima* product to cause confusion. This source of misidentification can be minimized by using higher-resolution techniques for visualization such as capillary or polyacrylamide electrophoresis of fluorescently-labeled products.

Relative to previously described molecular methods of larval identification, the multiplex PCR described here reduces costs in terms of expendable materials as well as greatly reducing processing time. The cost of expendable materials for a single multiplex reaction in this study was approx. 50 US cents, including materials used in electrophoresis and gel photography. This compares favorably to the $1.19 required to identify a single rockfish larva by multiplex PCR in Rocha-Olivares (1998) *after* DNA extraction. Our current method uses fewer reagents and is therefore less expensive than any identification method that uses probe hybridization to membrane-bound targets or restriction digestion. Allozyme markers might be less expensive than this multiplex PCR method, but allozymes have less resolution, versatility, and potential for automation (Graves et al. 1990; Hu et al. 1992). Allozyme methods are also impossible to use with ethanol-preserved material (Lavery and Staples 1990).

Our processing time is reduced many-fold by eliminating DNA extraction, probe hybridization, and restriction digestion steps in larval identification. A minimum of laboratory expertise is needed to perform the PCR reaction and gel electrophoresis for multiplex PCR identifications. Also, by using fluorescently labeled primers, this assay could be easily automated from the PCR step through the visualization of PCR products in a capillary electrophoresis robot. Automation would reduce the cost of expendables such as agarose and gel photography (9 US cents per larva) while increasing potential throughput and accuracy, as described above.

Conclusions

Progress in some areas of larval ecology is stymied by the inability to collect species abundance data from large numbers of samples collected at the necessary temporal or spatial scales. Larval dispersal in marine bivalves is even more challenging because most of the animals cannot be identified by morphological criteria alone. The taxonomic diversity of marine bivalves is daunting when attempting to target an assay toward select species, but at the same time, it is the great evolutionary depth of this molluscan clade that provides suitable DNA sequence diversity for an effective species identification assay using multiplex PCR. The relatively high efficiency of larval identifications using this method stems from its requirement for only a single molecular step and the ability to identify any one of five species (thus far) with that single step.

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