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REVIEW

Techniques for the identification of bivalve larvae

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ABSTRACT: Quantification of planktonic larval distributions has been limited by processing time, given the large numbers of samples generated by extensive field surveys. Until recently, the only technique available for reliable species identification of bivalve larvae was direct microscopic observation, but even this method is restricted to larval stages and species that can be distinguished morphologically. Molecular methods (e.g. antibody and oligonucleotide markers) show considerable promise for identifying bivalve larvae to species, regardless of developmental stage, alleviating ambiguity or subjectivity of some traditional, morphology-based taxonomy. Moreover, attaching species-specific molecular probes to fluorescent reporter tags, for example, has great potential for automated, expedited sample-processing. Optical identification techniques are promising, but probably not at the species level. Current methods of distinguishing bivalve larvae—morphological, molecular (i.e. immunological and DNA-based), or optical—are reviewed here to facilitate the selection of appropriate techniques for a given research problem and to stimulate the development of creative alternative approaches for rapid and accurate species identification.

KEY WORDS: Bivalve larvae · Species identification · Immunofluorescent probes · Oligonucleotide probes · Molecular markers

INTRODUCTION

Research that focuses on the role of the larval stage in population, community and ecosystems ecology has been greatly hindered by 2 major technological limitations in quantifying planktonic larval distributions. The first limitation has been obtaining large numbers of samples with adequate spatial and temporal coverage, especially in relation to the sampling of physical and chemical variables (Haury et al. 1978, Butman 1987, 1994, Levin 1990, Davis et al. 1992a, Garland & Zimmer 2002, Garland et al. 2002). Extensive sampling is required because larval distributions are notoriously dilute and patchy in both space and time (Gaines et al. 1985, Scheltema 1986, Davis et al. 1991, 1992b, Garland et al. 2002). The second limitation in quantifying planktonic larval distributions has been processing the large numbers of samples generated by extensive field surveys. Direct collections of invertebrate larvae in relatively long time series are now possible, for example, using a moored zooplankton pump (e.g. Butman 1994); however, processing these samples remains cumbersome. Weeks of intensive plankton sampling can lead to several years of full-time sample-processing. Until recently, the only technique available for reliable species identification was direct microscopic observation, but even this method is effective only for those larval stages and species that are distinguishable morphologically.

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Many of the youngest larval stages, particularly of bivalves (frequently the most abundant invertebrate larvae in coastal samples) are so similar in appearance during their early development that they cannot be identified to species definitively using gross morphological criteria alone (e.g. Loosanoff et al. 1966, Chanley & Andrews 1971, LePennec 1980). Ignoring these early larval stages or lumping them into supra-specific categories may limit the scientific or management questions that can be addressed meaningfully with field data. Species data are crucial because species-specific behavioral repertoires of planktonic larvae have been invoked to explain patchiness in adult distributions (Hannan 1981, Grosberg 1982, Caffey 1985, Shanks & Wright 1987). Shell-fisheries management, for example, is usually directed at a few key, commercially important organisms, and species-specific information on larval distributions of the targeted species is needed for understanding recruitment variation (e.g. Mann 1988, Weinberg 1999).

Molecular methods (e.g. antibody and oligonucleotide markers) hold considerable promise for identifying bivalve larvae to species, regardless of developmental stage, thereby alleviating some of the ambiguity or subjectivity of traditional morphology-based taxonomy and eventually expediting sample-processing. Yet there are tradeoffs between specificity and efficiency. For instance, the technology required to apply molecular methods is not as accessible as a light microscope. Moreover, morphological or molecular techniques are presently applied to reasonably intact specimens directly sampled in the field. Acoustic (e.g. Holliday 1980, Pieper & Holliday 1984, Greene & Wiebe 1990) and other remote-imaging technologies, such as the video plankton recorder and the optical plankton counter (e.g. Ortner et al. 1981, Davis et al. 1992a, Herman 1992, see review in Dickey 1988), were not designed to obtain detailed taxonomic information on bivalve larvae. Sound-scattering, for example, is unlikely to vary sufficiently among small, morphologically similar bivalve larvae to yield unique target strengths.

Recent leaps in technology development for obtaining time series of larval concentrations using automated direct samplers have considerably out-paced technological development for efficient and accurate enumeration and identification of these animals to species. Methods of distinguishing bivalve larvae – morphological, molecular (i.e. immunological and DNA-based), and optical – are reviewed here to facilitate the selection of the appropriate technique for a given research problem and to stimulate the development of creative alternative approaches for rapid and accurate species identifications.

TECHNIQUES

Morphological

Microscopic examination remains the most popular technique for distinguishing bivalve species, although the subjectivity associated with this approach can render it problematic. Detailed examination of morphological features requires comparisons between sampled larvae and voucher collections, e.g. preserved specimens, drawings, or photographs of larvae of known origin that have been identified by experts. Yet there are over 200 species of bivalves present off the east coast of the United States alone (Gosner 1971), and the larval stages have been described for less than ¼ of these species (Loosanoff et al. 1966, Chanley & Andrews 1971, Lutz et al. 1982). For example, there are ≥16 species of *Tellina* in West Atlantic waters, with larval descriptions of only 2: *T. agilis* and *T. tenera* (e.g. Sullivan 1948, Chanley & Andrews 1971). Moreover, certain genera contain many closely related and morphologically similar species. Even genera in disparate families may be virtually indistinguishable (e.g. Savage & Goldberg 1976, Lutz et al. 1982). Detailed comparison of morphological features among sympatric bivalves is required for definitive identification, which is problematic given the paucity of published larval descriptions.

Early research on bivalve larvae from Europe (Lebour 1938, Werner 1939, Jørgensen 1946, Rees 1950), Japan (Yoshida 1953, 1957, Miyazaki 1962), and North America (e.g. Stafford 1912, Sullivan 1948) was largely descriptive, whereas subsequent keys (Loosanoff et al. 1966, Chanley & Andrews 1971) provided detailed information and comparisons among various species. According to these more recent guides, bivalve larvae can be classified based on shape, dimensions, hinge-line length, umbo character and color (Loosanoff et al. 1966, Chanley & Andrews 1971). Most bivalve larvae appear morphologically similar at the early, straight-hinged stage, however, and some groups remain morphologically indistinguishable even at later stages. Thus, the targeted morphological characteristics at early stages are generally insufficient for definitive species identification. Phenotypic plasticity also renders morphology-based discrimination questionable. For example, the expression of many salient morphological characters is dependent upon environmental conditions, such as food concentration and water temperature (e.g. Shirley et al. 1987, Boidron-Métairon 1988, Strathmann et al. 1992).

Taxonomic criteria using invariant morphological characters is desirable because of the high variability associated with the expression of morphological characters, targeted by Loosanoff et al. (1966) and Chanley

& Andrews (1971), e.g. color. Werner (1939) and Rees (1950) were the first to note the uniqueness of each species' hinge structure (i.e. shape and placement of hinge 'teeth'; Loosanoff et al. 1966), even at the straight-hinge stage. Visualizing the hinge structure became easier with the use of scanning electron microscopy (SEM; Turner & Boyle 1975, Lutz & Jablonski 1978, 1979, Lutz & Hidu 1979, LePennec 1980, Lutz et al. 1982, Fuller et al. 1989). These techniques require, however, the disarticulation of shells from individual larvae, and the meticulous leveling of shells before viewing under the SEM. Only a limited number of larvae can be examined because this method is very time-consuming.

Regardless of the chosen resolution—relatively high for SEM or low for light microscopy—there will always be a certain degree of subjectivity associated with morphology-based taxonomy. Moreover, there is a general tendency to assign names that exist in taxonomic keys as opposed to leaving an organism unidentified. Reliance on morphological criteria alone means that the accuracy of identifications depends on the level of expertise of the identifier, and that both accuracy and precision may be sacrificed when larval identifications within a given sample or region are made by several taxonomists. In contrast, molecular methods potentially decrease subjectivity and increase both the accuracy and precision of taxonomic determinations.

Immunological

Immunological techniques for recognizing species in mixed populations capitalize on the occurrence of unique, diagnostic, 'signature' molecules—often proteins or portions thereof—within a given species (e.g. Beltz & Burd 1989). These molecules, when injected into a vertebrate host such as a mouse or rabbit, are regarded as 'foreign' and an immune response is triggered within the host. During this response, the host produces antibodies in order to confer immunity against the foreign substances, or 'antigens', in the blood stream. These newly expressed antibodies recognize and bind to their homologous signature antigen, in this case a portion of the larval protein. The target region on a larval antigen is referred to as an 'epitope,' and its exposure to the antibody can be extremely sensitive to conformational changes in the protein. Repeated injections of the epitope into the vertebrate host (hyperimmunization) results in accelerated antibody production within the host. Immunostimulants and protein-expression vectors are sometimes added to the injection mixture to maximize the immune response.

Using well-documented biochemical techniques, antibodies formed in this manner can be isolated from the host, purified, and tagged with fluorochromes or other appropriate reporter markers that can be detected visually (e.g. Harlow & Lane 1988, Beltz & Burd 1989). Under standardized reaction conditions, these tagged antibodies will recognize the complementary epitope(s) against which they were produced. Obtaining species-specific antibody probes is contingent, however, on finding an epitope that is unique to only 1 bivalve species. Targets include epitopes that are conserved within a single species, regardless of its developmental stage or physiological state, but that are not present among other closely related species of bivalves. Thus, it is necessary to compare proteins among sympatric bivalves in a voucher collection. Conformational changes in a protein that would mask exposure of the epitope are common, however, even resulting from differences in specimen freshness and method of preservation.

Immunological techniques have been used to address research questions in biological oceanography and larval recruitment (see reviews by Bohlool & Schmidt 1980, Yentsch et al. 1988, Powers et al. 1988, 1990, Ward 1990). The most extensive, early immunochemical applications were for food-web analyses, i.e. identifying taxa in macerated gut contents (Feller et al. 1979, Feller & Gallagher 1982). Although trophic groups were usually distinguished, the antibodies produced in these studies provided limited taxonomic resolution; because of extensive antibody cross-reactions among species, the resolution rarely extended below the ordinal or familial taxonomic levels.

Improvements in protein isolation and antibody purification procedures have provided greater taxonomic resolution for both single-cell (e.g. Dahle & Laake 1982, Campbell et al. 1983, Ward & Carlucci 1985) and multi-cellular (e.g. Feller et al. 1979, Feller & Gallagher 1982, Shapiro et al. 1989, Ohman et al. 1991, Campbell et al. 1994) organisms, including planktonic larvae of benthic invertebrates (e.g. Feller 1986, Miller et al. 1991, Demers et al. 1993, Hanna et al. 1994). However, species-specificity was not always attained. The ultimate degree of specificity remained low for antibodies produced in both the early food-web studies and the studies on invertebrate larvae because none of this research targeted a species-specific epitope for use in the production of antisera. Rather, whole organisms (i.e. containing multiple proteins versus a signature antigen) were homogenized and used to inoculate the vertebrate host. Within the protein complement of the homogenized organism, the majority of proteins are shared across supra-specific taxonomic groups and only a small number of proteins are species-specific. Thus, multiple-antigen injections trigger immune

responses, producing numerous antibody types that must be purified further and screened for effectiveness (Feller & Gallagher 1982).

After multiple-antigen injections were made in the food-web and early larval studies, polyclonal (Feller et al. 1979, Feller & Gallagher 1982, Demers et al. 1993) and monoclonal (Miller et al. 1991, Hanna et al. 1994) antibodies were selected that showed the least reactivity with other, non-targeted, organisms. The resulting polyclonal antibodies were successful as generic and higher-taxon-specific markers, commensurate with the initial goals of these groundbreaking studies, but were not reliable species-specific markers because of substantial cross-reactivity with other species (Feller & Gallagher 1982). The monoclonal antibodies were more successful in terms of species-specificity, but at considerable initial cost in terms of production and screening time. The monoclonal procedure involves culturing large numbers of isolated cell lines—each producing antibodies toward a single epitope—and assaying for the clones showing minimal reactivity with non-targeted species. Distinguishing barnacle larvae is the most successful case thus far (Miller et al. 1991), yet application of monoclonal antibodies required 2 steps to separate 3 species.

The advantages of polyclonal antibodies are higher affinity, wider reactivity, longer shelf life, simpler production techniques once the antigen has been purified, and lower overall production costs in terms of both time and expense. However, unless the injected antigen is species-specific, polyclonal antibodies are generally inadequate for species identifications unless they are purified further by adsorption, affinity chromatography or blocking techniques (e.g. Harlow & Lane 1988, Buchmann et al. 1992, Hockfield et al. 1993, Mendoza et al. 1995, Costas & Lopez-Rodas 1996). The advantages of monoclonal antibodies are large-scale production (using tissue culture methods) and high specificity. Purification of the antigen is also unnecessary because initial screening for species-specificity occurs after antibody production in the host cell-line (Beltz & Burd 1989). However, the binding characteristics of monoclonal antibodies are often unreliable. Because they are so specific, the effectiveness of monoclonal antibodies can be compromised by any slight degradation of the epitope. Refinements to the monoclonal antibody technique, such as creating multiple monoclonal antibody 'cocktails', may well yield the desired species-specificity and shelf life for larval probes (Demers et al. 1993, but see Gallagher et al. 1988 and references cited therein).

Many studies have applied polymorphic allozyme electrophoresis techniques to discriminate among groups of adult bivalves (e.g. Beaumont et al. 1989, McDonald et al. 1991, Benzie & Williams 1998). Hu et al. (1992) adapted these techniques to distinguish successfully among larvae of 3 oyster species. If 1-, 2-, or 3-dimensional electrophoresis can be used to isolate species-specific general proteins or allozymes, then these bivalve proteins or allozymes can be excised from electrophoretic gels and used to inoculate a vertebrate host (e.g. Crowle et al. 1972, Caldwell et al. 1975, Diano et al. 1987, review in Anderson 1983). Early studies using this approach noted favorable results on invertebrate larvae (Feller 1986) and adults (Gallagher et al. 1988). More recently, this technique was coupled with adsorption purification techniques to identify scallop (*Pecten maximus*) larvae, although the probes were not tested on other scallop species (Paugam et al. 2000).

Immunofluorescent markers developed against a defined epitope may be devoid of the known disadvantages of antibody probes, such as limited yield of monoclonal antibodies, difficulty in producing species-specific antisera, high degree of cross-reactivity, and the effort required to maintain tissue cultures required for the production of monoclonal antibodies. Polyclonal antibodies developed in this manner should provide adequate sensitivity and specificity, and production is generally much less laborious than for monoclonal antibodies (Macario & Conway de Macario 1983, Harlow & Lane 1988).

Two major drawbacks of antibody probes may limit their potential effectiveness. Firstly environmental conditions, ontogenetic changes in the larvae, and sample preservation status may alter protein concentration or conformation of the protein's epitope, and these changes can increase variability in the antibody binding response (e.g. Feller 1986, Demers et al. 1993). Secondly larval proteins may be highly conserved and thus may not differ sufficiently among species for use as species-specific markers. Isolating species-specific, immunogenic and stable epitopes—those that do not change with age, stage, physiological state, preservation status, or reaction conditions—represents the ultimate challenge.

DNA-based

Using a combination of cytogenetics (examination of chromosomes and nuclei at the cellular level) and cytology (staining of structures inside cells), early molecular techniques involved karyotyping—differentiating amongst taxa using chromosome number and characteristics (e.g. size, type and morphology). Cytogenetic examination of bivalve larvae is possible once the shell has been dissolved (Stiles & Choromanski 1987). Although karyotyping has proven useful in some applications (e.g. Blaxhall 1983), and could potentially

be automated using other molecular methods such as fluorescent *in situ* hybridization (e.g. Zhang et al. 1999, Libertini et al. 2000), the technique may be somewhat cumbersome and perhaps not as specific as more recent molecular methodologies. Over the last decade, the focus in molecular technologies for species identification has shifted toward the fundamental information code in the cell, deoxyribose nucleic acid (DNA), and away from higher structural levels (e.g. genes, chromosomes, or proteins).

The DNA of an organism is largely invariant with age, stage, or physiological state, yet it varies among different taxonomic groups. For these reasons, DNA has been a target for species-specific probe development for a number of plants and animals (see reviews by DeLong et al. 1989, Stahl & Amann 1991, Amann et al. 1995), including larval invertebrates (Olson et al. 1991, Banks et al. 1993, Geller et al. 1993, Coffroth & Mulawka 1995, Medeiros-Bergen et al. 1995, Ó Foighil et al. 1995, 1998, Geller 1996, Bell & Grassle 1997, 1998, Toro 1998, André et al. 1999, Hare et al. 2000, J.P. Grassle & P. Nelson unpubl. data).

The design of both immunochemical and oligonucleotide probes involves a similar strategy in that 'signature molecules' unique to a particular species are identified and isolated. In oligonucleotide approaches, the signature molecule is a small sequence or piece of nucleic acid (DNA or RNA), whereas in immunochemical approaches the signature molecule is a product of gene expression (usually a protein or a portion thereof). Ultimately, the success of either technique hinges on knowledge of the protein or genetic makeup (i.e. gene sequences) of sympatric species, and the uniqueness and stability of the targeted molecules. Thus, it is necessary to consult a voucher collection of organisms of known origin that have been accurately identified.

Traditionally, the development of oligonucleotide probes involves identifying a DNA sequence that is conserved (in terms of nucleic acid sequence similarity and length) within a species, and does not occur in closely related species. DNA must first be extracted from adult or larval tissue of as many sympatric species as possible, the DNA amplified using the polymerase chain reaction (PCR), the nucleic acids sequenced, and unique genetic signatures identified by comparing sequences (Rice 1990, Rice et al. 1993). Finally, 'specific primers' (complementary nucleic acid sequences) are designed to target the unique sites and labeled for probe production (Hockfield et al. 1993, Dieffenbach & Dvekster 1995).

Both mitochondrial (mtDNA) and nuclear ribosomal (rDNA) DNA have been targeted for bivalve probe production. In general, nuclear rDNA is more conservative than mtDNA because mutation rates are typically greater in mtDNA. Although mtDNA has remarkably stable gene order and content, variations occur, mainly as length differences, especially in the 'non-coding' or control regions (regions that do not code for proteins). Thus, the non-coding regions of mtDNA tend to be useful in phylogenetic studies of species and populations, whereas nuclear rDNA tends to be more useful in phylogenetic studies of genera and families.

The DNA probes developed by Bell & Grassle (1997, 1998) targeted a sequence within nuclear 18S rDNA which was family-specific for mactrid bivalves (in this case, the surfclam *Spisula solidissima* and the coot clam *Mulinia lateralis*). A 2-step PCR-restriction fragment length polymorphism (RFLP) technique was used to differentiate between the 2 species (Bell & Grassle 1998). In RFLP analysis, restriction enzymes are used to cleave bonds between specific nucleotides in the PCR amplification products, resulting in fragments of nucleotide chains (e.g. Silberman & Walsh 1992). Fragment lengths vary among taxa and are quantified using electrophoresis. Other studies involving 18S rDNA yielded family-level discrimination (e.g. Kenchington et al. 1994, Adamkewicz et al. 1997).

Mitochondrial DNA coding for the small ribosomal subunit (16S rDNA) has been targeted in oysters and mussels, but the resulting probes did not differentiate among congeners (Banks et al. 1993, Geller et al. 1993, Ó Foighil et al. 1995). These studies also applied a 2-step PCR-RFLP analysis for species-specific discrimination, yet restriction fragments were obtained (as in the Bell & Grassle 1998 study), indicating that at least 1 base pair differed in the targeted bivalves. In theory, only a single base pair difference is required to discriminate between 2 species using a single-step assay called the ligase chain reaction (LCR; reviewed in Wiedmann et al. 1995). In the LCR, 2 primers are ligated together only when they occur adjacent to each other, and are used to probe for single base-pair differences in the targeted sequence. Thus, application of LCR may have led to successful discrimination in these studies.

The mitochondrial cytochrome-c oxidase subunit I DNA (mtCOI) has been effective for resolving species (Palumbi & Benzie 1991). Targeting variation in the mtCOI gene, primers were developed by Folmer et al. (1994). Mitochondrial COI gene sequences were used to identify the origin of adult oysters suspected of being transferred as larvae in ballast water (Ó Foighil et al. 1998). Probes targeting the mtCOI gene were also used to differentiate amongst 5 species of freshwater mussels using the combined PCR-RFLP approach (Baldwin et al. 1996). A single-step DNA assay involving the mtCOI gene has been developed recently for identifying larvae of 5 species of coastal bivalves (Hare et al. 2000). Rather than utilizing DNA

extractions or restriction digestions, primers were designed to amplify species-specific size products from the mtCOI gene of individual larvae. Several species-specific primer pairs were multiplexed in a single reaction so that all 5 target species were assayed simultaneously.

Both the PCR-generated probes (e.g. Heath et al. 1996, Hare et al. 2000) and the PCR-RFLP methods (e.g. Silberman & Walsh 1992, Bell & Grassle 1998) require sequence information about and primers designed for the targeted genome. An alternative approach utilizes PCR-generated randomly amplified polymorphic DNA (RAPDs). In the RAPD technique, DNA is first extracted from the target specimen and then amplified using the PCR. Multiple 'random primers' (not specific to any gene sequence) are used to generate many fragments of different lengths (Welsh & McClelland 1990, Williams et al. 1990). Next, electrophoretic molecular weight separation techniques identify fragments (called 'polymorphic markers') that are species-specific. Subsequently, the individual primer(s) that produced polymorphic markers amplify and probe DNA from unknown larvae. PCR-RAPD markers differentiated, for example, among 5 species of gorgonian coral larvae (Coffroth & Mulawka 1995), and between larvae of 2 congener oyster species (André et al. 1999). The main advantage of PCR-RAPD probes is that their production requires much less technology and time than other DNA-based probes because no knowledge is required of nucleotide sequences in the target organism or in sympatric species. One disadvantage, however, is that PCR-RAPD techniques are sensitive to reaction conditions, such as temperature and DNA concentration (see reviews by Burton 1996, Grosberg et al. 1996).

Once the proper genetic signature has been targeted, oligonucleotide probes can be produced to any degree of taxonomic specificity. Thus far, however, larvae have been manually sorted from plankton samples and analyzed individually or in small groups via gel electrophoresis, rendering this technique somewhat laborious. Still, this approach shows great promise, especially if the techniques become more automated in the future (see Hare et al. 2000).

Optical

Within the field of marine science, image-analysis techniques have been used to determine the biomass of planktonic organisms (e.g. Bjørnsen 1986, Sieracki & Viles 1990, Bittner et al. 1998) and to determine their sizes and shapes (Gevirtz 1976, Jeffries et al. 1984, Estep & MacIntyre 1989, Beaulieu et al. 1999, reviews in Fawell 1976 and Berman 1990). Furthermore, optical-digital methods have been successful for identifying certain groups of phytoplankton (e.g. Pech-Pacheco & Alvarez-Borrego 1998, Culverhouse et al. 1996, McCall et al. 1996) and zooplankton (Gallager et al. 1996) based on their size and shape characteristics.

In their present state of development, optical techniques cannot distinguish the majority of larval bivalves because species-specific characters are generally found at the microscopic (i.e. morphological) or molecular (i.e. proteins or nucleic acids) level. For example, imaging morphological characters and dimensions is highly dependent upon the orientation of the specimen—a factor not easily controlled *in situ* (Fuller et al. 1989). Unique and stable macroscopic optical features have yet to be identified.

Automated optical techniques are useful when higher taxa are targeted (e.g. identification to class Bivalvia rather than to species) or in coupling optical techniques with molecular tagging procedures (i.e. as in Amann et al. 1990a). Different species within a sample can be color-coded by attaching species-specific molecular probes to fluorescent reporter tags that, once excited, emit at a given wavelength of light. For example, Species A can be labeled with a fluorescein-conjugated probe, Species B with a rhodamine-conjugated probe, and Species C with a AMCA-conjugated probe (Jackson Immunology Research Laboratories, West Grove, PA). These probes are visualized as green-yellow, red and blue emitted light, respectively, using a single wide-band excitation source (Harlow & Lane 1988, Recktenwald 1992; Molecular Probes, Eugene, Oregon). Thus, Species A, B and C can be readily differentiated by their colors, and an image-analysis or flow-cytometry (e.g. Radbruch 1992) system can be used to automate the process. A major challenge is detecting an adequately expressed signal, either by targeting a tagged molecule on the shell surface or by amplifying the signal of a molecule tagged within bivalve tissue.

Perspective

Microscopic techniques are advantageous because, like most invertebrates, much of the traditional taxonomy of bivalve molluscs has been based on morphological differences. Yet, as in many other taxonomic groups, molecular techniques potentially provide new criteria for more reliable identification of bivalve larvae, as well as distinguishing larvae that cannot be differentiated using morphological characters alone. Although comparisons among studies using morphology-versus molecular-based identifications may initially be problematic, the conceivable gain certainly merits the effort.

Immunofluorescent and oligonucleotide probes have their advantages and disadvantages. Some applications are complementary (e.g. Herrera Medina 1982, Macario & Conway de Macario 1983, Powers et al. 1990), especially when used in concert with traditional assays. Ideally, the most time- and cost-effective marker technique for use in automated processing of large numbers of field samples should: (1) involve no direct sorting of organisms from a sample (instead, the probe would be applied to a multi-species assemblage in a small dish); (2) be effective for intact, whole organisms (so that specimens can be saved for other analyses); (3) result in a sufficiently detectable surface expression on the organism for detection via image-analysis techniques (for automated counting and sizing); (4) be relatively inexpensive to develop (to generate probes for a large number of species); (5) be relatively inexpensive to produce once developed; (6) produce accurate and repeatable results.

Immunofluorescent probes can be applied to whole organisms within a sample, which can then be sized simultaneously with an image-analysis identification system and saved in voucher collections for other analyses (e.g. basic morphometrics). The main disadvantages of developing and implementing this technique include: the requirement to extract protein from the larvae of numerous bivalve species that are often difficult to raise or acquire; the potential lack of species-specificity; and the conceivably highly variable results (because protein expression is dependent upon a suite of endogenous and exogenous factors).

Oligonucleotide probes have the advantage of being developed using adult tissue that is much easier to obtain than larval tissue, especially considering the number of species required to build a voucher collection. Moreover, DNA varies less than morphology, proteins and optical characters. The potential specificity of oligonucleotide probes also ranges from individuals (e.g. human fingerprinting, as in Jeffreys et al. 1985) to higher taxonomic levels, which can be viewed as an asset or a liability depending upon the scientific question. On the downside, application of most oligonucleotide probes (e.g. dot blot methods, as in Silberman & Walsh 1992; PCR methods as in Cary et al. 1993, Olson et al. 1991, Medeiros-Bergen et al. 1995) requires destructive processing of the organisms or parts of the organisms. Although *in situ* oligonucleotide probes do not destroy the organisms (DeLong et al. 1989; Amann et al. 1990b, 1995), they must still be individually isolated from samples before testing. Finally, to assess fully the accuracy of oligonucleotide probes, more documentation is needed on how the DNA of local populations is affected by interspecific hybridization (Gaffney & Allen 1993) and the introduction of non-endemic congeners. For example, contamination from non-endemic aquaculture hatchery stocks (Naylor et al. 1998) and ballast water (e.g. Carlton 1985, Carlton & Geller 1993) is becoming widespread worldwide, and could lead to the corruption of local population gene pools (e.g. Geller 1996).

The high degree of specificity makes oligonucleotide probes desirable for many applications: tracking the dispersal of organisms originating from a particular population (e.g. Bucklin et al. 1992, Martin et al. 1992); difficult species identifications or tracking relatively rare larvae that can be easily sorted from plankton samples (e.g. Olson et al. 1991, Bell & Grassle 1998); biodiversity studies (DeLong et al. 1993); and whenever targeted material is available in limited supply and the DNA must be amplified by the PCR (e.g. Giovannoni 1991, Cary et al. 1993), such as when a single larva is isolated from the deep sea (e.g. Berntson 1998). Oligonucleotide probes may be inefficient, however, for large-scale identification of a species across its geographic range. In this case, immunochemical methods may be more efficient (e.g. for determining planktonic larval distributions) when it is not feasible or practical to sort larvae from samples and when targeted material is available in large amounts or can be easily cultured in the laboratory during development of the probe. Immunofluorescent tagging methods are operable on whole organisms that need not be sorted from the sample individually.

All approaches—morphological, molecular and optical—are sensitive to organism damage and preservation artifacts (France & Kocher 1996, Dawson et al. 1998). In addition, all approaches require information on sympatric species in order to identify characters (e.g. morphological features, proteins, nucleotide sequences or optical qualities) that are unique to a given species. Thus, it is critical to maintain a voucher collection consisting of accurately identified organisms of known origin. A voucher collection of larval bivalves (spawned and reared in the laboratory and expertly identified) is required in morphological and optical techniques as well as for the development and testing of immunofluorescent probes. For oligonucleotide probes, the voucher collection could consist largely of adult bivalves because, in theory, larvae and adults should have identical DNA. However, oligonucleotide probes must also be applied to larval tissue to test for reaction effects such as differences in DNA concentration.

The ability to detect optically, distinguish amongst, and enumerate dissimilarly colored dots (i.e. the tagged larvae) is a straightforward, well-described application of image-analysis technology (e.g. Bjørnsen 1986, Sieracki & Viles 1990, Amann et al. 1990a, reviews by Inoué 1986 and Berman 1990) and flow cytometry (e.g. Yentsch 1990, Radbruch 1992). It is the

next logical step to couple these technologies with the types of molecular probes discussed here. The greatest challenges in these fields are to pinpoint species-specific and stable signatures—morphological, molecular and optical—and to streamline the technology involved in the application of molecular probes.

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