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Electrophoretic identification and genetic analysis of bivalve larvae*

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Abstract. Taxonomic identification and genetic analysis of larval marine invertebrates have been vexing problems. We describe a polyacrylamide mini-gel electrophoresis technique for resolving proteins from individual larval bivalves (shell length 250 to 350 μm) and apply this technique to three species of laboratory-cultured larval oysters [*Ostrea edulis* L., 1758, *Crassostrea gigas* (Thunberg, 1793) and *C. virginica* (Gmelin, 1791)] reared during summer 1989. Electrophoretic patterns of proteins clearly discriminate among the three species and allow genetic analysis of a polymorphic allozyme locus (*Pgi*) in field-collected larvae and juveniles of *C. virginica*. This technique provides an economical tool for large-scale taxonomic, ecologic, and genetic studies of meroplanktonic stages of various species.

Introduction

Basic studies of the ecology and population genetics of marine invertebrates have long been hampered by an inability to identify and analyze early life history stages of species isolated from planktonic or benthic samples. For example, assessing the impact of natural or anthropogenic disturbances on marine ecosystems, predicting recruitment for fisheries management, optimizing the timing of substrate placement for aquaculture, and conducting basic ecological surveys all depend on identifying the seasonal and spatial abundance of meroplanktonic stages of various species. Similarly, many unanswered questions regarding population structure, breeding systems, and natural selection await development of techniques for genetic analysis of individual larvae and early juveniles. Taxonomic assignment of larval marine bivalves is particularly difficult because differences in shell morphology that typically serve as systematic tools are notoriously unreliable. Simultaneously, genetic analysis has been hampered by the small quantity of soft tissues present for protein electrophoretic or other genetic studies. Recently, polymerase chain reaction (PCR) techniques were used to identify two species of larval sea cucumbers (Olson et al. 1991) Although PCR techniques are not limited by the small size of specimens, application to large-scale meroplanktonic surveys might be limited by great expense in terms of materials, time, and effort. Furthermore, in the absence of suitable DNA primers for polymorphic genes in most marine invertebrates, the PCR approach will not be useful for basic population genetic studies of breeding systems and natural selection.

Protein electrophoresis has rarely been applied to planktonic marine organisms. Bucklin and Marcus (1985) used polyacrylamide gel electrophoresis (PAGE) to study differentiation among three populations of the copepod *Labidocera aestiva* (ca. 1 mm body length), and Mork et al. (1983) identified pelagic eggs (diameter 0.6 to 2.7 mm) of 11 gadoid and six flatfish species by isoelectric focusing. Compared to whole copepods and fish eggs, larval bivalves are minute, usually less than 400 μm shell length and composed mostly of shell. Here we describe a new technique for preparing minute specimens of marine bivalves for PAGE analysis of proteins and enzymes and apply this technique to individual larvae (250 to 350 μm in length) of three commercially important species of oysters: *Ostrea edulis*, *Crassostrea gigas*, and *C. virginica*.

Materials and methods

Adult specimens of *Ostrea edulis* L., 1758, *Crassostrea gigas* (Thunberg 1793), and *C. virginica* (Gmelin 1791) were obtained from the following sources: *O. edulis* from the University of Maine, Darling Center, Walpole, Maine, in July 1989; *C. gigas* from the hatchery stock of Coast Oyster Co., Quilcene, Washington, in July 1989; *C. virginica* from the Virginia Institute of Marine Sciences, Eastern Shore Laboratory, Wachapreague, Virginia, in July 1989. Ca. 20 oysters of each species were induced to spawn under laboratory conditions, and larvae were reared using standard culture methods (Loosanoff and Davis 1963). In addition, on 5 September 1990, eyed-larvae of *C. virginica* were isolated by pumping natural seawater in Wachapreague, Virginia, through a plankton net (100 μm

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mesh). On the same day, shell substrates were placed in the open water and natural larvae were allowed to settle. Two weeks later, we sampled juvenile oysters (shell length 3 to 7 mm) from the shell substrates. Eyed-larvae of all three species were placed in seawater in 1.5 ml polypropylene centrifuge tubes and frozen at −60 °C. To date, frozen larvae have been held at −60 °C for 3 yr without substantive loss of enzyme activity or electrophoretic resolution.

Upon thawing, larvae were held in a watch glass on ice, and whole individuals were isolated into 400 µl microcentrifuge tubes with 1 to 2 µl of "grinding solution". For general proteins, the grinding solution contained: 62.5 m*M* Tris-HCl pH 6.8, 2% w/v sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 12% glycerol, and 0.001% w/v bromophenol blue. For allozymes, the grinding solution contained: 1 m*M* Tris-HCl pH 6.8, 0.1 m*M* EDTA, 0.1 m*M* $MnCl_2$, 0.04% NADP, 12% glycerol, 0.4% Triton-100, and 0.001% w/v bromophenol blue. Tissues were homogenized with a glass rod that was tapered to fit closely the diameter of a microfuge tube. Extracts were spun for 3 to 5 s at 12000 *g* in a Beckman 152 microcentrifuge at 5 °C.

A thin-layer polyacrylamide gel electrophoresis (PAGE) system (Model 2050, Pharmacia LKB, Piscataway, New Jersey, USA) was modified to reduce the size of sample insertion wells. New sample insertion combs were fabricated from 0.5-mm thick polyethylene film. For general proteins, we cast 15 wells (0.5 × 1.5 mm), each holding 2 µl of tissue extract. Gels were made of 10% SDS-polyacrylamide gels in a 0.375 *M* Tris-HCl, pH 8.8, buffer (Laemmli 1970). A stacking gel (3% acrylamide in 0.125 *M* Tris-HCl, pH 6.8) was necessary for obtaining tight bands. Constant amperage of 13 mA was applied for 45 to 55 min at 20 °C. Silver staining (National Diagnostics, Manville, New Jersey, USA) was used to reveal soluble proteins.

For allozymes, we cast 24 wells (0.5 × 1.0 mm), each holding 1.5 µl of tissue extract. Gels were 5% w/v acrylamide-bisacrylamide gels in 0.025 m*M* Tris, 0.129 m*M* glycine, pH 8.5. A 3% stacking gel (as above) was necessary. Gels were run at 5 °C for 55 min, with a starting voltage of 150 V, then a constant voltage of 200 V. Enzyme staining procedures (Hebert and Beaton 1989) were modified by substituting 2.5% agarose for agar in the stain overlays. To be consistent with earlier studies of oysters (Buroker et al. 1979, Buroker 1983), we assigned allelic designations based on their percentage mobility relative to the allozyme encoded by the common allele (designated as *100*) in *Crassostrea gigas*.

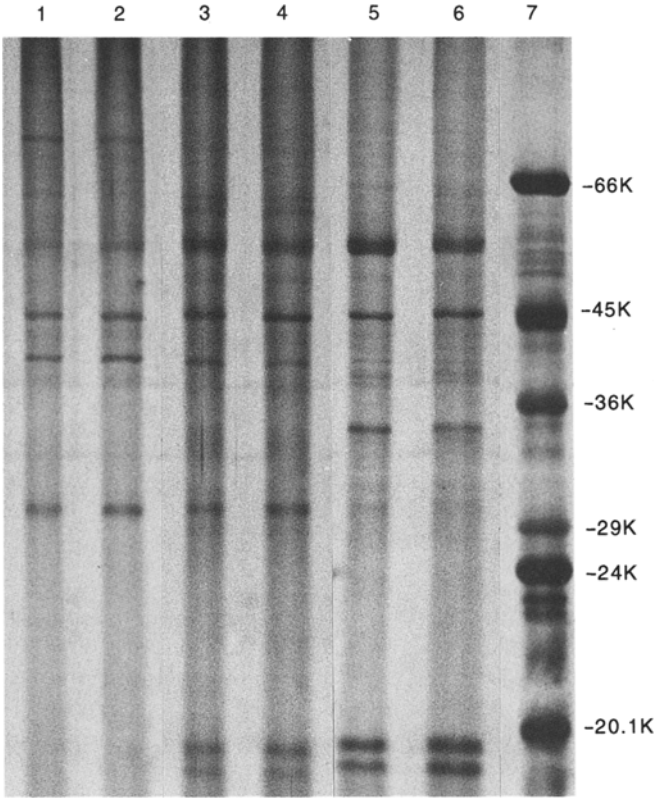

Fig. 1. *Ostrea edulis*, *Crassostrea gigas*, *C. virginica*. General proteins of eyed-pediveligers of three laboratory-raised oyster species. Lanes 1 and 2 are *O. edulis*; Lanes 3 and 4 are *C. gigas*; Lanes 5 and 6 are *C. virginica*; Lane 7 contains the SDS(sodium dodecyl sulfate)-7 molecular weight markers (Sigma Chemical Co., St. Louis, Missouri, USA). The origin is at the top of the gel and anodal migration is downwards. K: kilodalton

Results

Eyed pediveliger larvae of *Ostrea edulis* ($N=43$), *Crassostrea gigas* ($N=58$), and *C. virginica* ($N=63$) exhibited diagnostic differences in their general protein patterns (Fig. 1). Three protein bands [55.3 (kilodalton), 45.3 kDa and 40.6 kDa] were present in all individuals of each of the three species. One band (75.2 kDa) was diagnostic for *O. edulis*, and two bands (19.5 and 19.0 kDa), shared by *C. gigas* and *C. virginica*, were diagnostic of the *Crassostrea* species. Three bands discriminated between *C. gigas* and *C. virginica* larvae: 32.2 and 28.4 kDa in *C. gigas* vs 34.2 kDa in *C. virginica*. We observed no variation in protein pattern within samples of these three species, however, caution should be exercised before concluding that these phenotypes are strictly diagnostic.

Extracts from individual oyster larvae provided sufficient enzyme activity to reveal the products of several gene loci. Malate dehydrogenase (MDH), glucose-phosphate isomerase (PGI), malic enzyme (ME) and leucyl-alanine peptidase (PEP) showed scorable bands in single larvae; however, phosphoglucomutase (PGM), α-naphthol esterase (EST), and aspartate aminotransferase (AAT) did not exhibit sufficient activity to resolve scorable bands with the present techniques. To illustrate the utility of enzyme electrophoresis for studies of larval oysters, we report on the products of two gene loci, *Mdh-2* and *Pgi*.

Allozymes encoded by the *Mdh-2* locus clearly discriminated among the three species of larval oysters (Fig. 2a). In our samples, *Ostrea edulis* was fixed for a *108* allele, *Crassostrea gigas* for *100*, *C. virginica* for *106*. Earlier studies of adult *C. gigas* and *C. virginica* revealed species diagnostic patterns encoded by *Mdh-2* and also reported a second, more anodally migrating locus, *Mdh-1*, which did not discriminate between the two *Crassostrea* species (Buroker et al. 1979, Buroker 1983). Although low activity *Mdh-1* products were sometimes visible in eyed-larvae of *O. edulis*, products of this locus generally were not visible in the *C. gigas* and *C. virginica* larvae.

Allozymes encoded by the *Pgi* locus are useful for both taxonomic and population genetic analyses. *Pgi* allozymes completely discriminated between eyed-larvae of *Ostrea edulis* and the two *Crassostrea* species (Fig. 2b). Although *C. gigas* and *C. virginica* larvae shared *Pgi* alleles, frequencies differed markedly in the two samples (Table 1). The specimens of *C. virginica* examined in the present study revealed the presence of three alleles at the

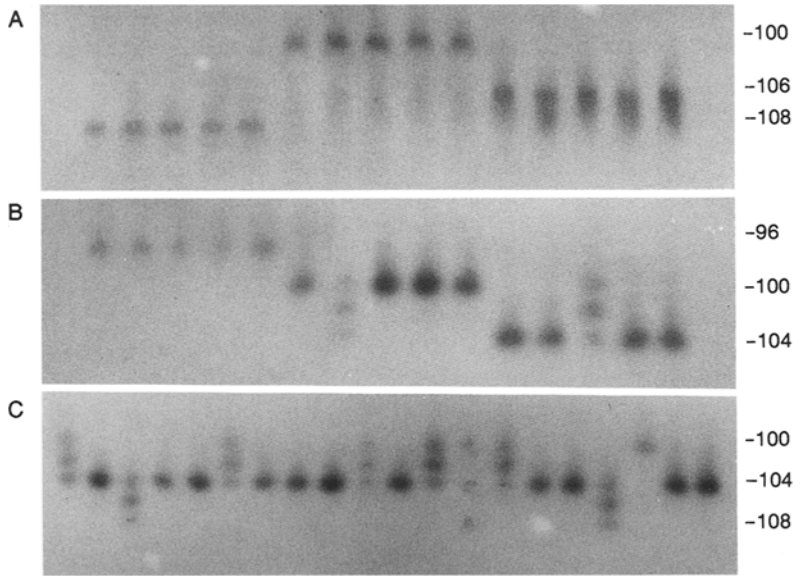

Fig. 2. *Ostrea edulis, Crassostrea gigas, C. virginica.* Allozyme phenotypes of larval oysters. (A) *Mdh-2* patterns. (B) *Pgi* patterns. For both gels, Lanes 1 to 5 are *O. edulis*; Lanes 6 to 10 are *C. gigas*; and Lanes 11 to 15 are *C. virginica*. (C) *Pgi* patterns of natural larval *C. virginica* collected from Wachapreague, Virginia. The origin is at the top of the gels and anodal migration is downwards

Table 1. *Ostrea edulis, Crassostrea gigas, C. virginica. Pgi* allelic frequencies in oysters from hatchery stocks, and from field-collected larvae, juveniles and adults of *C. virginica*. H_{obs}: level of observed heterozygosity. (–): no data or not applied to those cells

	O. edulis Hatchery larvae	*C. gigas* Hatchery larvae	*C. virginica*			
			Hatchery larvae	Natural larvae	Natural juven.	Natural adults[a]
N	50	70	249	104	85	122
Alleles						
92	0.02	–	–	–	–	–
96	0.98	–	–	–	–	–
100	–	0.986	0.175	0.236	0.218	0.283
104	–	0.014	0.779	0.716	0.729	0.672
108	–	–	0.046	0.048	0.053	0.037
Others	–	–	–	–	–	0.008
H_{obs}	–	–	0.373	0.404	0.430	0.467
f	–	–	−0.039	0.065	0.099	0.003
χ^2_{2DF}[b]	–	–	0.871	0.954	0.955	0.998

[a] Data from Vrijenhoek et al. (1990)
[b] Test of Hardy-Weinberg hypothesis for genotypic frequencies

Pgi locus. Genotype frequencies in the hatchery sample of eyed-larvae were not significantly different from expectations for a randomly mating population (Table 1). Also, natural samples of eyed-larvae, juveniles, and adults conformed with Hardy-Weinberg expectations. The observed levels of heterozygosity (H_{obs}) and fixation indices ($f = 1 - H_{obs}/H_{exp}$) are given in Table 1. Although none of the *f* values were significant, the only sample showing a slight heterozygote excess ($f = -0.039$) was the hatchery sample of larval *C. virginica*. Natural samples of larvae and juveniles showed slight heterozygote deficiencies for the *Pgi* locus, but the wild adults were essentially in equilibrium ($f = 0.003$).

Allelic frequencies were marginally heterogeneous across the four samples of *Crassostrea virginica* ($\chi^2 = 12.221$, df = 6, $P = 0.057$). Much of the heterogeneity was due to differences between the hatchery and natural samples ($\chi^2 = 9.328$, df = 2, $P = 0.009$). Deviant allelic frequencies in the hatchery sample might be due to the small number of parents (10 males × 10 females) used to found this population. No significant differences ($\chi^2 = 2.930$, df = 2, $P = 0.297$) in allelic frequencies were found among natural larvae, juveniles and an earlier sample of adults (Vrijenhoek et al. 1990). In fact, the frequencies of major *Pgi* alleles are relatively homogeneous along the entire Atlantic coast of the United States (Buroker 1983). Thus past and present studies of this species provide no evidence for significant population structure or differential selection among major life stages for *Pgi* genotypes in this region.

Discussion

The electrophoretic technique described in the present study discriminates unambiguously among larvae of

three species of oysters. Silver staining of general proteins provided a sensitive technique for revealing diagnostic markers for these species. Although we observed no variation in protein patterns within the present samples of these species, caution should be exercised before concluding that these phenotypes are strictly diagnostic. Local and broadscale geographical surveys must be undertaken to determine whether the patterns are fixed within each species.

Allozyme phenotypes also were useful in discriminating among the three oyster species. Phenotypes produced by the *Mdh* locus were completely diagnostic of the three species. Although *Pgi* phenotypes completely separated *Ostrea edulis* from the two *Crassostrea* species, some allozymes were shared by *C. gigas* and *C. virginica*. More closely related species might require a multi-locus allozyme approach if no single locus is completely diagnostic. Multi-locus screening of individual larvae is possible because eyed-larvae provided sufficient tissue extract and enzymatic activity to stain both MDH and PGI on separate gels. Furthermore, enzymes with great differences in electrophoretic mobility (e.g. PGI and PEP) could be stained on the same gel.

Although both general proteins and allozymes can be used to discriminate among larval oyster species, development of these tools requires different approaches. It is most efficient to first screen for species diagnostic differences among adults of the species under study, and then try to identify these differences in the larvae. However, general protein patterns of adult oysters exhibit tissue specificity, and ontogenetic shifts in gene expression create different patterns in various life stages. In contrast, the *Mdh-2* and *Pgi* phenotypes were expressed in larval, juvenile, and adult tissues. Use of these ontogenetically conservative allozyme phenotypes facilitated development of discriminant markers in larvae.

Population genetic analysis of larvae may prove useful in resolving the "heterozygosity paradox" for marine bivalves – many loci exhibit substantial heterozygote deficiencies while simultaneously providing evidence for heterozygote advantage in growth and survival of adults (Fujio et al. 1983, Zouros and Foltz 1984, Gaffney et al. 1990). Although the present analyses of *Pgi* in *Crassostrea virginica* provided no evidence for statistically significant deviations from random mating, slight deficiencies of heterozygotes were observed in the natural sample of larvae and juveniles. It would be desirable to have information from additional polymorphic loci in larvae to better assess possible deviations from panmixia in this species. Comparisons of gene frequencies in natural larvae throughout the breeding season, and of pre- and post-settlement larvae in known cohorts also are possible with the present techniques. Such studies could reveal differential viability among multilocus heterozygosity classes or genotypes if they occur.

In conclusion, micro-electrophoresis of proteins provides an economically feasible technique for large-scale studies of the ecology, genetics, and adaptation of small larval and early juvenile stages of many invertebrate species.

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