Chapter 29
Bivalve Genomics: Complications, Challenges, and Future Perspectives

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"Looked at as a question in natural history, the oyster problem is very simple. The demand has outgrown the natural supply, but it is easy to increase the supply indefinitely by oyster culture, and this is all that is needed."

William K. Brooks on the overharvesting of oysters in Chesapeake Bay, 1905

Introduction

The world's marine fisheries are in severe decline, primarily as a result of overharvesting. As worldwide population expands, stress on resources will only increase; indeed, because of overfishing, some fisheries are now tightly regulated and others have been closed altogether. In addition to the loss of a fishery, severe declines in fishery species can have radiating impacts on ecosystems (Pauly and Maclean 2003). One iconic example of a species that has suffered from overfishing and whose loss has led to significant impacts on its native habitat is that of the Eastern oyster, *Crassostrea virginica*, in Chesapeake Bay.

The overwhelming significance of the oyster—economically, ecologically, and as a cultural icon—is highlighted by the devastation of the oyster population in Chesapeake Bay to what is currently estimated as 1% of its original abundance. The collapse of the oyster industry in Maryland and Virginia has led to controversial proposals to introduce a nonnative oyster species as a solution to the environmental and economic crises (National Research Council 2004). The loss of oysters is a loss of their capacity to filter and to help control algal populations in these coastal estuarine waters (Newell 1988, Wetz et al. 2002). Although fishing pressure has decreased from its peak in the early twentieth century, diseases (primarily Dermo and MSX) have maintained an intense pressure on populations, in such a way that abundance has continually declined. The reestablishment of a sustainable, vigorous oyster fishery on the East Coast of the United States may be aided by genomic approaches to understand the devastating effects of diseases and stress.

As early as 1891, Brooks argued for the use of aquaculture as a means to relieve the fishing pressure on the natural populations in Chesapeake Bay (Brooks 1905). Although growth in molluscan aquaculture production has slightly lagged behind that of overall aquaculture growth, in 2002 worldwide molluscan aquaculture accounted for 11.8 million tons of production, second only to freshwater fish, with a value of $10.5 billion (FAO Fisheries Department 2004). Altogether, bivalve aquaculture accounted for 26.1% of total aquatic production (Table 29.1), with other molluscs adding an additional 9.4%. In addition, freshwater molluscs accounted for 633,000

Table 29.1. Aquaculture production of various categories of molluscs as reported by FAO Fisheries Department.

Species	Production (million tonnes)	Proportion of total (%)	Annual growth (APR)
Oysters	4.32	10.8	3.9
Misc. marine molluscs	3.74	9.4	14.3
Clams, cockles, arkshells	3.43	8.6	14.1
Mussels	1.44	3.6	2.7
Scallops, pectens	1.23	3.1	3.1
Freshwater molluscs	0.013	0.03	14.6

tons of inland capture fisheries, nearly equivalent to that of cichlids (including tilapia) and freshwater crustaceans. Clearly there is a substantial economic incentive for increasing molluscan aquaculture production.

In the paragraph immediately following Brooks' simple answer to the oyster problem, he suggests that, as a practical matter, increasing oyster production is anything but simple. Despite this difficulty, aquaculture production of oysters has clearly grown. The Pacific oyster *Crassostrea gigas* has the highest annual worldwide production of any marine or freshwater species, at about 4.2 million tons in 2002. As worldwide capture fisheries plateau (or decline), growth in aquaculture is necessary if worldwide seafood demands are to be met without destroying fisheries stocks (FAO Fisheries Department 2004).

Aquaculture and restoration are two of the primary motivating factors underlying the use of genomics for the oyster model. As additional bivalve fisheries grow, they will face the same questions and difficulties faced by the oyster community. Indeed, the market for geoduck (*Panope abrupta*) has recently and rapidly grown (Davis 2006), but genetic and genomic resources for this species lag. In this chapter, we will review past and present genomic efforts and issues unique to bivalves and make suggestions for future researchers to avoid pitfalls.

Complications as a Result of Extremely High Fecundity

The enormous fecundity (10^6–10^8 eggs per female per season) and high larval mortality of most marine animals, including bivalves, makes them fundamentally different from the more familiar and well examined animal models (10–10,000 eggs per female, lifetime). Thirty years ago, Williams (1975) argued in his Elm-Oyster model that sexual reproduction and genetic diversity are favored to a much greater degree in high fecundity species than in low fecundity species. Species with high fecundity likely generate more mutations than low fecundity species, because of the large number of cell divisions required to produce millions or billions of gametes (*cf.* to the argument for male-driven evolution in humans) (Li et al. 2002). The consequences of high fecundity were observed in the earliest genetic studies of bivalve populations, which discovered high levels of protein polymorphism, heterozygote deficiency, and correlations of individual heterozygosity with fitness-related traits, such as size-at-age. These unique biological phenomenon remain at the heart of bivalve genetics, with recessive

lethals leading to distortion of Mendelian ratios in controlled crosses and a high frequency of null alleles leading to inaccurate genotyping.

Challenges as a Result of High Rate of Polymorphism

Protein polymorphism for bivalves was long ago found to be among the highest for animals: average allozyme heterozygosity is greater than 20%, three to four times the mammalian average (Buroker et al. 1979, Fujio 1982, Hedgecock and Sly 1990). The complications of high genetic polymorphism manifest as nonreactive, null allozyme alleles (Mallet et al. 1985, Foltz 1986), and abundant nonamplifying, PCR-null alleles for many DNA markers (Hu et al. 1993, McGoldrick et al. 2000, Vadopalas and Bentzen 2000, Launey and Hedgecock 2001, Reece et al. 2001, Sekino et al. 2003, Reece et al. 2004, Astanei et al. 2005, Sato et al. 2005). In the Pacific oyster, null alleles are segregating at over half of the approximately 100 loci tested in mapping families, even though microsatellites and families were from the same natural population (Li et al. 2003, Hedgecock et al. 2004a). In addition, cross species transfer of DNA markers is limited, as cross-specific polymerase chain reaction (PCR) amplification decays rapidly with evolutionary distance, so that only one in eight Pacific oyster markers amplifies from the Eastern oyster, which diverged >5 million years ago. This decay in cross-specific amplification exceeds that observed across genera or even families of vertebrates (Schlötterer et al. 1991, Garza et al. 1995, Pépin et al. 1995, Fitzsimmons et al. 1995, Rico et al. 1996) or species of Drosophila (Harr et al. 1998, Colson et al. 1999, Noor et al. 2001, Huttunen and Schötterer 2002), suggesting rapid rates of sequence evolution in PCR primer binding sites.

In the context of controlled crosses, null alleles are a minor complication. Nonamplifying alleles are often identified when the expected Mendelian ratio (based on parental genotypes) does not fit with the predicted genotypes of the offspring from a controlled cross. A simple and common case is the progeny of AB × Aø parents. The parents present as an AB heterozygote and an AA homozygote, and, as expected, one-half of the progeny are phenotypically A (AA or Aø); however, only one-quarter are AB and the remaining quarter show only the B allele indicating that a null allele must be segregating. In contrast, null alleles are a significant issue for population genetic work in bivalves, because failure to identify null heterozygotes (e.g., the Aø heterozygote mentioned above) leads to inaccurate genotyping and an, often significant, excess of homozygous classes. Thus, they will likely be an issue for natural population association studies that are common for model organisms. Whether single nucleotide polymorphisms (SNP) present in coding loci will prove more robust is unclear at this time, but many oyster biologists hope that SNPs will overcome the uncertainties of null alleles.

Inbred Lines, Phenotypes and Pedigree Analysis

Controlled crosses (particularly using inbred lines) provide an opportunity to manipulate the genotype and, combined with genomic approaches, provide powerful means for dissecting complex, multifactorial phenotypes, such as disease and stress resistance, growth, and survival. Disease resistance is of great interest in restoration of the

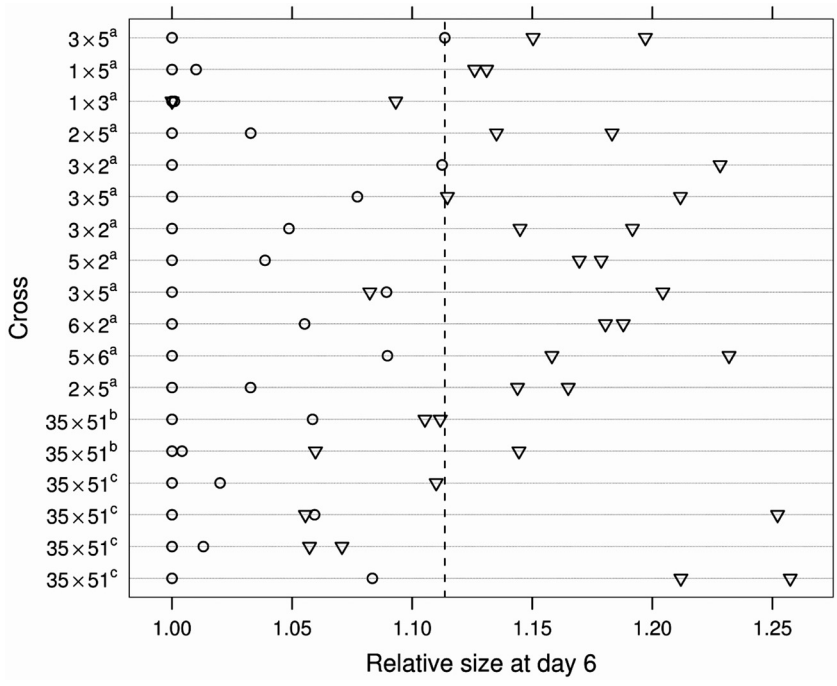

Figure 29.1. Size variation in inbred and hybrid *C. gigas* larvae. Upper graph: Size at day 6 for 18 crosses relative to the slowest growing family in each cross. Δ-hybrid larvae, o-inbred larve. Data from Pace et al. (2006); Curole unpublished data; Meyer and Curole unpublished data.

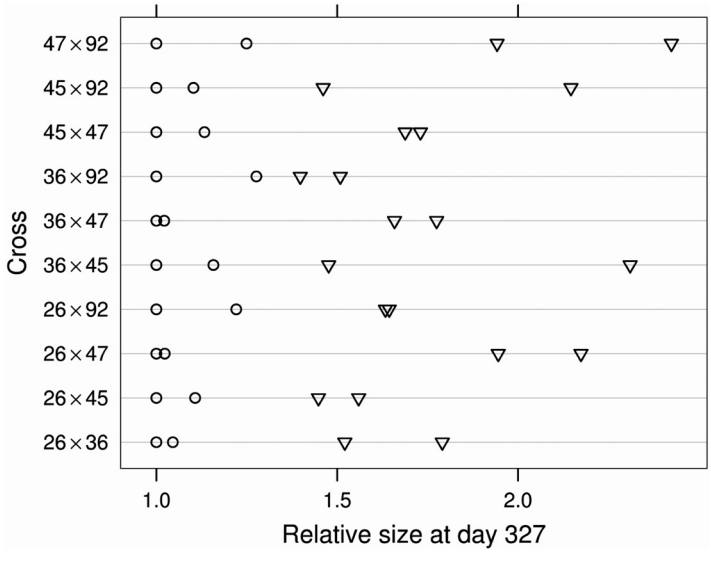

Figure 29.2. Size at day 136 post-fertilization for 10 families of inbred and hybrid *C. gigas* relative to the slowest growing family for in cross. Δ-hybrids, o-inbreds. Data from Hedgecock and Davis (2006).

American oyster; stress resistance, growth, and survival are of greatest interest in the Pacific oyster, the primary bivalve aquaculture species (Figures 29.1 and 29.2).

Growth heterosis, or hybrid vigor, in bivalve molluscs has a long and storied history, founded primarily on early observations of correlations between allozyme heterozygosity and particular fitness-related traits in wild-caught populations (Zouros and Foltz 1987, Britten 1996). Size-at-age is the most commonly used metric, with significant correlations between heterozygosity and shell length or gross weight at a particular time point in the life cycle (Fujio 1982, Gaffney and Scott 1984, Koehn and Gaffney 1984, Foltz and Chatry 1986, McAndrew et al. 1986, Koehn et al. 1988, Zouros et al. 1988, Gosling 1989, Gaffney 1990, Pogson and Zouros 1994, Zouros and Pogson 1994). The Pacific oyster provides an animal model for studying heterosis, a phenomenon more evident in plants and underlying the improvement of most crops (Gowen 1952, Crow 1998); however, the fundamental genetic cause and underlying mechanisms of heterosis have eluded researchers for decades (Birchler et al. 2003). An experimental genomic approach now offers the possibility to understand this phenotype and harness this understanding to increase production.

In an effort to establish a breeding program, investigators have developed approximately 50 inbred lines for experimental crosses from the naturalized population of *C. gigas* in Dabob Bay, Washington (Hedgecock 1994), using self and brother-sister mating (Hedgecock et al. 1995). The third inbred generation (G_3) of inbred lines was propagated in 2004 to produce the G_4, which has an expected inbreeding coefficient of 0.59. Factorial crosses of inbred lines showed that oysters exhibit heterosis for both growth and survival (yield), experimentally validating the aforementioned years of work associating growth with heterozygosity (Hedgecock et al. 1995). Heterosis for yield and size-at-age has been observed repeatedly, across generations and inbred lines (Figures 29.1 and 29.2) (Hedgecock and Davis 2006). In addition, 24 of the 34 hybrids (71%) presented in Figure 29.1 show a greater relative size at day 6 than the largest inbred across all crosses. Controlled crosses have revealed other similarities with agricultural crops (in particular, maize). Notably, hybrid populations show significantly less variance in size (analysis of variance [ANOVA] of standard deviations at day 6, $F = 23.2$, $P = 5.8 \times 10^{-6}$; Figure 29.3), which means that for larvae a majority of the population is simultaneously reaching the settlement stage, therefore maximizing yield at seed-set. Controlled crosses have also facilitated the elucidation of the physiological mechanisms of heterosis (Hedgecock et al. 1996, Pace et al. 2006), an aspect of this phenotype that has eluded researchers.

Crossbreeding inbred lines of oysters to produce hybrids holds great promise for increasing the yields of farmed Pacific oysters (Hedgecock and Davis 2000). Factorial crosses among inbred lines produce F_1 hybrids for yield traits (progress reports at http://www.hmsc.orst.edu/projects/wrac) (Hedgecock and Davis 2006), and the most promising crosses are then reproduced at a commercial scale. Hybrids are currently in commercial production on the U.S. West Coast (JP Davis, Taylor Resources, personal communication).

Disease-resistant selected strains have been developed for the Eastern oyster. Several strains are available (National Research Council 2004), including the DEBY strain, which has been deployed in the Chesapeake. Many of these strains show significant increases in survival after disease challenge, with the greatest increase following the first generation of selection. In field trials, the DEBY strain shows anywhere from one-quarter to one-half the mortality level of local controls (Ragone Calvo et al.

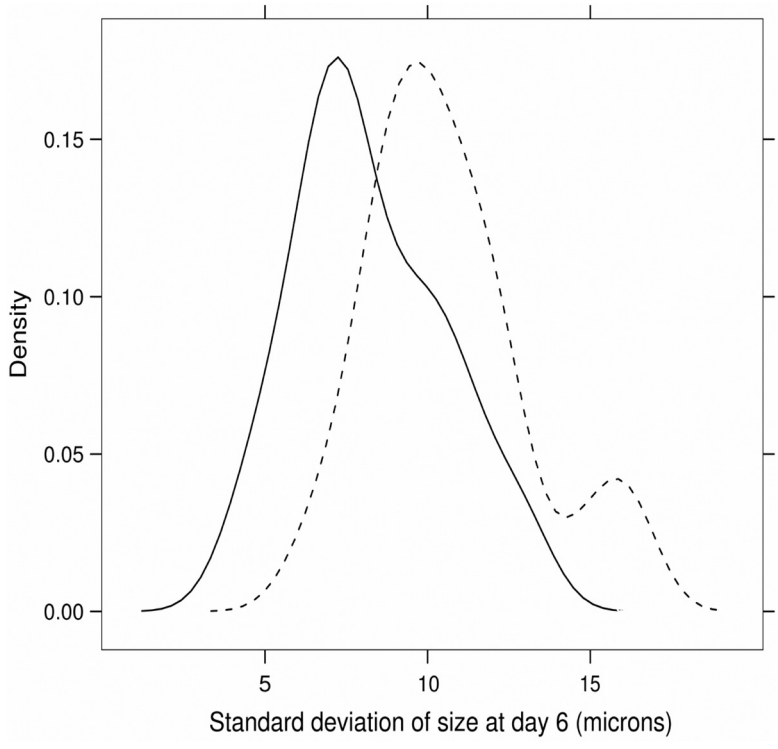

Figure 29.3. Density estimate of standard deviation of size-at-age for crosses presented in Figure 29.1. Solid line-hybrids, dashed line-inbreds. Standard deviations were subjected to kernel density estimation using the density function in the statistical program R (R Development Core Team 2006).

2003). Strains are generally maintained through a mass spawn of selected individuals as opposed to pairwise crosses. This approach is haphazard and can lead to the unintentional loss of genetic diversity in these lines (Hedgecock et al. 1992), which may ultimately result in inbreeding depression.

The greatest difficulty in dealing with inbred oyster lines or selected broodstock is maintaining separate populations. Although great effort is taken to keep lines separate by growing the animals in different tanks, wellers, and bags, cross contamination is a fact (Mallet et al. 1985, Foltz 1986, Li and Guo 2004, Zouros et al. 1994). Over 4 years of genotyping for WRAC broodstock, an average of 14.7% of broodstock animals were excluded from crosses because they were either verified contaminants or insufficient genotype information was available to determine provenance (Table 29.2). Based on these data, it should be clear that an unlucky choice of animals could result in crossing of full-sibs when a hybrid cross is intended, leading to significantly reduced yield. Even worse, contamination of a line or broodstock is a possible outcome; this has serious implications for a broodstock program, especially if lines have been specifically bred for disease resistance as they are for the Eastern oyster. The solution to this issue is broodstock genotyping, a must for any hatchery breeding program. Ideally, a set of highly variable hatchery markers is established, genotyping methods are standardized, and tissues are preserved for all broodstock. Prior to

Table 29.2. Broodstock contamination. Contaminants have genotypes at several loci that are incompatible with parent or control genotype. Uncertain are individuals with insufficient genotype information to determine provenance. Six microsatellite markers were used in 2001 and 2002. Eleven microsatellite markers were used in years thereafter.

Year	Tested	No. of contaminants	Uncertain
2001	164	8	1
2002	203	11	11
2003	339	37	53
2004	272	23	0
Total	978	79	65
Percent rejected		8.1%	6.6%

spawning, potential broodstock candidates are genotyped to validate their pedigree and only spawned after validation. As a rule of thumb, 10–12 individuals per line should be screened and genotyped to guarantee at least one ripe male and female. The importance of pedigree validation cannot be overstated.

Linkage Maps

One of the most fundamental genomic approaches is the generation of a genetic linkage map, as described in Chapter 10, because linkage maps are ideal for identifying quantitative trait loci. (See Chapter 11.) Prior to the advent of DNA markers, allozymes were used in the hope that an allozyme locus might be linked with a particular phenotype of interest. Minimal linkage groups were generated from the handful of allozymes that co-segregated; full linkage maps were not feasible with allozymes because of the paucity of loci. Early efforts with allozyme loci in crosses of wild bivalves discovered significant distortions of Mendelian segregation ratios. These distortions are the result of a high genetic load at least in the Pacific oyster, but likely in other bivalves as well with a minimum of 15–20 recessive lethal mutations per oyster, about 5 times the genetic load of a human or fruit fly (Launey and Hedgecock 2001, Bucklin 2002). A high genetic load resolves two aforementioned issues in bivalve genetics: distortions of Mendelian inheritance ratios in lab-reared progeny of wild parents (Wada 1975, Beaumont et al. 1983, Foltz 1986, Hu et al. 1993, McGoldrick and Hedgecock, 1997, McGoldrick et al. 2000, Reece et al. 2004) and the correlation of heterozygosity with fitness related measures in natural populations. These phenomena are uncommon or nonexistent in terrestrial animals (Houle 1989, Britten 1996). High mutational load further accounts for severe inbreeding depression and its converse, hybrid vigor (heterosis), in experimental crosses (Lannan 1980; Hedgecock et al. 1995, 1996; Evans et al. 2003).

Distortion of Mendelian ratios (the result of recessive lethals) at individual loci does not significantly affect linkage mapping, but in combination, two linked loci that affect viability can cause difficulties. See Hackett and Broadfoot (2003) for details. Linked lethal recessives have been observed in the Pacific oyster (Bucklin 2002), but are less frequent than unlinked lethals. Despite these potential complications, framework linkage maps of more than 100 microsatellite DNA markers have been

published for the Pacific oyster (Li et al. 2003, Hubert and Hedgecock 2004). Consensus maps have 10 linkage groups, in accord with the haploid chromosome number, cover 70–80% of the Pacific oyster genome, and have marker densities so that the expected distance of a new gene to the nearest marker on a map is 4 to 6 map units (centiMorgans [cM]). Consistent with the high levels of genetic polymorphism, linkage maps for the Pacific oyster show significant differences among families in recombination rate and, more significantly, gene order. These differences suggest polymorphism for the distance between markers as well as for chromosomal rearrangements in the Pacific oyster (Hubert and Hedgecock 2004). An advantage of maps from multiple families is the ability to map a locus by synteny (e.g., if marker x is between markers y and z in family 1, then one can infer that it is between markers y and z in other families where marker x is not segregating); however, because of the polymorphism in marker location, one should be cautious in inferring the location of markers across families.

Amplified fragment length polymorphism (AFLP) maps of 341 markers (Hedgecock et al. 2004b) and 119 markers have also been generated (Li and Guo 2004). Because of the high levels of Mendelian distortion in the Pacific oyster, AFLPs are not as useful as in species with limited distortion (Li and Guo 2004). An AFLP map with three microsatellites and one Type I marker is also available for the Eastern oyster (Yu and Guo 2003). This map has good coverage because of the use of AFLPs, and revealed lower levels of segregation distortion than in the Pacific oyster. Unfortunately, AFLPs have major weaknesses because they are anonymous dominant markers that are specific to a cross and, because of their anonymous nature, are nontransferable to other crosses. Each cross also produces two maps, one for each parent, which can be difficult to reconcile. The dominant nature of these markers also makes it impossible to estimate genetic effects at quantitative trait loci, a significant complication if the goal is understanding complex production traits. Despite the relative ease of generation of a large number of AFLP markers (compared with microsatellites), codominant microsatellite loci are superior for map construction.

Tissue Preservation

Preservation of tissue has emerged as an important issue, because genomic work requires the use of intact, good-quality DNA. Adult tissues are easily preserved in ethanol for later DNA extraction, but it is best to perform high-quality DNA extractions as soon as possible because the tissue degrades and loses structure over time. Although more expensive, commercially available kits produce high-quality DNA extractions that are stable over time; this cost may be justified if the sample is important (e.g., broodstock parents or mapping family tissues).

Larvae and spat present a much greater obstacle. If placed directly in ethanol, larvae and spat will simply close their valves and undergo anaerobic metabolism, essentially digesting themselves until death, leaving poor quality tissue and DNA. This effect is minimized to some extent if larvae are killed immediately by adding formalin or bleach directly to seawater containing swimming larvae and then preserved in ethanol. Again, it is best to extract DNA from larvae as soon as possible, because tissue degradation likely begins immediately after preservation, and after time,

extractions produce little to no DNA. We have had the greatest success by killing larvae and then immediately aliquoting individual larvae into extraction buffer in a 96-well plate and preserving at −80°C. Plates prepared in this manner show greater PCR amplification success.

QTL Mapping of Performance Traits

By consolidating mapping efforts, the oyster community has initiated the establishment of F_2 families as reference material for genotyping. As indicated above, the order and location of markers may vary across families, so that a map should be made for each family. The availability of DNA and a linkage map for a reference family, onto which loci can be mapped, should reduce the number of orphan loci (loci that have been genotyped but can not be placed on a linkage group).

With the development of genetic linkage maps, mapping of quantitative trait loci has moved forward. QTL have been identified for three phenotypes in the Pacific oyster: shell shape, shell color, and growth. A single, additive quantitative trait loci (QTL) explains 32% of the variance in pigment saturation of the left valve (Hedgecock et al. 2006a), supporting the observation that shell and mantle edge color are heritable in *C. gigas* (Brake et al. 2004). Two QTL, one of which also explains 32% of the variance, were identified for a left bend in the dorsal-ventral growth axis (Hedgecock et al. 2006a). Four QTL residing on three linkage groups have been identified for initial size, growth curve inflection, and growth rate (Hedgecock et al. 2004b). Dominance appears to play a role in growth, as it does in survival via recessive lethals, with all growth QTL showing dominance. There is also some evidence for epistasis between QTL on two different linkage groups. At least two significant QTL for growth map to linkage groups with significant Mendelian distortion. Although Mendelian distortion does not appear to impact the ability to identify QTL, separating the effects of a QTL for the phenotype of interest from the effects of recessive lethals may prove a formidable task. At loci tightly linked to the recessive lethal, the frequency of one homozygote class is significantly less than the other genotypic classes (or possibly completely absent), reducing the power to detect differences between genotypic classes. Although it might be reasonable to expect that the recessive lethal is the locus responsible for differences in growth, proving this may be very difficult. Additionally, this is probably not a reasonable expectation for other phenotypes, such as shell color or sex.

Disease-resistance QTL have also been identified for the Eastern oyster (Yu and Guo 2006). At this time, phenotyping for disease resistance involves genotyping a subset of animals prior to exposure, exposing them to disease in the field, and genotyping a subset of survivors. Deviations from Mendelian ratios before and after disease exposure are then used to identify putative disease-resistance QTL. Using this approach, 12 QTL across two families were identified. MSX infections were light for these families, but Dermo infections were heavy; as such, it is likely that these are disease-resistance QTL for Dermo. A majority of these QTL were identified in a cross between a wild individual and a selected male from the Rutgers NEH stock. In contrast, only two QTL were identified in a cross between parents both from the NEH stock, suggesting that most disease-resistant QTL are fixed in this line. This possibility is disconcerting

given that the NEH × NEH family experienced 53% mortality. Lab-based challenges would be ideal, as well as the genotyping of all individuals prior to field exposure and monitoring of individual mortality.

Cytological Maps

Aquaculture bivalve species generally have low chromosome numbers, with *Mytilus* species having 14 pairs of chromosomes (Thiriot-Quiéveux and Ayraud 1982, Moynihan and Mahon 1983) and cupped oysters having 10 pairs of metacentric or submetacentric chromosomes (Ahmed and Sparks 1967, Longwell et al. 1967, Leitão et al. 1999). In *C. gigas* there are 1.0–1.3 chiasmata per chromosome, implying a genetic map length of 500–650 cM (X Guo, personal communication). Chromosomal banding and FISH techniques with P1 clones, ribosomal RNA (rRNA) genes and repetitive sequences have recently been applied to chromosome identification and mapping (Wang et al. 2001). A repeat that accounts to 1–4% of the genome has been isolated and mapped to centromeric regions of several chromosomes in the Pacific oyster (Clabby et al. 1996, Wang et al. 2001). Major rRNA genes have been mapped to 10q in the Pacific oyster and 2p in the eastern oyster (Xu et al. 2001, Wang and Guo 2004).

Physical Maps

Bacterial artificial chromosome (BAC) libraries have been constructed by Clemson University Genomics Institute and are publicly available. These are deep coverage libraries (12× and 10× coverage for the Pacific and Easter oysters, respectively), with average insert sizes of 134 kilobase (kb) and 150 kb, respectively. The libraries were constructed from sperm cells, which, in the case of *C. gigas,* were taken from two F_1 hybrid males (Cunningham et al. 2006b).

Assays of gene copy number for *C. gigas* suggest the possibility of gene duplication in this species (Cunningham et al. 2006b). Estimates of gene copy number for *C. virginica* indicate one to two copies in the BAC library. In contrast, copy numbers of four to five were estimated for two genes in the *C. gigas* library. Whether the high estimates for copy number are a sampling artifact or truly represent duplications is of great interest, because gene duplications can complicate genomic efforts.

With these resources in hand, the oyster community is poised to generate a physical map. Efforts are under way to secure funding for BAC fingerprinting and the generation of a physical map. In addition, a large number of ESTs that will be sequenced by the Department of Energy's Joint Genome Institute (JGI), see EST libraries below, will be from the same inbred lines crossed to make the hybrid males whose DNA was represented in the BAC library. These lines have also been the subject of extensive genetic mapping work. JGI is also sequencing approximately 60 clones from these BAC libraries, which hybridized to probes for eight selected genes (Cunningham et al. 2006a). The BAC sequencing effort will also quantify single nucleotide and gap nucleotide polymorphism, which could interfere with assembly of whole genome shotgun sequences.

Expressed Sequence Libraries: Gene and SNP Discovery

Cloning and sequencing of ESTs are in progress, with the ultimate goal being gene discovery. Two large-scale projects are being performed by the JGI. The larger of these is the agreement by the JGI to sequence 150,000 complementary DNAs (cDNA) from several *C. gigas* libraries. This sequencing effort will produce a wealth of data, including SNPs for at least a couple of inbred lines. One technical issue regarding the construction of the library is the presence of mitochondrial transcripts. Transcriptomic analysis (see below) revealed that the mitochondrial, large ribosomal subunit accounts for 25% of transcripts in 6-day larvae, although this may change with life stage (Anisimov 2005). Removing these transcripts prior to library construction is important for reducing the redundancy of the library. The smaller-scale effort is the sequencing of 25,000 clones from *Mytilus* cDNA libraries. This is directed at identifying genes important in thermal tolerance.

On a smaller scale, pilot EST collection programs for the Pacific and Eastern oysters using hemocyte and embryo cDNA libraries from *C. virginica* and a hemocyte cDNA library from *C. gigas* have been completed (Jenny et al. 2002, Gueguen et al. 2003, Tanguy et al. 2004). Although EST collections are small, the gene discovery rate is excellent for genes important in stress and immune challenge function. In addition to the traditional oyster EST collections described, a library of 4.6 million Pacific oyster ESTs is available from the Massively Parallel Signature Sequence (MSSS) comparative transcriptomic analysis described below. A subset of these ESTs has been sequenced to identify candidate loci for heterosis (Meyer 2006).

Several labs are also working to identify SNPs in a subset of previously sequenced loci. In particular, SNP discovery is being done for a subset of loci in both the Pacific and Eastern oysters for the purpose of comparative mapping, which has thus far been limited by poor cross-amplification of microsatellite loci. See the Challenges as a Result of High Rate of Polymorphism section. An additional effort is taking place in the Pacific oyster to identify SNPs for candidate loci identified in the transcriptomic analysis of hybrid vigor (see below). SNP frequencies are relatively high (1 every 50 base pairs [bp]), and gap nucleotide frequencies are very high (1 every 33 bp) (Curole and Hedgecock 2005).

Transcriptomics

Transcriptome analyses have rapidly grown in recent years, and expression analyses of bivalves have been propelled by this explosion in growth. The early genetic and physiological work on hybrid vigor in the Pacific oyster attracted collaboration with Lynx Therapeutics, Inc. (now Solexa), which provided MPSS profiles of gene expression in inbred and hybrid larval oysters. These profiles quantify genomic expression with great depth, to the equivalent of a few mature RNA (mRNA) molecules per cell, for all expressed genes simultaneously (Jongeneel et al. 2003). Expression analysis by MPSS produced 4.6 million 17-bp sequences, comprising 23,275 unique signatures whose expression is greater than 3 transcripts per million. Expression less than this level is not significantly different from zero (Hedgecock et al. 2006b). Statistical contrasts among genotypes of MPSS expression data identified approximatley 350 candidate heterosis

genes for further genetic or functional analysis. Observed patterns of gene expression are more complex than predicted from the classical dominance and overdominance explanations of heterosis (Gowen 1952, Crow 1998), in that hybrids show dominance for low expression and even underexpression. These expression patterns are consistent, on the other hand, with the metabolic efficiency hypothesis for growth heterosis (e.g., reduced rates of protein turnover in hybrid compared to inbred oysters) (Hawkins et al. 1986, Hedgecock et al. 1996, Meyer 2006). These results are similar to work in maize, which has identified over- and underdominant expression in hybrids relative to inbreds (Swanson-Wagner et al. 2006).

In addition to the MPSS work, the Oyster Microarray Consortium has constructed a microarray with *C. gigas* and *C. virginica* ESTs (Jenny et al. 2006). The microarray includes duplicate spots for 4,460 *C. virginica* clones and 2,320 *C. gigas* clones, as well as 188 various control ESTs, with two arrays per slide (for 27,496 features per slide). Approximately 70% of the sequences show no significant match to the Gene Ontology database; of the remaining 30%, approximately 70% are annotated as biological processes, 7% as molecular function, and approximately 20% as cellular component for the two *Crassostrea* species (Jenny et al. 2006). The availability of a standardized microarray platform should significantly advance transcriptomic work. Disease-resistance efforts will likely receive a large boost, as many of the clones are derived from libraries made with disease-challenged animals. One note of caution is necessary; although microarrays have the ability to generate large amounts of data relatively easily, their use for bivalves must be evaluated carefully. The high levels of DNA polymorphism, in particular indels, may lead to biases in hybridization signal. Tempering this caution is the observation that indels are less frequent in coding regions (Curole unpublished data).

Linking Candidate Genes to QTL

Several factors have motivated the identification of SNPs in coding loci and their use in genetic mapping. High levels of marker polymorphism are ideal for intraspecific work because of a greater number of segregating loci, but the breakdown in interspecific cross-amplification of highly polymorphic loci has hindered comparative genomic efforts. The importance of comparative genomic approaches has been exemplified by the success of the vertebrate community, where syntenic maps facilitate the identification of QTL candidate genes for nonmodel organisms. With just a few coding loci mapped, one can quickly compare model and nonmodel organisms, identify the region of synteny, and then scan for possible candidate genes. Lastly, the identification of candidate genes for several phenotypes of interest has motivated an effort to genetically map these loci to test candidacy.

Efforts are under way to map factors responsible for growth in the Pacific oyster and disease in the Eastern oyster. See the QTL Mapping of Performance Traits section above. In addition to mapping microsatellite loci to localize QTL, the goal of one SNP discovery effort is testing the candidates for linkage to QTL. Approximately half of the candidates for hybrid vigor that have been sequenced have SNPs segregating between the two lines crossed to generate the F_2 mapping family, despite the relatively short sequences (generally 300 bp). Thus, with short sequences one can expect

that half of the loci will have SNPs segregating in any particular family. Seven candidates have been genotyped in the F_2 population, two of which show linkage to QTL for growth (Curole and Hedgecock 2006). Using these preliminary data, approximately 100 (28%) of the MPSS candidates will require additional evaluation as possible genetic contributors to the heterosis phenotype.

Tools for the Future

Advances in the genomics field are occurring so rapidly that it is likely any specifics given in this section will be out of date by the time it is published (or shortly thereafter). With this in mind, we focus our discussion on principles that are likely not to be out of date. Primary among these is the development of SNPs, because these represent the most fundamental level of genetic polymorphism in an organism. (See Chapter 6.) SNP discovery is set to take a large step forward with the extensive EST sequencing that is ongoing for the Pacific oyster, and the smaller-scale efforts for the American oyster and the California mussel (*Mytilus californianus*) will help to round out the Pacific oyster work. Of interest are exonic SNPs and, in particular, SNPs located in protein-coding regions of genes. There is hope that these SNPs will prove more stable and show a much lower frequency of null alleles.

Sequencing technologies are rapidly advancing, and at some point, genome sequencing will likely require small-scale efforts as opposed to the large-scale sequencing efforts currently required. (Also see Chapters 25 and 26.) Such a change in efficiency would clearly open up vast opportunities in bivalve aquaculture and restoration. What is currently a slow process of moving from candidate EST, to gene identification, SNP discovery, and finally genetic mapping, would move much more rapidly. In addition, comparative genomics efforts would be significantly bolstered by inexpensive genome sequencing of candidate species.

Multiple genome sequences would facilitate SNP discovery, enabling high-density genetic maps so that QTL could be narrowed to much smaller candidate regions as opposed to the current tens-of-centiMorgans. This would also require high throughput SNP genotyping, but platforms are currently available. As with most other molecular techniques, development will be driven by model species and then, with time, will trickle down to nonmodel species; systems that are well developed, such as the oyster, will be in an excellent position to capitalize on these techniques.

Probably the greatest challenge facing genomics in nonmodel organisms is bioinformatics. (See Chapter 27.) The ability to produce large amounts of data requires the development of tools to organize and analyze this data. Again, tools developed for model organisms will have to be adapted to these nonmodel species. In contrast to molecular tools, adapting bioinformatic tools should be relatively inexpensive because they only require adequate computing power.

Understanding the System

The primary goal of genomic work, and in particular genomic work in oysters, is focused on understanding the genetic, biochemical, and physiological system that

produces a particular phenotype. In the case of the Pacific oyster, there is great interest in understanding the hybrid vigor phenotype to capture this potential for aquaculture. Disease resistance is of interest for the American oyster, with the ultimate goal being the reestablishment of a healthy oyster fishery, particularly in the Chesapeake Bay.

Transcriptomic studies offer the potential to identify the genes contributing to these phenotypes, but with one particular caveat. If these phenotypes are the result of a complex set of genic interactions or pathways, as they likely are, transcriptomics will produce candidates that may be important in development of the phenotype (proximate causal factors), but are not necessarily the segregating genetic variation leading to the phenotype (the ultimate causal factor). Recent developments, in particular the approach of mapping expression as a quantitative trait, offer a potential bridge between these proximate and ultimate factors. See Gibson and Weir (2005) for a review.

Predicting the Future: Biomarkers

The ultimate goal of much of the oyster genomics work is predictive. The large number of lines and potential hybrid crosses make it impractical to grow to harvest all possible pairwise hybrid combinations. Ideally, identifying the specific pairwise cross, or crosses, that will produce the greatest yield would be done early in the larval life stage (e.g., at day 2 or 3 when feeding begins). Early prediction of yield would allow a hatchery to separate from the hundreds of possible crosses (Hedgecock 2005), the handful that will produce the greatest growth, and to focus its efforts on these crosses, significantly increasing the efficiency of oyster production and moving one step closer to fulfilling Brook's desire to increase the supply indefinitely.

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