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HYBRIDIZATION OF TETRAPLOID AND DIPLOID *CRASSOSTREA GIGAS* (THUNBERG) WITH DIPLOID *C. ARIAKENSIS* (FUJITA)

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ABSTRACT Three replicates of hybrid crosses of tetraploid and diploid *C. gigas* (Thunberg) with diploid *C. ariakensis* (Fujita) were produced with controls. Larval survival and growth were documented. Cytological events were also monitored in oocytes from hybrid crosses following insemination. Among the four types of hybrid crosses, diploid *C. gigas* (female) × diploid *C. ariakensis* (male) (GA) was the most successful. Survival of GA was about the same as that of controls in two of three replications, although its growth rate was 25–30% lower. Crosses of tetraploid *C. gigas* (female) and diploid *C. ariakensis* (male) (GGA) had poor yield at day 2 post-fertilization (0.05%), but grew nearly as well as controls subsequently. The other two types of hybrids (i.e., diploid *C. ariakensis* [female] and tetraploid *C. gigas* [male] [AGG], diploid *C. ariakensis* [female] and diploid *C. gigas* [male] [AG]) suffered very low yield at day 2 (0.01% and 0.003%) and grew very slowly. Spat were obtained from all replicates of GA crosses and one of three replicates of GGA, and proved to be hybrids by polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) diagnosis. GGA hybrids were confirmed to be triploid by flow cytometry. No larvae survived to eyed stage in AGG or AG crosses. Cytological examination revealed that the vast majority (>99%) of oocytes from hybrid crosses had a prolonged meiotic prophase I or metaphase I at least through 180 min post-insemination.

KEY WORDS: *Crassostrea gigas, Crassostrea ariakensis,* diploid, hybrid, tetraploid, oyster, breeding, polyploidy

INTRODUCTION

There are numerous reports of attempted interspecific hybridization in the genus *Crassostrea* (Gaffney & Allen 1993). However, most should be viewed with caution because these reports were unaccompanied by genetic confirmation of putative hybrids. Even a modest amount of contamination may account for the majority or all of surviving progeny in hybrid crosses in which fertilization rate and viability are normally low or nil (Allen & Gaffney 1993). One case seems clear: Pacific oyster *C. gigas* (Thunberg) and Suminoe oyster *C. ariakensis* (Fujita—formerly *C. rivularis* Gould) can be crossed to produce viable hybrids (Allen & Gaffney 1993).

The production of hybrids is interesting because they may possess qualities that improve commercial traits. Hybrids also could be back-crossed to introgress certain traits into either of the parental species; for example, disease resistance. Introgression of disease resistance into *C. virginica* (the Eastern oyster) from *C. gigas* was the rationale for the extensive hybrid trials undertaken by Allen et al. (1993). Later, many more hybridization trials were attempted, using bridging crosses between races of *C. virginica*, using (only slightly) fertile *C. gigas* × *C. ariakensis* hybrids (GA), and using polyploidy (Lyu 1996). However, under no circumstances tested in the lab did *C. virginica* hybridize with *C. gigas* or *C. ariakensis*.

Although they failed as a bridging cross to *C. virginica*, GA hybrids are still of interest for several other reasons. First, no work has been done on the qualities of diploid GA hybrids as an aquaculture product, although this work might more appropriately be carried out where there is on-going commercial culture of these two Asian species. For the East coast, they are nonnative. Second, because diploid hybrids are possible, production of polyploid hybrids should also be possible. Polyploid hybrids are potentially useful for improvement of commercial traits (Longwell 1986). Virtually no work on polyploid hybrids of shellfish has been done. Third, and most apropos to research on the East Coast, is the issue of testing nonnative species as an alternative to the native Eastern oyster because of the decline in the fisheries there.

Trials of nonnatives were begun in Delaware Bay several years ago (Allen 1993) and have been conducted for *C. gigas* in the Chesapeake Bay (Calvo et al. 2000). Trials with *C. ariakensis* have been (Calvo et al. 2001) and continue to be conducted. *C. gigas* seems more suitable for higher salinity environments and *C. ariakensis* seems suitable for more estuarine conditions. In all field trials up to this point, triploids have been used to effect population control because of their sterility (Allen & Downing 1990, Gaffney & Allen 1992, Guo & Allen 1994a).

Triploid hybrids then are of interest because they are expected to be sterile, more so than diploids because of the added burden of gametogenesis in hybrids (Thorgaard & Allen 1986, Thorgaard & Allen 1992). Triploid hybrids may also have characteristics intermediate to the two parental species, for example, salinity preference. The genotypes that might be available for culture in an estuary as varied as the Chesapeake Bay, for example, could range from triploid *C. gigas* (GGG) through two types of triploid hybrids—either tetraploid *C. gigas* × diploid *C. ariakensis* (GGA) or diploid *C. gigas* × tetraploid *C. ariakensis* (GAA)—to triploid *C. ariakensis* (AAA), with phenotypes potentially encompassing the full range of estuarine and marine conditions.

To date, all hybrid crosses between *Crassostrea* species have been made between diploids. Diploids are also used in the production of triploid hybrids using ploidy induction techniques (Allen et al. 1989). For example, triploid hybrids were attempted between *C. virginica* and *C. gigas* by inhibiting polar body 2 with cytochalasin B treatment (Allen et al. 1993). However, this hybrid seems to be inviable in any form. Triploid hybrids were also attempted (S. K. Allen, Jr., unpublished data) between *C. gigas* and *C. ariakensis*. These, too, were unsuccessful for another reason: fertilization in this cross is protracted, taking more than 3 h. As a

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consequence, polar body 2 formation is asynchronous and treatments to inhibit polar body 2 are useless. The availability of tetraploid *C. gigas* (Guo & Allen 1994b), tetraploid *C. ariakensis*, or both, provides a new opportunity to produce triploid hybrids by crossing (Guo et al. 1996), rather than induction and to further investigate the fitness of them. As a first step, we examined the feasibility of hybridizing tetraploid *C. gigas* and diploid *C. ariakensis*. At the same time, we repeated crosses of diploid *C. gigas* with diploid *C. ariakensis* as controls and also examined early development of eggs cytogenetically.

MATERIALS AND METHODS

Oyster and Gametes

Sexually mature oysters used in this study were 2 years old and obtained from stocks held at the Cape Shore Laboratory, Haskin Shellfish Research Lab. Ploidy of tetraploid Pacific oysters was confirmed in all individuals by flow cytometry prior to spawning. Gametes were obtained by strip spawning. All surfaces and instruments contacting the oysters were cleaned with dilute bleach and rinsed with fresh water between handling and opening of different individuals. Sex was determined by gonad biopsy under a light microscope. Once the sex was determined, the animals from different sexes were removed to separate containers. Gametes from each oyster were dissected into individual beakers. Eggs were passed through a 60-μm Nytex screen to remove the large tissue debris and rinsed on a 25-μm screen, then suspended in filtered (2 μm) seawater at 23–25°C for at least 30 min to confirm that the eggs were not self-fertilized. Sperm were separated from debris by passing the suspension through a 15-μm screen.

Experimental Design

Abbreviations for gamete contributions of the two oyster species are as follows: G = diploid *C. gigas;* GG = tetraploid *C. gigas;* and A = diploid *C. ariakensis*, with female listed first. Eight types of crosses were conducted (Table 1) overall, although not all crosses were possible in all three replicates. For each replication, an individual female and male were used. After spawning, parents were frozen at −80°C for subsequent genetic confirmation of the progeny.

TABLE 1.

Experimental Design for Crosses Among 4n *C. gigas*, 2n *C. gigas*, and 2n *C. ariakensis*.

	♂			
	G	**GG**	**A**	
♀				
G	GG		GA	
GG			GGA	Rep 1
A	AG	AGG	AA	
♀				
G	GG	G/GG	GA	
GG	GG/G		GGA	Rep 2
A	AG	AGG	AA	
♀				
G	GG	G/GG	GA	
GG			GGA	Rep 3
A	AG	AGG	AA	

Individual females and males were used for each replication, and three replicates were made. Gamete contribution is represented by GG, G, or A, respectively, with female listed first.

Embryonic and Larval Development

Insemination was conducted at 23–25°C and for hybrids, high densities of sperm were used (Lyu & Allen 1999). Fertilization rate was assessed by directly examining at least 100 oocytes under the light microscope at 60–90 min post-insemination for controls and up to 180 min post-insemination for hybrid crosses. After determining fertilization rate, oocytes were transferred to culture vessels whether fertilization was observed or not. Fertilization was considered successful if the oocyte was at or beyond polar body 1 formation.

Yield at 48 h post-insemination was estimated by directly counting straight-hinge larvae with normal appearance. Yield was calculated as

(no. of straight-hinge × 100)/no. of eggs incubated

Temperature and salinity for larval cultures of crosses of GG, GA, GGA, G/GG, and GG/G, where *C. gigas* was the egg source, were 25°C, 22–23 ppt (Breese & Malouf 1975). For crosses of AA, AG, and AGG, where *C. ariakensis* was the egg source, temperature and salinity were 26°C and 20 ppt (Breese & Malouf, 1977). Seawater in the larval cultures was renewed every 2 days. In all hybrid cultures, densities of larvae were sufficiently low to prevent density-related growth effects; densities in parental culture were within those used in standard larval culture, beginning at 10/mL and winnowing out to 1–2 /mL. During water changes, numbers of remaining larvae were estimated and shell length was measured for 20 individuals for each cross. When larvae reached eyed stage, eyed larvae were collected and treated with a solution of 10^{-4} M epinephrine for 16 h (Coon et al. 1986). Following treatment, metamorphosed larvae were held in a downweller system until they reached a shell length of approximately 1 mm, when they were transferred to an upweller silo. We took great care to eliminate all sources of contamination throughout the culture process.

For cytological observations, eggs from each hybrid cross were sampled and fixed with Carnoy's solution (1:3 glacial acetic acid and absolute methanol) at 90, 120, 150, and 180 min post-insemination. Fixatives were changed twice following light centrifugation. Chromosomes were observed by acetic orcein stain (Guo et al. 1992).

Genetic Confirmation

We randomly sampled 28 spat from each replicate of GA crosses and all GGA spat. In progeny, the whole body was prepared for DNA extraction, whereas mantle tissue (2–8 mg) from corresponding parental species was prepared using a commercial kit (PureGene, Gentra, Minneapolis, MN). An additional gill tissue sample from GGA spat was taken and stored in DAPI/DMSO (Sigma, St. Louis, MO) solution at −80°C for flow cytometric analysis.

An approximately 550-bp region of the nuclear rDNA genome was amplified via polymerase chain reaction (PCR) using primer ITS-1. (The primer pair was designed by Dr. Patrick M. Gaffney, University of Delaware [Hedgecock et al. 1999]). Reaction volume of 25 μL contained 50 MM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer, 5 U/μL polymerase (Taq DNA polymerase, Sigma) and 1 μL DNA extraction. DNA amplifications were performed in a programmable thermal cycler (PTC-100, M.J. Research, Inc., Walthram, MA) using a 2-min initial denaturation at

94°C and then 34 cycles of 45-sec denaturation at 94°C, 1-min annealing at 52°C, 1-min extension at 72°C, and finally a 5-min extension at 72°C.

Restriction enzyme digestion of PCR products was made with restriction endonuclease Hinf I (Sigma). Digestion volume of 20 μL contained 1 × buffer (supplied by New England Biolab Inc., Beverly, MA), 5 units Hinf I, and 8 μL PCR product. Digestion mix was incubated at 37°C for 3–4 h, followed by 5 μL 10× loading dye to stop the digestion.

All PCR products and restriction digest fragments were electrophoresed in a 3% agarose (Sigma) gel in 1× TBE (0.089 M Tris-borate, pH 8.3, 0.002 M ethylenediamine tetraacetic acid [EDTA]) buffer. A molecular weight marker (pUC 18, digested with Hae III, Sigma) was loaded along with the product of interest. The gel was run at 60–90 V, stained with ethidium bromide (0.2 mg/mL) for 10–15 min, and visualized by transillumination.

Statistical Analyses

All data were analyzed with the computer program SYSTAT (Wilkinson 1990). Fertilization rates and yield data were arcsine transformed prior to statistical analysis (Sokal & Rohlf 1981). To compare the performance of hybrids to their controls, a two-way ANOVA was used. Paired *t*-tests were conducted to compare certain crosses to their reciprocals.

RESULTS

Fertilization Rate and 48-h Yield

Mean fertilization rates in the parental (nonhybrid) crosses were 94% (GG), 77% (AA), 88% (G/GG), and 85% (GG/G) (Table 2), with no statistically significant difference among them ($F = 3.118$, $P = 0.132$) by ANOVA. In hybrids, signs of fertilization did not appear until 180 min after insemination, precluding estimates of fertilization rate.

Yield at 48 h post-insemination varied significantly among crosses (Table 2) ($F = 3.964$, $P = 0.018$). Yield in GG was significantly greater than that in AA ($t = 4.162$, DF = 4, $P = 0.014$) but there was no difference between other parental crosses. Yields were similar in GGA and AGG ($t = 1.010$, DF = 4, $P = 0.369$), whereas GA had higher yields than AG ($t = 5.364$, DF = 4, $P = 0.006$). Yields of GA and AA crosses were about the same. GGA and AGG crosses produced many fewer larvae than controls. AG crosses suffered extremely low yield (0.003%).

TABLE 2.

Mean fertilization rates and yields ± SD (*n*) at 48-h in parental and hybrid crosses combined from three replicates.

Cross	Fertilization Rate (%)[a]	Yield (%)
GG	94 ± 4.9 (3)	21 ± 7.5 (3)
GA	ND	4 ± 1.2 (3)
GGA	ND	0.05 ± 0.01 (2)
AA	77 ± 13.9 (3)	3 ± 2.5 (3)
GG/G	85 (1)	4 (1)
G/GG	88 ± 5.0 (2)	18 ± 24.4 (2)
AGG	ND	0.01 ± 0.02 (3)
AG	ND	0.003 ± 0.006 (3)

ND = no data.

[a] Fertilization rate was observed at 60–90 min post-insemination for pure crosses; 180 min post-insemination for hybrid crosses.

Larval Survival and Growth

After 48 h post-insemination, survival of GA crosses was about equal to controls in two of three replicates (Fig. 1). Larvae of GGA crosses had high survival, although the number of eyed larvae was small (of 450 larvae on day two, 280 survived to eyed stage). For AGG and AG crosses, mortality was severe and steady for 13 days, with no survival to eyed stage. In GG crosses, mortality was generally density related, moderating at lower densities at about day 6–10. For AA crosses, survival was generally poor, making this cross a poor control. In general, survival ranked GG, GA, and AA, in descending order. Survival of G/GG was variable: better than GG in replicate 2 but worse than AA in replicate 3. Survival of

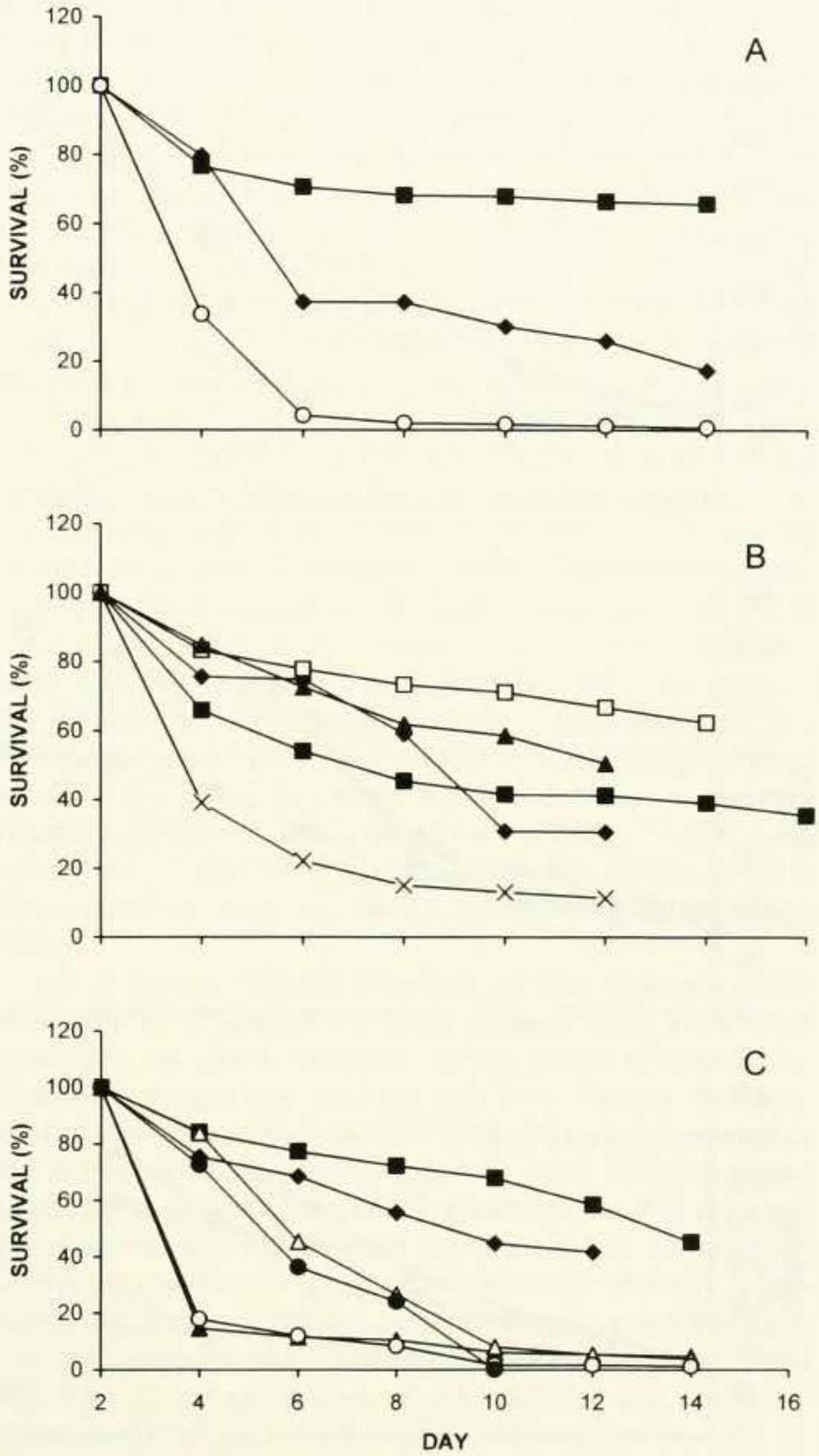

Figure 1. Mean survival of hybrid larvae and their respective controls from day 2 up to day 16 in crosses of diploid and tetraploid *C. gigas* with diploid *C. ariakensis*. (A) Six matings were made in replicate 1. No larvae survived to day 2 in AG, AGG, and GGA. (B) Eight matings were made in replicate 2. No larvae survived to day 2 in AG, AGG, and AA. (C) Seven matings were made in replicate 3. No larvae survived to day 2 in GGA. Counts were terminated when harvesting of eyed larvae was begun. GG (♦), GA (■), G/GG (▲), GG/G (×), GGA (□), AGG (△), AA (○), AG (●).

G/GG crosses was better than its reciprocal, GG/G. Larvae survived to setting in all replicates of GG and GA, 2 of 3 replicates of G/GG and 1 of 3 replicates of GGA. Spat were obtained from all of these.

Larvae of GG, G/GG, GG/G, and GGA crosses grew at similar rates. Crosses of GA grew slower than GG controls but faster than AA (Fig. 2). GA larvae were generally smaller than GG larvae. Both AG and AGG crosses grew very slowly (AG larvae died at day 10). AA crosses grew slowest of all controls. Mean size of eyed larvae was 350 μm for GG, 336 μm for GA, 360 μm for GGA, 361 μm for G/GG, and 361 μm for G/GG. ANOVA showed no significant difference in eyed larvae size among crosses ($F = 1.712$, $P = 0.199$). After 90 days post-setting, spat from GGA reached 12.0–17.5 mm in shell size compared with 2.87–8.0 mm in the corresponding GA cross ($t = 8.49$, DF = 6, $P < 0.001$).

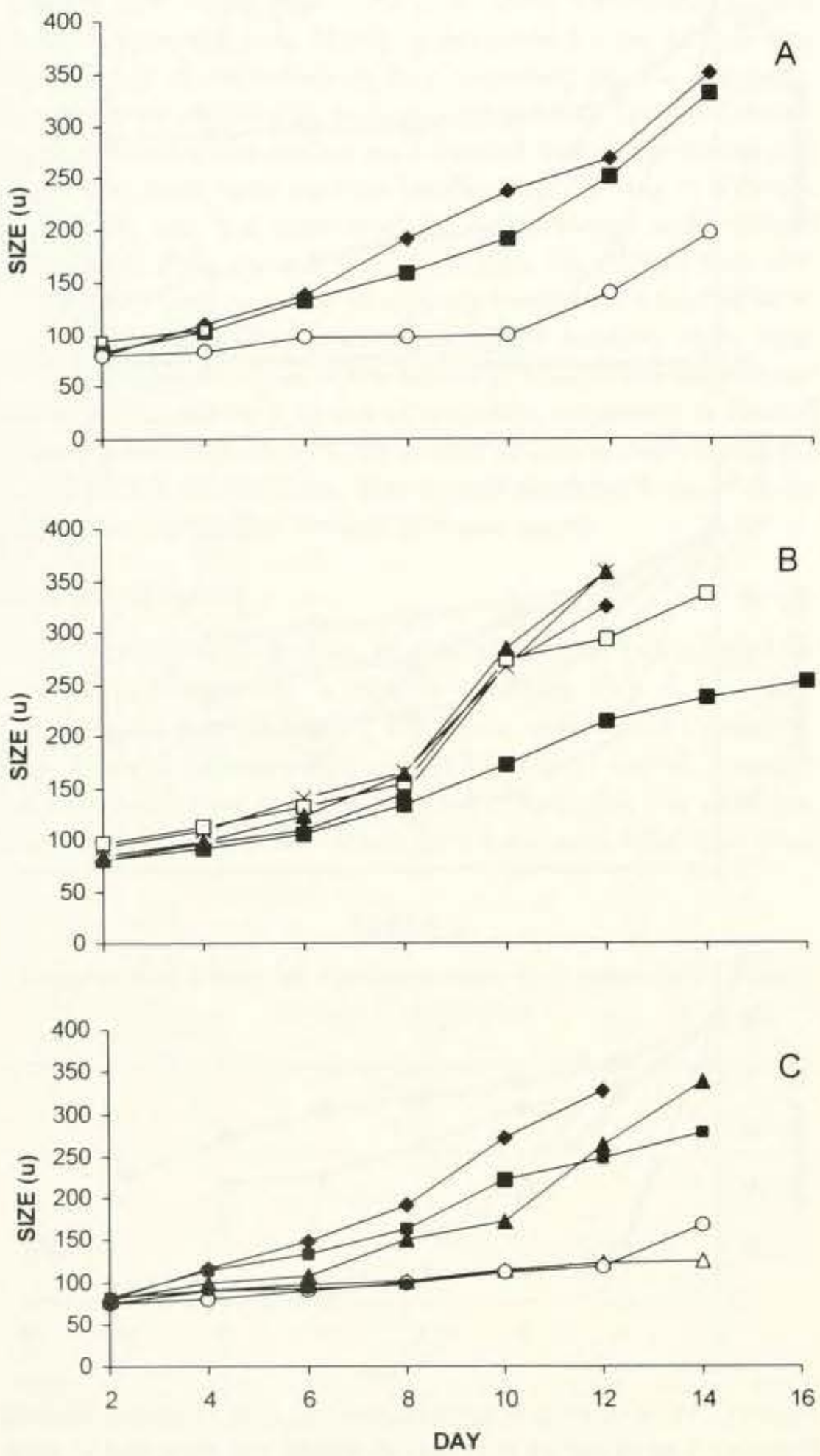

Figure 2. Mean size (μm) of hybrid and control larvae from day 2–16 in crosses of diploid and tetraploid *C. gigas* with diploid *C. ariakensis*. (A) Larvae from GG, GA, GGA, and AA in replicate 1; (B) larvae from GG, GA, GG/G, and G/GG in replicate 2; and (C) larvae from GG, GA, G/GG, AGG, AA, and AG in replicate 3. Measurement of surviving larvae was made until eyed larvae appeared. GG (♦), GA (■), G/GG (▲), GG/G (×), GGA (□), AGG (△), AA (○), AG (●).

Cytological Observation of Eggs from Hybrid Crosses

The vast majority of eggs from hybrid crosses were delayed at prophase I or metaphase I at least through 180 min post-insemination (Fig. 3A–E). In fact, of all eggs examined at 180 min post-insemination (≥150 observations from each of GA, AG, GGA, and AGG), only 2% of eggs from GGA had entered anaphase I (Fig. 3F). In GA, AG, and AGG, 10 bivalents were still observed at this time. Chromosome aggregation was much more complicated in eggs from GGA crosses. In general, eggs contained an average of 10 quadrivalents, although other types of synaptic chromosomes were also present, i.e., univalents, bivalents, and trivalents (Fig. 3D, E).

Genetic Confirmation

Agarose gel electrophoresis of the PCR products consistently revealed two bands, one at around 587 base pairs (bp) and the other at around 527 bp (Fig. 4, top). Restriction digest fragments resolved distinct bands in both parental species. Two bands were resolved, one at around 434 bp and the other around 138 bp in *C. gigas* females, both tetraploid and diploid. In contrast, two bands were resolved at around 267/257 bp and 174 bp in *C. ariakensis* males (Fig. 4, bottom). The hybrids (GA and GGA) expressed all four bands corresponding to their parental species. An additional band was detected at around 458 bp in hybrids. Identical band resolution was observed in replicates 1 and 2 of GA crosses, whereas an extra band was resolved at 587 bp consistently in both parental species and hybrids in replicate 3 (Fig. 4, bottom, last four lanes). All progeny samples (28 individuals from each replicate of GA and a total of 4 individuals from GGA) were hybrids. Three of four GGA hybrids were triploid as confirmed by flow cytometry; the other hybrid was diploid.

DISCUSSION

In hybrid crosses, fertilization was not apparent even as late as 180 min post-insemination. These same observations were reported previously (Miyazaki 1939, Imai & Sakai 1961), both

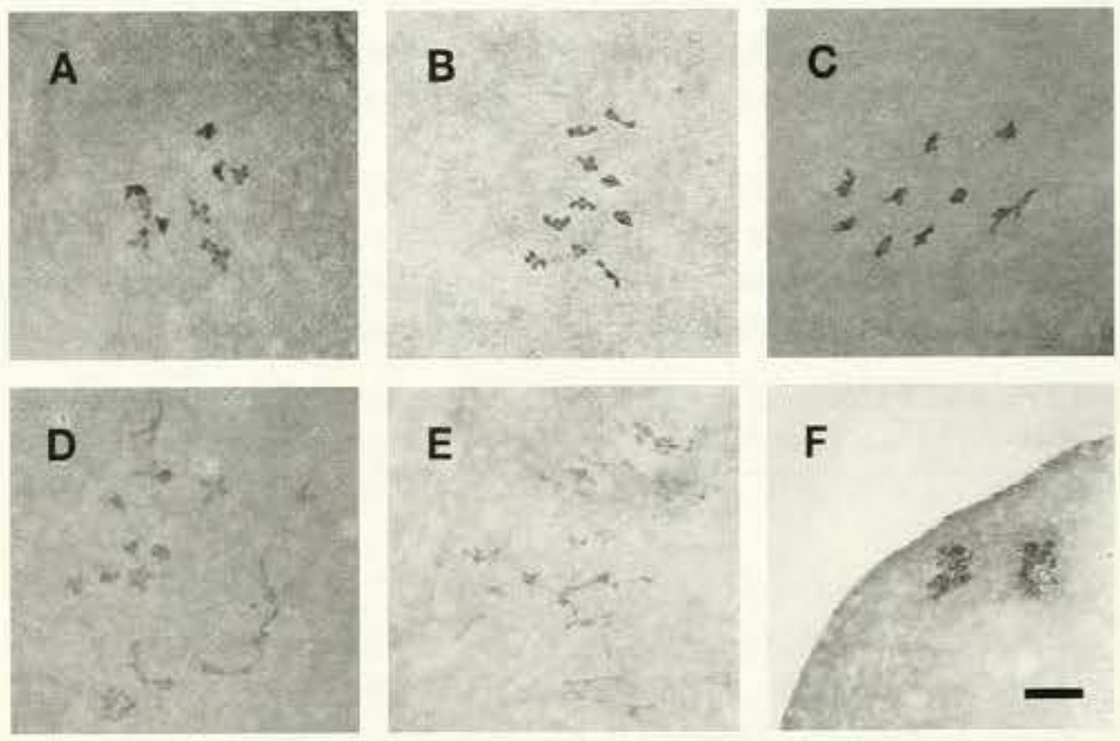

Figure 3. Chromosome observations in crosses of diploid and tetraploid *C. gigas* with diploid *C. ariakensis* at 180 min post-insemination. (A) Prophase I in oocytes from GA; (B) prophase I in oocytes from AG; (C) prophase I in oocytes from AGG; (D) and (E) prophase I in oocytes from GGA; and (F) anaphase I in oocytes from GGA. Scale bar on (D): 10 μm.

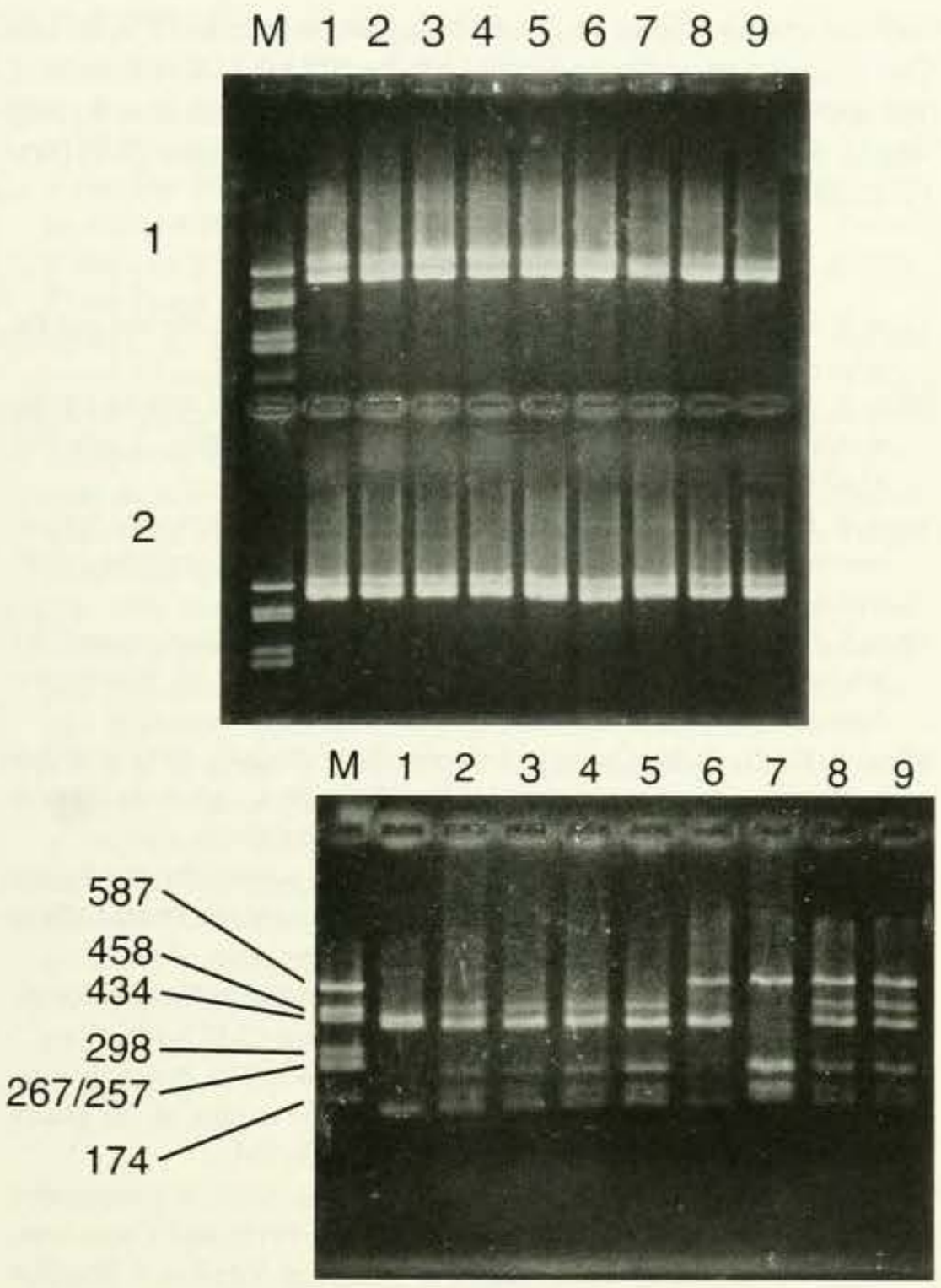

Figure 4. Top: Electrophoretic separation of undigested DNA products resulting from PCR amplification of genomic DNA, using primer of ITS-1. rDNA originated from parental species and their hybrid progeny. Row 1: lane 1 = diploid *C. gigas* (♀); lane 2 = diploid *C. ariakensis* (♂); lanes 3–4 = G × A progeny; lane 5 = diploid *C. gigas* (♀), lane 6 = diploid *C. ariakensis* (♂), lanes 7–9 = G × A progeny. Row 2: lane 1 = tetraploid *C. gigas* (♀), lanes 2–5 = GG × A progeny; lane 6 = diploid *C. gigas* (♀), lane 7 = diploid *C. ariakensis* (♂), lanes 8–9 = G × A progeny. Lane M = molecular weight marker. Bottom: Electrophoretic separation of PCR amplified rDNA products from GA and GGA crosses digested with restriction endonuclease Hinf I. M = molecular weight marker; lane 1 = tetraploid *C. gigas* (♀; lanes 2–5 = GG × A progeny, lane 6 = diploid *C. gigas* (♀), lane 7 = diploid *C. ariakensis* (♂), lanes 8–9 = G × A progeny. The molecular weights of bands resolved from the marker are, from largest (slowest migrating) to smallest: 587, 458, 434, 298, 267/257, 174, and 102 base pairs, respectively.

of whom found little or no fertilization between *C. gigas* and *C. ariakensis* in their studies of Japanese oysters. However, we observed 8%–9% fertilization rate in GA crosses in other experiments (data not shown). In a previous study, a mean fertilization rate of 12% was reported for GA crosses (Allen & Gaffney 1993). Zhou et al. (1982) revealed that fertilization rates in *C. gigas* (♀) × *C. ariakensis* (♂) were 0–52.6% and its reciprocal, 2.3%–18.8%. These data indicate that the fertilization rate in these hybrids varies widely. Success of hybridization should not be assessed solely on the fertilization rate. In contrast, fertilization between other species of *Crassostrea* occurred readily, and often showed relatively high rates, but larvae survived for only a short time before complete mortality (Menzel 1986; Allen et al. 1993).

Despite the apparent lack of fertilization (observed up to 180 min post-insemination), replicates of GA yielded viable spat, which reconfirms the compatibility of the gametes from *C. gigas* (♀) and *C. ariakensis* (♂). Buroker et al. (1979) reports a relatively high genetic similarity between these two species. Other studies also demonstrate the feasibility of hybridization between these two species (Allen & Gaffney 1993; Downing 1988; Downing 1991; Zhou et al. 1982), although only Allen & Gaffney (1993) confirmed hybrids genetically. In contrast to the success of GA, the reciprocal AG failed to produce any spat, although no morphological deformities were observed in the larvae. AG larvae were previously shown to be much less viable compared with the reciprocal although a few spat were obtained (Allen & Gaffney, 1993). The diploid control AA (three replicates) consistently survived poorly and grew slowly in this study. This may partly account for the failure to obtain any spat from the AG cross. According to published accounts (Breese & Malouf 1977; Langdon & Robinson 1996), the survival and growth of *C. ariakensis* are similar to *C. gigas* under appropriate culture conditions. It is not clear why larvae of *C. ariakensis* performed so poorly in this study, because we have routinely cultured *C. ariakensis* on other occasions.

Only one of three replicates of GGA yielded spat. The failure of the other two replicates might be attributable to low fecundity of tetraploid *C. gigas* used here (data not shown), although generally tetraploids have shown high fecundity (Guo et al. 1996; B. Eudeline, Taylor United, Inc. and S.K. Allen, Jr., unpublished data). In all three replicates, yield at day 2 was low. We suggest that the major barrier for GGA production on a pilot- or production-scale is low yield at day 2. Afterward, larvae of GGA crosses survived well (virtually no mortality). While it is clear that more GGA progeny could be obtained by using more parents, the real challenge is to find factors that lead to high levels of fertilization for the gametes that are available. We also suggest that there is a difference in growth rate between triploid (GGA) and diploid (GA) hybrids. First, GGA eyed larvae appeared 5–7 days earlier than GA and right after those of controls GG, G/GG, and GG/G. Second, the size of spat from GGA was greater than the size of GA at 90 days post-insemination, although the number of GGA spat was small.

All 28 progeny sampled from each of three replicates of GA were hybrids. With respect to GGA progeny, some eyed larvae attached to the culture containers, leading to loss of eyed larvae. Consequently, only four culchless spat were obtained. However, the fact that three of the spat were triploid hybrids demonstrates that hybridization between tetraploid *C. gigas* (♀) and diploid *C. ariakensis* (♂) was successful. Triploid hybrids of *C. gigas* and *C. ariakensis* cannot be obtained in any other way. In particular, the use of cytochalasin B (or other polar body inhibitor) is precluded in GA crosses because of the prolonged period leading to syngamy and polar body formation. It is not possible to create triploids without some level of predictability and synchrony among developing eggs (Allen et al. 1989).

PCR restriction fragment length polymorphism (RFLP) diagnosis was an effective means to verify putative hybrid progeny. ITS-1 amplification/Hinf I digestion successfully distinguished among *C. gigas*, *C. ariakensis*, and hybrids, which show bands present from both parental species. Application of this method is based on availability of an appropriate primer: ITS-1 in this study. Other methods have been used to confirm hybrid status (Allen & Gaffney 1993; Allen et al. 1993; Nakamura et al. 1990; Jiang et al. 1988). Karyotype analysis has been useful for hybrids among the

pearl oyster genus *Pinctada* (Jiang et al. 1988). For species within the genus *Crassostrea*, the great similarity in their karyotype renders this type of analysis useless in hybrid documentation until more discriminating markers are designed. Flow cytometry was equivocal in discriminating between hybrid and pure crosses using dissociated cells of pooled larvae (Allen et al. 1993).

Cytological examination of newly fertilized eggs from hybrid crosses revealed that either fertilization was severely delayed or early development of oocytes was extremely slow. The duration of meiotic maturation in hybrid crosses was at least triple that of controls. The delay of meiosis was probably not due to the quality of gametes because eggs in controls were normal and reached first cleavage at around 60 min post-insemination consistently (data not shown). Another hybridization attempt between *C. gigas* and *C. ariakensis* indicated there was no apparent difference in the time of development from eggs to the straight-hinge larvae (Zhou et al. 1982). Apparently, fertilization was also highly successful in the study by Zhou et al. leading us to suspect contamination. An alternative explanation is that Zhou et al. used a different population of *C. ariakensis*. The *C. ariakensis* used here are from a population derived from Ariake Bay in Japan. Those of Zhou et al. are likely from mainland China. In this study, despite the delay in fertilization, healthy larvae were obtained and cultured through metamorphosis in all replicates of GA and one replicate of GGA. Furthermore, hybrid spat were obtained. Success in obtaining larvae suggests that meiosis in GA and GGA crosses must have resumed at some time, despite being delayed for an uncommonly long time. A similar observation was made in *C. angulata* oocytes fertilized with sperm from *C. virginica*, where 47% of them exhibited metaphase I nearly 4 h after insemination. However, no parental crosses were made for comparison (Stiles 1973). Oocytes in hybrid crosses of *C. gigas* and *C. ariakensis* with *C. virginica* proceed through meiosis and early mitosis in fairly normal fashion (Scarpa & Allen 1992). The timing of meiotic and mitotic landmarks was the same for all crosses, parental and hybrid. However, hybrids of *C. gigas* and *C. ariakensis* with *C. virginica* were inviable (Allen et al. 1993) after a short larval period despite normal meiotic and early mitotic behavior. *C. gigas* × *C. ariakensis* crosses were not included in Scarpa and Allen's work.

Overall, this study reveals a new potential application for oyster breeding: triploid *C. gigas* × *C. ariakensis* hybrids. We were limited to relatively few tetraploid brood stock for this study, but since that time, tetraploids have become commercialized on the West coast of the United Staes. We were also limited to making the triploid hybrid in one direction; that is, using only *C. gigas* as the tetraploid, enabling experiments on GGA (4n♀) or AGG (4n♂). We were unable to explore GAA (4n♂ *C. ariakensis*) or AAG (4n♀ *C. ariakensis*). Presumably the former would behave like GA and GGA cultures, and the latter would behave like AG and AGG cultures. These experiments will have to wait for the tetraploid *C. ariakensis* that are currently under development. At present, GGA hybrids can be made only at a high cost of gametes from the parental species. Even with high fecundity, the losses are so severe that commercial production would be a problem. Studies on factors that promote higher fertilization rate, syngamy, development rates, or all of these would contribute to efforts to produce pilot-scale quantities of GGA for future testing.

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