

An Induced Mass Spawn of the Hermaphroditic Lion-Paw Scallop, *Nodipecten subnodosus*: Genetic Assignment of Maternal and Paternal Parentage

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Abstract

The Pacific lion-paw scallop is commonly propagated for aquaculture by induced mass spawns of few individuals. Parentage of a mass spawn of this species has not been evaluated nor has the maternal and paternal contribution of each of these functional hermaphrodites to the progeny. Genotypes of 6 spawners and 374 resulting progeny at 6 microsatellite loci were coupled with mitochondrial DNA sequencing to assign maternal and paternal parentage. After the identification of a high proportion of null alleles (9.7%), microsatellite data revealed that 51.7% of the progenies were full siblings, with a significant, unequal contribution of the 6 spawners to the progeny. Three progenies were the result of self-fertilization. All spawners contributed paternally (though unequally); however, 2 spawners were the maternal parents of all but 7 progenies resulting in a variance effective population size of 3.52. DNA sequencing confirmed 4 microsatellite mutations within 4476 alleles scored, all in the paternal germ line. With minor exception, the loci conformed to Mendelian rules of segregation when null alleles were accounted for, and 2 loci were found to be linked. These results lend insight to the genetic composition of induced mass spawns and provide a basis for the development of more effective spawning techniques.

Natural populations of the lion-paw scallop (*Nodipecten subnodosus*) are distributed along both the Pacific and Gulf of California coasts of the Baja California Peninsula, Mexico, southward to the western coast of Peru (Keen 1971; Masso-Rojas et al. 2000). The largest scallop in the tropical Eastern Pacific, the lion-paw has been the subject of commercial fisheries for decades. A recent increase in economic value of the scallop (Masso-Rojas et al. 2000), relative ease of culture (Garcia-Esquivel et al. 2000; Koch et al. 2005), and the decline of local abalone fisheries (Hobday and Tegner 2000) have led to the development of aquaculture companies specific to the lion-paw scallop. The Mexican government is currently issuing aquaculture permits for this scallop around the Baja California Peninsula, almost all of which are within Guerrero Negro (Figure 1) (SAGARPA 2003).

As a functional hermaphrodite, the lion-paw scallop releases both eggs and sperm during each annual spawn. The potential for self-fertilization, coupled with a high

fecundity of more than 20 million eggs per spawn per individual (Maldonado-Amparo et al. 2004), contributes to an increased possibility of variance in reproductive success (Hedgecock 1994; Hedgecock et al. 2007) as well as inbreeding depression in later generations. This reproductive variance in highly fecund marine organisms has been referred to as a sweepstakes reproductive effort (Hedgecock 1994), where some individuals have substantial success in spawning, whereas others have little or none. In such a scenario, genetic drift becomes a considerable force in shaping the genetic diversity of future generations. In aquaculture situations, where spawning is already restricted to a select set of individuals, any variation in reproductive effort may significantly affect current and future stocks.

Aquaculture companies produce millions of progenies for harvest by replicating the lion-paw scallops' natural method of propagation through induced mass spawns. However, unlike natural spawns, which potentially involve

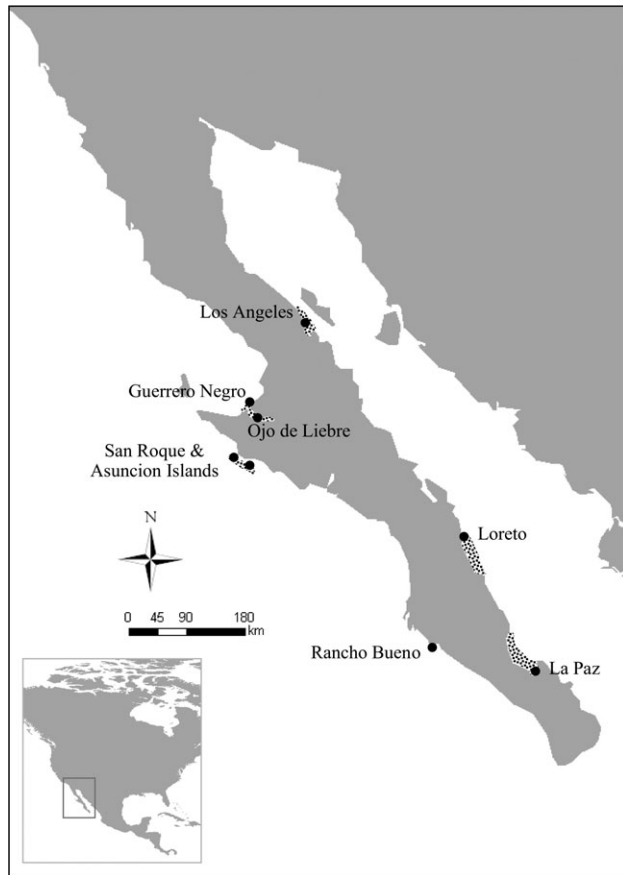


Figure 1. The Baja California Peninsula, Mexico, illustrating locations where sampling, spawning, and grow out of lion-paw scallop occurred. Dotted areas indicate known aggregations of the lion-paw scallop.

thousands of individuals, as few as 6 scallops are used during each spawn in aquaculture. Although significant genetic drift has been shown to result from mass spawns of other species (Withler 1988; Gaffney et al. 1992; Benzie and Williams 1996), it has not been determined whether progenies of mass spawns of *N. subnodosus* represent an equal and random contribution of each spawner.

In this study, a mass spawn of 6 lion-paw scallops was carried out in a means similar to that which would be used in an aquaculture setting. The primary objective of this study was to utilize microsatellite DNA markers in conjunction with mitochondrial DNA (mtDNA) to identify maternal and paternal parentage of a sample of progeny from this mass spawn and therefore determine the extent of contribution of each spawner. This was performed with the purpose of using these data to discuss the implications of propagation through mass spawns for both aquaculture and natural populations. This is the first instance in which parentage of progeny resulting from such a mass spawn has been evaluated in this species and additionally is the first example in which parentage in this functional hermaphrodite has been broken down into maternal and paternal

components. Besides addressing the primary objective mentioned above, these data generated information valuable to future research of this species regarding allelic inheritance, incidence of null alleles, and microsatellite mutation rates within the species and will contribute to its improved management in the aquacultural setting as well as in the wild.

Methods

Controlled Mass Spawn

Thirty adult lion-paw scallops (shell length 15 cm) were obtained in September 2004, by diving in Guerrero Negro lagoon, Baja California, Mexico (Figure 1), and transported to the Aquaculture Genetics Laboratory at Centro de Investigaciones Biológicas del Noroeste S.C. for maturation conditioning (Ramírez et al. 1999; Maldonado-Amparo et al. 2004) for 30 days prior to spawning. After this time, 6 scallops were chosen by visual inspection of the gonad for an advanced maturation stage. The act of spawning was performed in a means similar to that used in aquaculture. Each scallop was induced to spawn by injecting 0.2 ml of serotonin (0.5 mM) in the adductor muscle (Ruiz-Verdugo et al. 2001). To avoid polyspermy, the scallops were first placed separately in a 20-l bucket for 60 min, exchanging the water as needed to discard sperm. They were then placed together in a 1500-l tank to allow for further gamete release. However, because the amount of sperm released in this tank still reached undesired levels, all scallops were transferred to a second 1500-l tank, which was used for data collection. The scallops were determined to have completed spawning when visual inspection of the female portion of the gonad showed a change in coloration from bright red-orange to translucent light orange. Once spawning was complete, the scallops were removed from the spawning tank and a 1-cm² sample of adductor muscle from each spawner (referred to as G1–G6) was collected for genetic analysis.

After 24 h, the D-larvae were collected from the tank using a 45- μ m screen sieve, washed with 1- μ m filtered seawater, counted by taking the average count of larvae within 3 samples of a known volume, and extrapolating the calculated density to the total volume. All larvae were grown to the pediveliger stage (15 days) by stocking of 10 larvae/ml and feeding *Isochrysis galbana* and *Chaetoceros calcitrans* (1:1) at an initial concentration of 30 000 cells/ml, increasing to 80 000 cells/ml by day 14. The number of pediveliger larvae was estimated using the same method described for D-larvae.

All pediveliger larvae were placed in shallow (0.50 m deep) fiberglass curved-bottom tanks at a stocking density of 0.5 larvae/mm² for settling. Feeding continued using the same diet at concentrations up to 200 000 cells/ml. After settling and growing for another 30 days, the population size of the spat (approximately 3 mm) was estimated once again.

A random sample ($N = 2000$) of spat was taken from the laboratory to the grow-out facility at Estero de Rancho

Bueno, south of Magdalena Bay in Baja California Sur Mexico. At approximately 6 months of age (April 2005), 1 cm² of adductor muscle was sampled from 374 progenies and stored in 70% ethanol. All samples were shipped to the Genomic Variation Laboratory at the University of California Davis, CA, for genetic analyses.

DNA Extraction and Amplification

DNA was extracted from each individual with the Promega Wizard SV96 extraction kit and quantified with a 595 Fluorimager (GE Healthcare Piscataway, NJ). Each individual was genotyped at 6 microsatellite loci consolidated into 2 multiplex polymerase chain reactions (PCRs) (Multiplex 1—NsubA274, NsubB278, NsubC262; Multiplex 2—NsubA223, NsubA227, NsubC023; Ibarra et al. 2006). PCR protocol was as follows: Multiplex 1—10 ng template DNA, 1 µl Promega 10× buffer, 1–2 µl of each primer (10 µM), depending on relative fluorescence in relationship to other primers in the multiplex, 1 µl MgCl (25 mM), 0.8 µl deoxynucleoside triphosphates (dNTPs) (20 µM), 0.075 µl Promega *Taq* polymerase, and nanopure water to bring the total reaction volume to 10 µl; Multiplex 2—15 ng template DNA, 1 µl Promega 10× buffer, 1–2 µl of each primer (10 µM), 0.75 µl MgCl (25 mM), 0.8 µl dNTPs (20 µM), 0.075 µl Promega *Taq* polymerase, and nanopure water to bring the total reaction volume to 10 µl. Forward primers were fluorescently end labeled with 6-FAM, VIC, or NED for visualization. The thermal cycler profile for both multiplexes consisted of an initial denaturation at 94 °C for 2 min followed by 24 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 2 min.

Electrophoresis took place with 5.5% denaturing polyacrylamide gels run at 2600 V for 75 min on a Bio-Rad BaseStation in genotyping mode (Bio-rad, Hercules, CA). A ROX-labeled GeneScan 500 internal size standard (Applied Biosystems Foster City, CA) was run with each sample. Individual genotypes were scored using Cartographer software (Bio-Rad), and all allele sizes were verified manually. PCR product from the 6 parents was run on each gel for scoring consistency.

Analyses

Null Alleles and Microsatellite Parentage

The presence of null (nonamplifying) alleles was determined by microsatellite inheritance patterns. Initially, a subset of progeny was manually assigned to parents using only 1–3 loci. Assignment based on this subset of the loci allowed for the analysis of inheritance patterns at the other loci. For example, in the case where a progeny with a single locus genotype of A/A was known to be the offspring of parents with genotypes A/B and C/C, the parent with genotype C/C was concluded to have a null allele. The presence of null alleles in each parent at each locus was confirmed by evaluating the remaining offspring of that parent in question.

Progenies found to have alleles not present in the parent population were rerun and rescored to confirm the

genotype. In some cases, DNA was reextracted from the original tissue and resequenced to confirm the allele sizes.

After putative null alleles were assigned to parents, manual assignment of progeny was confirmed using CERVUS 3.0 (Marshall et al. 1998; Kalinowski et al. 2007) with null alleles designated as 999 bp. CERVUS employs a likelihood-based approach for parentage assignment based on comparison of real genotypes to those simulated from the allele frequencies of the data. Analyses accounted for the possibility of self-fertilization, genotyping error rate was kept at the default value of 1%, and 10 000 offspring were simulated. Parentage assignment of individual parents was based on significant log of odds ratio (LOD) scores at the 95% confidence level. The polymorphism information content (PIC) value of each locus was also determined in CERVUS.

Mendelian Inheritance and Gametic Combination

After parsing the largest family (G1 × G3) into maternal and paternal components, each locus of spawners G1 and G3 was tested for deviation from Mendelian 1:1 allelic segregation ratio using a chi-square test. The *P* values were evaluated for significance after a sequential Bonferroni adjustment for multiple tests (Holm 1979). Random combination of gametes within the progeny was also tested in families G1 × G3 and G3 × G1 (maternal × paternal) using chi-square tests. The expected ratio of genotypes present in the offspring was calculated both by using the observed segregation ratios of the parental alleles at each locus for that family as well as by assuming a 1:1:1:1 or 1:1 genotypic ratio.

Gender-Specific Parentage Assignment

Two *N. subnodosus* mtDNA sequences for the 16S rRNA subunit (GenBank accession numbers AJ972441, AJ972442) (Saavedra and Peña 2006) were aligned using SEQUENCHER v4.7 (Gene Codes, Ann Arbor, MI). The consensus sequence was input into PRIMER3 (Rozen and Skaletsky 2000) to design PCR primers specific for this species. The resulting primer pair amplified approximately 415 bp of the 16S rRNA subunit using the forward and reverse oligos: Nsub16SF (5'-GGGTAACATGGGGAGTCGT-3') and Nsub16SR (5'-CAAACCCGCCCATCTATAAG-3'). The PCR protocol for amplification of mtDNA was as follows: 20 ng template DNA, 2 µl 10× Promega buffer, 1.5 µl each of forward and reverse primers (10 µM), 1.5 µl MgCl (25 mM), 1.5 µl dNTPs (20 µM), and 0.375 U Promega GO *Taq* polymerase. Nanopure water was added to bring the total reaction volume to 20 µl. PCR product was purified using the QIAquick PCR cleanup kit (Qiagen, Chatsworth, CA), and sequencing using the primer Nsub16SR was performed at the University of California Davis' Department of Biological Sciences sequencing facility. The resulting sequences were aligned, and the electropherograms were visually checked for any ambiguities. All sequence visualization, editing, alignment, and evaluation were performed in the program SEQUENCHER v4.7 (Gene Codes).

A similar procedure was employed for primer development to amplify and sequence a 340-bp fragment of the 12S rRNA subunit. Oligos Nsub 12SF (5'-AGTG-CAGCTGCTTGGGTACT-3') and Nsub 12SR (5'-CA-CAAGTTAGATTTCACGTTTCAGG-3') were designed in PRIMER3 (Rozen and Skaletsky 2000) after alignment of 2 *N. subnodosus* sequences in GenBank (accession numbers AM039777, AM039778) (Saavedra and Peña 2006). PCR conditions, cleanup, and sequencing procedures were identical to those used for the Nsub16S fragment.

Microsatellite Sequencing

The allele from each progeny that did not match that of either assigned parent was sequenced along with the parental alleles of the parent in which the mutational event was suspected to occur. Each individual was amplified at the locus of interest in single locus PCRs consisting of 10 ng template DNA, 1 μ l Pfu 10 \times buffer (Stratagene, La Jolla, CA), 0.5 μ l of each primer (10 μ M), 0.75 μ l MgCl (25 mM), 0.8 μ l dNTPs (20 μ M), 0.025 μ l Go Taq Flexi polymerase (Promega, Madison, WI), 0.7 μ l Pfu polymerase (Stratagene), and nanopure water to bring the total reaction volume to 10 μ l. The thermal cycler profile consisted of an initial denaturation at 94 $^{\circ}$ C for 2 min followed by 34 cycles of 94 $^{\circ}$ C for 30 s, 62 $^{\circ}$ C for 30 s, 72 $^{\circ}$ C for 1 min, and a final extension at 72 $^{\circ}$ C for 10 min. Each individual was amplified in replicate at the locus of interest.

The PCR product was diluted 1:1 with 98% formamide loading dye, and 3.5 μ l was loaded into a 5% denaturing polyacrylamide gel for electrophoresis at 45 W for 90 min. The gel was stained using the SYBR-green agarose gel overlay technique of Rodzen et al. (1998) and visualized on a Fluorimager 595 (GE). The alleles of interest were excised from the gel and placed into 150 μ l of nanopure water for elution overnight at room temperature. About 8 μ l of the eluted DNA was utilized in PCR reamplification using the same protocol for the initial reaction except increased to 40 μ l reactions. Resulting PCR product (1 μ l) was run on a 5% denaturing polyacrylamide gel as previously described to check for amplification of the allele of interest. PCR product was cleaned up using either an AMPure (Agencourt, Beverly, MA) or QIAquick (Qiagen) kit. Cleaned product was sent to the University of California Davis' College of Agricultural and Environmental Sciences Genomics Facility for sequencing in both directions. Each allele of interest was sequenced at least 2 times in each direction from each of the 2 PCR amplifications. Resulting sequences were visualized and aligned using SEQUENCHER v4.7 (Gene Codes).

Effective Population Size

The inbreeding and variance effective population sizes were calculated using the observed gamete contribution of each spawner (Crow and Denniston 1988), developed for monocious organisms with the potential for self-fertilization. The mean number of gametes (k) contributed per individual was adjusted to 2, to account for a bias in the calculation of effective population size caused by an inflation of the

variance in gamete contribution as the mean number of gametes contributed increases. This phenomenon results in an inflated "index of variability" (V_k/k) as explained in Crow and Morton (1955) and Hedrick et al. (1995). Calculation of inbreeding effective population size was performed considering only the microsatellite data, without the knowledge of maternal/paternal contribution (equation 1), as well as accounting for the type of gamete contributed (equation 4) (Crow and Denniston 1988).

Linkage Analysis

LINKMFEX ver 2.3 (Danzmann and Gharbi 2001) was employed to compute linkage and maternal/paternal recombination rates by examining allelic segregation. Potential linkage was evaluated for all parents in families where the spawner contributed to at least 25 progenies either maternally or paternally. Significant evidence of linkage was indicated by minimum LOD score of 3.0 ($P < 0.001$).

Results

Spawning

An estimated 14.4 million D-larvae were present at 24 h after spawning. This number decreased from 3.4 million at 15 days to 602 492 at 45 days of age. A census of the population 6 months after deploying 2000 scallops to the field found approximately 1500 surviving.

Null Allele Frequency, Mendelian Tests, and Gametic Combination

The evaluation of inheritance patterns in the spawners revealed a total of 7 null alleles present within 5 of the 6 loci genotyped for each spawner (9.7%).

With null alleles identified, analysis of allele segregation within family $G1\text{♀} \times G3\text{♂}$ showed significant deviation from the expected 1:1 ratio in spawner G3. No significance was found evaluating the same locus in the reciprocal cross with G3 as the maternal parent (Table 1). Gamete combination in the progeny did not differ from that expected at random when utilizing mtDNA to break family $G1 \times G3$ into maternal and paternal sibships, with the exception of NsubA274 and NsubC262 in parent G3 when acting paternally and when assuming a 1:1:1:1 expected genotypic ratio (Table 1). Only the deviation at locus NsubC262 remained significant after correction for multiple testing. Due to small family size, tests of Mendelian inheritance and random association of gametes were not performed on other crosses.

Microsatellite Parentage Assignment

The PIC value of the 6 loci ranged from 0.69 to 0.82 with a mean value of 0.77 and allowed for assignment of the progeny using only these markers (parent pair nonexclusion probability = 6.9×10^{-5} for these data). Parentage assignment was confirmed by significant LOD scores in

Table 1. *P* values resulting from chi-square test for Mendelian segregation at each locus and test of random gamete combination for both reciprocal crosses of G1 × G3 Under the progeny, genotypes are the observed and expected (in parentheses) numbers. Tests of gametic combination were performed using the observed segregation ratio of each individual to determine the expected number (first) of each genotype as well as by assuming a 1:1:1:1 or 1:1 genotypic ratio (second)

Family Maternal × paternal	Locus	Individual locus segregation				Gametic combination				
		Spawner G1		Spawner G3		Progeny genotypes				
		Genotype	<i>P</i> value	Genotype	<i>P</i> value	Observed (expected)				<i>P</i> value
G1 × G3	NsubA274	272/275	0.93	272/293	0.005 ^a	272/272	275/272	272/293	275/293	0.85/0.05 ^a
						34 (36.3/28.75)	38 (36/28.75)	24 (21.4/28.75)	19 (21/28.75)	
	NsubB278	186/202	0.64	204/242	0.78	186/204	186/242	202/204	202/242	0.48/0.42
						31 (26.8/28.75)	24 (28.2/28.75)	25 (29.2/28.75)	35 (30.8/28.75)	
	NsubC262	222/234	0.93	218/238	0.00005 ^b	222/218	222/238	234/218	234/238	0.98/0.0009 ^b
						19 (17.7/28.25)	39 (39.3/28.25)	16 (17.3/28.25)	39 (38.7/28.25)	
NsubC023	272/318	0.31	360/380	0.19	272/360	272/380	318/360	318/380	0.99/0.45	
					28 (27.4/28.5)	34 (35.1/28.5)	22 (22.6/28.5)	30 (28.9/28.5)		
NsubA223	178/178	n/a	184/Null	0.93	178/184	178/Null			1/0.93	
					58 (58/57.5)	57 (57/57.5)	206/Null	242/Null		
NsubA227	206/242	0.93	266/Null	0.64	206/266	242/266	206/Null	242/Null	0.94/0.88	
G3 × G1	NsubA274	272/275	0.36	272/293	0.82	272/272	275/272	272/293	275/293	0.44/0.32
						13 (16.6/19)	24 (20.4/19)	21 (17.4/19)	18 (21.6/19)	
	NsubB278	186/202	0.17	204/242	0.82	186/204	186/242	202/204	202/242	0.995/0.57
						22 (22.6/19)	22 (21.4/19)	17 (16.4/19)	15 (15.6/19)	
	NsubC262	222/234	0.56	218/238	0.36	222/218	222/238	234/218	234/238	0.42/0.22
						19 (15.7/18.75)	16 (19.3/18.75)	14 (17.9/18.75)	26 (22.1/18.75)	
NsubC023	272/318	0.65	360/380	0.30	272/360	272/380	318/360	318/380	0.80/0.52	
					18 (19.9/18.75)	18 (15.6/18.75)	24 (22.1/18.75)	15 (17.4/18.75)		
NsubA223	178/178	n/a	184/Null	0.82	178/184	178/Null			1/0.82	
					37 (37/38)	39 (39/38)	206/Null	242/Null		
NsubA227	206/242	0.30	266/Null	0.56	206/266	242/266	206/Null	242/Null	0.42/0.22	

^a Significant before correction for multiple testing.

^b Significant after a sequential Bonferroni correction.

CERVUS 3.0 in 373 of the 374 offspring. One offspring failed to assign to any of the 6 parents (8/12 alleles were not found in any spawner) and was likely a contaminant from other spawns occurring simultaneously in the laboratory. This individual was eliminated from all further analyses. All other progeny assigned at the strict (95%) level of confidence to only one parent pair.

Five progenies were observed to have 1 allele that did not match that of either parent. In each case, parentage was confirmed by assignment at the other 5 loci and confident assignment in CERVUS. Each progeny had one allele that matched an allele of one parent. That parent was therefore excluded from sequence analysis to identify the source of the mutation. Sequencing of the other parent alleles and progeny alleles suspected to be the result of mutations confirmed 4 mutational events within the microsatellite repeats (Table 2). Two mutations were the loss of 2 repeats units, whereas 1 showed a gain of 1 repeat. The origin of the suspected mutational event at locus NsubB278 is unclear as the parent had a null allele. Sequencing of the available parent allele showed that it differed from that of the progeny by a disruption of the motif along with an overall gain of 3 repeats.

On amplification for sequencing the fifth progeny that was scored as having a null mutation, a previously unseen allele amplified in 2 of 3 replicates of the PCR. This allele was sequenced and found to be identical to the parent in whom the mutation was suspected. This allele did not amplify in the 3 genotyping runs at this locus for this individual.

All 4 confirmed microsatellite mutations occurred in the paternal germ line, 2 in spawner G3 (rate per allele scored = 1.1×10^{-3}), 1 in G5 (2.1×10^{-3}), and 1 in G1 (6.1×10^{-4}). The per locus mutational rate varied from 2.9×10^{-3} for NsubC262 to 1.3×10^{-3} for NsubC023 and NsubB278 with the overall microsatellite mutation rate observed for these data at 8.9×10^{-4} (4/4476 alleles). Microsatellite sequencing also revealed several single-base polymorphisms present in the flanking regions of loci NsubC262 and NsubC023 (Table 2).

Parentage assignment showed that spawners G1 and G3 contributed to 272 and 291 of the 373 offspring, respectively, or 36.9% and 39.1% of the total gametes (Table 3). The contribution of spawners G2, G4, G5, and G6 was 6.4%, 1.3%, 10.9%, and 5.5%, respectively, and the contribution per individual was significantly different than that expected if all contributed equally to the progeny ($P = 4 \times 10^{-132}$).

Of the 15 possible outcrossed families, 11 were observed in this sample (Table 3). However, more than half (51.7%) of all individuals were full siblings belonging to family G1 × G3. Only 3 of the 373 progenies were not related to either spawner G1 or G3. Three other progenies (0.8%) resulted from self-fertilization, all of spawner G1.

Gender-Specific Parentage Assignment

Maternal parentage was determined using mtDNA sequencing for 364 of the 370 offspring resulting from matings of spawners G1 and G3 (99.2% of all progenies). Analysis of

Table 2. Mutational events observed in the progeny. Bold type shows the parent allele in which the mutation was likely to have occurred. In each progeny, one allele was identical to that of the other assigned parent and is not shown in the Table. Polymorphisms in regions outside the repeats are shown and a deletion is designated by “—”

GenBank accession	Individual (allele size)	Microsatellite sequence ^{a,b}
Locus NsubB278		
EU350106	Spawner G5 (171)	(ATGT) ₈ ATG
n/a	Spawner G5 (null)	—
EU350107	Progeny P290 (185)	(ATGT) ₆ GT(ATGT) ₅ ATG
Locus NsubC023		
EU350114	Spawner G3 (361)	G...C...A...A...(ATGG) ₅ (GTGG) ₂ (ATGG) ₆ ATA(GATA) ₁₄ GAT(GGAT) ₆ (AGA) ₇ AG...T...(GAAA) ₅
EU350115	Spawner G3 (384)	T...T...T...T...(GATA) ₁₉ GAT(GGAT) ₉ AGAT(AGA) ₁₉ AG...C...(GAAA) ₄
EU350116	Progeny P311 (378)	T...T...T...T...(GATA) ₁₉ GAT(GGAT) ₉ AGAT(AGA) ₁₇ AG...C...(GAAA) ₄
Locus NsubC262		
EU350108	Spawner G1 (291)	G...T...AGA(TAGA) ₁₃ (CAGA) ₂ A...A...A
EU350109	Spawner G1 (227)	T...A...AGA(TAGA) ₇ (CAGA) ₁₀ A...A...A
EU350113	Progeny P51 (223)	G...T...AGA(TAGA) ₁₄ (CAGA) ₂ A...A...A
EU350110	Spawner G3 (214)	T...A...AGA(TAGA) ₈ (CAGA) ₆ C...G...—
EU350111	Spawner G3 (231)	G...A...AGA(TAGA) ₁₆ (CAGA) ₂ A...A...A
EU350112	Progeny P354 (223)	G...A...AGA(TAGA) ₁₄ (CAGA) ₂ A...A...A

^a Locus NsubC023: Bases given denote polymorphisms between sequences. Spaces in the sequence denote regions identical between individuals. The sequence length between polymorphisms in the sites of consensus are (from left to right) 16, 3, 15, 14, 2, and 9 bp.

^b Locus NsubC262: Polymorphic bases outside of the repeat region are shown. Spaces in the sequence denote regions identical between individuals. The sequence length between polymorphisms in the sites of consensus are (from left to right) 1, 18, 23, and 5 bp.

Table 3. Progeny produced for each reciprocal mating shown by origin of gamete and overall. Numbers in parentheses indicate progeny from that cross that were unassigned to maternal/paternal parents.

	Male						Total as female	Total progeny	% contribution
	G1	G2	G3	G4	G5	G6			
Female									
G1	3	12	115	2	43	11	186	272	36.9
G2	0	0	0	0	(1)	0	0	48	6.4
G3	76	35	0	6	28	26	171	291	39.0
G4	2	0	0	0	0	0	2	10	1.3
G5	4	(1)	1	0	0	(2)	5	81	10.9
G6	0	0	0	0	(2)	0	0	41	5.5
Total as male	85	47	116	8	71	37	364		

376 bp of the 16S rRNA subunit was unable to distinguish spawners G1, G2, and G6. However, combined with 306 bp of the 12S rRNA subunit, a total of 5 haplotypes allowed for the discrimination of the maternal parent in all families of interest (Table 4).

Of the 269 progenies sequenced from spawner G1, 186 (69.4%) were the result of that scallop contributing maternally. In the case of the 287 progenies that attributed to spawner G3, 241 (84%) were due to G3 acting maternally. With the exception of family G1♂ × G3♀, where G3 acted maternally in 39.8% of the offspring, spawner G3 contributed as the maternal parent to all but one of its 96 progenies in the remaining 4 families. Spawners G2 and G6 did not contribute maternally to any progeny genotyped (Table 3). However, all spawners did contribute male gametes to the progeny. Of 36 families possible when considering all combinations of maternal and paternal gametes to the progeny (including selfing), 15 were represented (Table 3).

Effective Population Size

Variance effective population size was 3.52 for these data. The inbreeding effective population size was 3.64, using

only microsatellite parentage assignment data without the consideration of gamete type. Employing mtDNA data to assign male and female gametes, the inbreeding effective population size increased slightly to 3.68.

Linkage

Family size allowed for linkage analysis of paternal families of spawners G1, G3, G5, and G6 as well as maternal families of spawners G1 and G3. LINKMFEX uncovered significant LOD scores (>3.0) in 10 of the 11 families evaluated for markers NsubA274 and NsubC262 (Table 5). Recombination rates between these loci ranged from 0.0 to 0.153.

Discussion

Genotypes from the parents and progeny at the 6 loci employed in this study successfully assigned the progeny to parent pairs. A high incidence of null alleles in this sample is not surprising given similar reports in *Crassostrea gigas* (McGoldrick et al. 2000; Hedgecock et al. 2004; Appleyard and Ward 2006; Li and Kijima 2006), *Crassostrea virginica* (Reece et al. 2004), and *Haliothis discus* (Li et al. 2003; Li and

Table 4. Variable sites within the sequenced 16S (top) and 12S (bottom) mtDNA haplotype fragments for each spawner relative to that spawner G1. A dot indicates no change from the uppermost sequence (G1), whereas “—” designates a single base pair deletion in the sequence.

GenBank accession	Spawner (haplotype)	16S rRNA—sequence position (bp)										
		32	55	74	106	122	125	141	143	156	158	
EU040036	G1 (A)	T	C	A	T	T	C	G	C	A	C	
EU040036	G2 (B)	
EU040037	G3 (C)	.	T	G	.	—	T	A	T	C	—	
EU040038	G4 (D)	C	
EU040039	G5 (E)	.	.	.	C	.	T	
EU040036	G6 (B)	

GenBank accession	Spawner (haplotype)	12s rRNA—sequence position (bp)									
		36	99	100	109	110	113	121	148	184	193
EU040033	G1 (A)	A	A	C	G	T	C	A	C	A	T
EU040034	G2 (B)	.	G
EU040035	G3 (C)	G	.	T	A	C	A	C	T	G	C
EU368733	G4 (D)	.	G	.	A
EU040034	G5 (E)	.	G
EU040034	G6 (B)	.	G

Table 5. Results of microsatellite linkage analysis performed in LINKMFx for loci NsubA274 and NsubC262. No other pair of loci showed evidence of linkage.

Loci	Family (female × male)	Parent evaluated	LOD score	Recombination rate ± SE	N
NsubA274–NsubC262	G1 × G3	Female	26.51	0.035 ± 0.017	115
	G1 × G5		12.94	0	43
	G3 × G1		18.57	0.026 ± 0.018	76
	G3 × G2		22.62	0.061 ± 0.022	115
	G3 × G5		12.73	0.092 ± 0.033	76
	G3 × G6		4.64	0.121 ± 0.056	35
	G3 × G1	Male	3.44	0.142 ± 0.066	28
	G1 × G3		2.98	0.153 ± 0.07	26
	G1 × G5		12.94	0	43
	G3 × G5		8.43	0	28
	G3 × G6		5.99	0.038 ± 0.037	26

SE, standard error.

Kijima 2005). This elevated occurrence of null alleles in marine mollusks is often attributed to their high fecundity and thus high mutational load (Williams 1975). Though some of the nonamplifying alleles may have been able to be resolved with further sequencing and primer redesign, this was not necessary as the null alleles did not affect the overall ability to assign parentage in this controlled cross.

Sequencing of the microsatellite alleles that were suspected to be mutational events confirmed 4 mutational events within the microsatellite regions of the loci and also revealed some interesting polymorphisms between alleles in the microsatellite flanking regions. All observed mutations took place in the paternal germ line. Of the 4 microsatellite mutations observed, 2 were losses of 2 repeat units, whereas 1 was a gain of 1 repetition. Two alleles at locus NsubC262 mutated to alleles identical in the number of repeats of each motif, demonstrating size homoplasy. However, polymorphisms in the flanking region distinguished these alleles from one another and allowed for identification of the parent of origin.

The microsatellite sequence of the presumed mutational allele at locus NsubB278 showed a distinct difference from the parent allele sequenced. Although this could be a complex mutational event, we cannot say for certain that the sequenced parent allele resulted in the observed progeny allele because the parent also had a null allele. There is a possibility that the null allele mutated to yield the scorable progeny allele or it is also possible that it resulted from recombination.

Finally, a fifth individual was originally scored as having a mutation at locus NsubC262 with a homozygous genotype that was inferred to constitute a mutation to a null allele. On amplification for sequencing, 2 alleles amplified in 2 of 3 replicate reactions. This allele was never present in any of the 3 genotyping reactions. The second allele found in the latter amplifications sequenced to be identical to that of the parent; therefore, it does not appear to be a microsatellite mutation. However, we suspect that this allele could have experienced a mutation in a priming region, which caused preferential amplification of the opposing allele, making it appear null in some reactions. Following with the high

incidence of null alleles, the observed mutation rate of 8.9×10^{-4} is slightly greater than that reported in various fish (3.9×10^{-4} [Castro et al. 2006], 6.5×10^{-4} [Banks et al. 1999], 5.6×10^{-4} [Yue et al. 2007]) but fits within the general range of 5×10^{-3} to 5×10^{-5} established for microsatellite loci (Estoup and Cornuet 1999).

Sequencing of mtDNA in *N. subnodosus* is thus far limited to few individuals in a handful of phylogenetic studies (Barucca et al. 2004; Saavedra and Peña 2006), and overall, the mitochondrial genome of *N. subnodosus* is largely unstudied. The mtDNA in *N. subnodosus* is assumed to be maternally inherited although the case of the mussel *Mytilus* is an interesting exception as it demonstrates doubly uniparental inheritance (Zouros et al. 1994). Additionally, another pectinid, *Placopecten magellanicus*, is itself unique as it has the largest mitochondrial genome of any metazoan studied to date (Snyder et al. 1987; Smith and Snyder 2007) and also has been shown to be heteroplasmic in some individuals (Snyder et al. 1987; Fuller and Zouros 1993). No evidence indicates that mtDNA of *N. subnodosus* is not inherited maternally and that is an assumption made in this study. Furthermore, mtDNA sequence data from individuals in this study showed no indication of heteroplasmy.

The mtDNA sequencing did reveal a unique haplotype (C) in spawner G3, that is 17 bp divergent from any other haplotype and includes 2 single base pair deletions. With the exception of haplotype C, no other haplotypes differed from one another by more than 4 bp. Due to the lack of mtDNA studies on this species, there is no information about possible relationships between the mitochondrial lineages. However, haplotype C as well as a similar haplotype differing by only 1 bp was found in 4 of 21 wild lion-paw scallops sequenced from Ojo de Liebre, Mexico (Petersen JL, unpublished data). Interesting variation is also seen in the microsatellite sequence data. At locus NsubC023, the 361-bp allele of spawner G3 differed in repeat motifs as well as flanking region polymorphisms compared with the other alleles sequenced. This spawner showed similar variation in the flanking region of 1 allele (214) at locus NsubC262. Due to overall sequence similarity, there is no question that these alleles are the same locus. That said, the amount of

microsatellite sequence data is limited in this study but the microsatellite and mitochondrial variation within spawner G3 in the few samples studied here provide a strong argument for the need for further phylogenetic and population genetic work within *N. subnodosus*.

Spawner G3 again is included in the discussion of the reciprocal crosses of family G1 × G3. Allele segregation of spawner G3 deviated significantly from the expected 1:1 ratio at NsubC262 and was near significance in NsubA274, which was found to be linked (Table 5). However, these deviations were observed only when that spawner acted as a male. Further deviation from the expected genotype ratios of the progeny in family G1♀ × G3♂ when not accounting for observed individual locus segregation ratios suggests that this significance is real. Other studies have shown segregation distortion in oysters (McGoldrick et al. 2000; Launey and Hedgecock 2001; Li and Guo 2004); however, the cause of deviation within this parent when acting paternally and not while acting maternally is unknown.

The worst-case scenario for a mass spawn is that in which each scallop contributes only as a male or a female. In such a situation with 6 spawners, the possible number of outcrossed families (including both reciprocal crosses) is reduced from 15 to a maximum of 9 (3♂ × 3♀). In this spawn, 11 outcrossed families were represented. Additionally, 3 progenies resulted from self-fertilization of spawner G1. The limited observation of selfing in these data may be attributed to a low incidence of occurrence or to negative fitness effects shown in self-fertilized progeny of other bivalve mollusks (Beaumont and Budd 1983; Ibarra et al. 1995; Narvarte and Pascual 2003; Evans et al. 2004) as well as the effects of genetic load.

The familial variance uncovered using microsatellite genotyping was not surprising given the studies of *C. gigas* (Launey et al. 2001; Boudry et al. 2002) and due to the high fecundity of the species (Hedgecock 1994; Hedgecock et al. 2007). However, mtDNA haplotypes parsing the microsatellite families into maternal and paternal components revealed an additional level of variance in the type of gamete contributed per spawner. Two spawners dominated the contribution of oocytes, whereas all contributed paternally to the progeny. However, there was significant discrepancy between individuals, and the overall variance in maternal contribution outweighed the paternal variance 5.74:1.

The reason for the variance in success of each spawner is not understood, but several factors in each stage of the spawn are known to influence the success of reproduction in mollusks. Both natural characteristics and artificial factors brought about by the spawning situation likely contribute to explain the results observed in these data by acting on the processes of induction, gamete release, fertilization, and development.

Once injected with serotonin, *N. subnodosus* release sperm, then eggs, and finish by releasing another small aliquot of sperm (Velasco et al. 2007; Ibarra AM, unpublished data). The timing of gamete release after induction is undocumented in this species but found to deviate greatly in 3 *Chlamys* males induced by intramuscular

serotonin (O'Connor and Heasman 1995). Not only will the first sperm released be without eggs to fertilize but also evidence in *Haliotis* (Babcock and Keesing 1999; Baker and Tyler 2001) and *Strongylocentrotus franciscanus* (Levitan et al. 1991) shows sperm age is negatively related to fertilization success beginning as early as 30 min after discharge. In a controlled environment of limited size, the timing of gamete release by one spawner relative to that of the others may create a logistic advantage or disadvantage for fertilization success. In this spawn, this could explain the contribution of G1 if that scallop had begun to spawn earlier than the others. Although early spawning could be detrimental to the contribution of its sperm, it may allow more success maternally as its eggs were released before those of the others. The fact that G1 still contributed paternally could be due to sperm from the initial spawn or serve as an example of the benefit of releasing an additional aliquot of sperm during the final act of spawning. The concluding release of sperm may also help to explain the lesser amount of variance observed in individual contribution of male gametes relative to female gametes.

There is no means in which we could quantify the gametes released by each scallop without interfering with the spawning process. That said, the maximum success of each spawner is determined by its baseline fecundity, which likely varies. For instance, Maldonado-Amparo et al. (2004) found the average spawn of an individual lion-paw scallop to be 20.6 million eggs, ranging from 17.2 to 25.6 million ($n = 3$), whereas Villavicencio (1997) describes a thermally induced "partial spawn" in which only 5.3 million eggs were released. Beyond conditioning for spawning, and visual inspection of gonad maturity as was performed in this study, the productiveness of an individual in terms of quantity and quality of gametes is thus far not a characteristic that aquaculturists can control or even predict (example in *Chlamys*; Styan and Butler 2003). Some scallops may also suffer a loss of fitness from the means of induction itself as Velasco et al. (2007) have suggested that induced spawning may prompt the release of immature gametes.

Once gametes begin to be released, the concentration of sperm in a mass spawn is controlled to a limited extent by the initial disposal of excess sperm. However, no control exists as to the timing and quantity of eggs expelled and subsequent gamete interaction time or age of gametes at encounter. This is of note as the egg to sperm ratio is important in the success of fertilization in mollusks (Clotteau and Dube 1993; O'Connor and Heasman 1995; Desrosiers et al. 1996; Narvarte and Pascual 2003; Hodgson et al. 2007) and in minimizing problems associated with polyspermy (Desrosiers et al. 1996; Hodgson et al. 2007). In addition to the relative proportion of gametes, gamete interaction effects impact the number of abnormal larvae and early survivorship in other bivalve mollusks (Beaumont and Budd 1983; Gaffney et al. 1993; Boudry et al. 2002; Degremont et al. 2005) although this remains unstudied in *N. subnodosus*.

Fertilization may have been more random than our sample suggests, but individuals of some families may have

been eliminated early in development due to high genetic load, which is largely documented in oysters (Launey and Hedgecock 2001; Li and Guo 2004). After fertilization, maternal effects could also have given a fitness advantage to some families as shown by Cruz and Ibarra (1997) and Deng et al. (2005) in *Argopecten circularis* and *H. discus*.

Finally, the physical proximity between individuals influences success in some spawns (Levitan 2005). In this particular study, the position of the individuals, their movements, or the timing of gamete release were documented. The importance of these variables is clear and will be considered in future mass spawn experiments.

In this spawn, the inbreeding effective population size was 3.64 when calculated from the overall microsatellite data and 3.68 when parsing out male and female contributions of each spawner. This decrease in the effective population size relative to the actual number of scallops spawned is not surprising considering the sweepstakes hypothesis of reproduction as well as previous studies that show an effective population size significantly less than the census size in wild and hatchery populations (Hedgecock and Sly 1990; Gaffney et al. 1992; Hedgecock 1994; Boudry et al. 2002; Appleyard and Ward 2006; Hedgecock et al. 2007). Variance population size was similar to the inbreeding effective size at 3.52 and may be a better indicator of how well genetic diversity is maintained in a population as it measures the effect of genetic drift. Though Gaffney (2006) discusses that the introduction of hatchery stock with a reduced effective population size may have a negligible impact on relatively large wild populations, this statement is made in the context of reseeded or enhancing degraded wild populations. Furthermore, some data suggest that small effective breeding population may be a natural phenomenon in mollusks as a result of the sweepstakes reproduction hypothesis (Li and Hedgecock 1998; Moberg and Burton 2000; Hedgecock et al. 2007; but also see Flowers et al. 2002). However, scallops spawned in aquaculture may not be representative of the genetic diversity in the wild, are likely selected based on economically desirable characteristics such as size or maturation condition, and lack the potential for migration to supplement the gene pool.

Both aquaculture and natural populations of *N. subnodosus* could be negatively affected through a loss of genetic diversity. Selective breeding and unintentional inbreeding alter the composition of aquaculture stock relative to that of the wild populations from which they were derived (Hedgecock and Sly 1990; Smith and Conroy 1992; Li et al. 2004; Zhang et al. 2005; Hara and Sekino 2007). Not only is aquaculture of the lion-paw scallop taking place in waters contiguous with those in which natural populations occur but scallops grown in aquaculture undergo a sexual maturation and spawn before they are harvested. The mixing of hatchery produced spat or gametes with those produced naturally could alter the genetic makeup of the native populations (Allendorf and Ryman 1987; Bekkevold et al. 2006). Additionally, in some instances, aquaculturists may obtain their broodstock or spat from populations other than those in the area of

industry. Though the lion-paw scallop is distributed in aggregations on both coasts of the Baja California Peninsula, it has been shown that similarly distributed *Argopecten* populations are characterized by adaptations specific to their local habitat (Cruz and Ibarra 1997; Cruz et al. 1998). Any similar adaptations in *N. subnodosus*, which possibly took thousands of years to evolve, could be sacrificed if the native gene pools are altered by introgression.

Furthermore, the relatedness observed in this sample warns that the use of hatchery progeny as seed stock for wild populations should be taken with caution, especially when considering those areas destined for resource conservation. It is of note that the individuals spawned in this study were unrelated to one another. However, if progenies randomly selected from this spawn were subsequently spawned to perpetuate a captive population, it is likely that inbreeding would be significant and contributes to a decrease in effective population size.

Until more is known about the natural population structure and biology of this species, multiple, pairwise matings would ensure that each outcrossed event is represented and eliminate the possibility of self-fertilization. If it were shown that mtDNA haplotype is significant to fitness or that recombination events are unequal between genders, then it would also be wise to perform both reciprocal crosses of each spawner to maintain maximum genetic diversity. Conversely, the culture of sterile, triploid scallops would also provide a means to avoid unwanted introgression of hatchery stock into the wild (Beaumont 2000; Maldonado-Amparo et al. 2004).

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