SPERM, SPINES, SECONDARY CONTACT AND CYTOPLASMIC INTROGRESSION BETWEEN SIBLING SPECIES OF SEA STARS.

by

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ABSTRACT

Extensive studies of hybrid zone origin and formation have been conducted in the terrestrial environment, yet relatively few marine invertebrate hybrid zones have been well described. In the northwest Atlantic, a secondary contact zone has recently formed between two sibling species of sea stars, *Asterias rubens* and *A. forbesi*. I conducted a series of studies to determine the outcome of this contact.

Gametes of *A. rubens* and *A. forbesi* were reciprocally compatible in cross-fertilization studies, however the compatibility of heterospecific crosses was highly variable. Differential compatibility of heterospecific gametes was demonstrated in sperm competition studies in which I used a nuclear DNA marker to assign paternity to larval offspring. There was evidence of conspecific fertilization preference in *A. forbesi* and some *A. forbesi* sperm were competitively superior to *A. rubens* in fertilizing *A. rubens* eggs. A morphological survey of sympatric and allopatric *Asterias* populations did not quantitatively support the existence of a distinct group of intermediate phenotypes that might have been hybrids. However, evidence of hybridization and introgression in the contact zone was detected in a phylogenetic survey of mtDNA variation. *Rubens*-like haplotypes were found in three individuals with *forbesi*-like phenotypes. Asymmetric introgression may reflect differential compatibility of heterospecific gametes.

Asterias forbesi and *A. rubens* are not completely reproductively isolated in secondary contact. Asymmetric gamete compatibility appears to have led to asymmetric introgression of mtDNA from *A. rubens* into *A. forbesi*. This asymmetry suggests a prezygotic reproductive barrier not previously considered in these taxa, such as a gamete recognition protein system analogous to bindin in sea urchins. Further description of the *Asterias* secondary contact zone may enable future studies to test theories of speciation and hybrid zone dynamics using these closely-related species.

ABBREVIATIONS AND SYMBOLS USED

χ^2	Chi-square distribution: a probability density function whose values range from zero to positive infinity
°C	degrees Celsius
5'	the fifth carbon in the pentose group deoxyribose
3'	the third carbon in the pentose group deoxyribose
α	alpha, criterion for statistical significance
ANCOVA	analysis of covariance
ANOVA	analysis of variance
bp	base pair; 1 bp = 1 pair of nucleotides
B.P.	Before Present
cm	centimeter; 1 cm = 10^{-2} meters
COI	cytochrome *c* oxidase subunit I; a gene in the mtDNA genome
CTAB	cetyltrimethylammonium bromide
ddH_2O	distilled de-ionized water
df	degrees of freedom in a statistical analysis
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
F	test statistic in an analysis of variance or covariance
F_1	first generation of offspring resulting from an arranged cross
F_{ST}	fixation index; reduction in heterozygosity within subpopulations relative to the total population.
Glu	glutamic acid, an amino acid
h	hour
HKY + G	DNA substitution model of M. Hasegawa, H. Kishino, and T. Yano
IRD	infrared dye
IRD700	infrared dye that fluoresces at a wavelength of 700 nm
ITS	internal transcribed spacer, a sequence found in the nuclear genome
k	number of predefined clusters in a cluster analysis
KCl	potassium chloride

km	kilometer; 1 km = 10^3 meters
m	meter; 1 m = 10^3 millimeters
M	molar; unit of concentration; moles of solute per liter of solution
M13	filamentous bacteriophage of the bacterium *E. coli* used for cloning
min.	minute
$MgCl_2$	magnesium chloride
µg	microgram; 1 µg = 10^{-6} grams
µL	microliter; 1 µL = 10^{-6} liters
µm	micrometer; 1 µm = 10^{-6} meters
mL	milliliter; 1 mL = 10^{-3} liters
mm	millimeter; 1 mm = 10^{-3} meters
µM	micromolar; unit of concentration; 1µM = 10^{-6} M
mM	millimolar; unit of concentration; 1mM = 10^{-3} M
mol	mole; the amount of substance that contains the same number of formula units as there are ^{12}C atoms in 12 grams of ^{12}C (6.0225×10^{23}, or Avogadro's number)
m s^{-1}	meters per second
mtDNA	mitochondrial DNA
Mya	million years ago
N	effective population size
NE	northeast
ng	nanogram; 1 ng = 10^{-9} grams
NLIN	command in a statistical program to perform non-linear regression
NW	northwest
pmol	picomole; 1 pmol = 10^{-12} moles
^{32}P	the radionuclide phosphorous-32
PCR	polymerase chain reaction
p	probability of significant departure from the null hypothesis
PAUP*	Phylogenetic Analysis Using Parsimony
pUC19	engineered plasmid DNA used for cloning
r	correlation coefficient

R^2	square of the multiple correlation coefficient
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
SAS	a statistics computer program
SCUBA	Self Contained Underwater Breathing Apparatus
s.d.	standard deviation, equal to the positive square root of the variance
sec.	second
t	test statistic in a Student's *t* test
T_A	primer annealing temperature
TBE	Tris Boric acid Ethylenediamine tetra-acetic acid
TCS	a computer program that estimates gene genealogies
Thr	threonine, an amino acid
TMAP	the major acrosomal protein, unknown function found in *Tegula* snails
tRNA	transfer RNA
Tr:Tv	ratio of transitions (when a purine nucleotide is substituted for another purine or a pyrimidine is replaced by another pyrimidine) to transversions (when a pyrimidine is substituted for a purine or vice versa).

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CHAPTER ONE:

OUTCOMES OF SECONDARY CONTACT IN MARINE INVERTEBRATES: A COMPARISON WITH TERRESTRIAL SPECIES

Introduction

The repeated glaciations of the Pleistocene epoch had a significant impact on the phylogeography and genetic differentiation of species. At the peak of the last glacial maximum 20 000 B.P., the Northern Hemisphere ice complex covered most of North America, northern Eurasia and the polar seas (CLIMAP 1976). As the ice advanced, terrestrial species retreated into refuges or became extinct. Sea levels dropped, affecting near-shore marine species (Valentine and Jablonski 1986, Palumbi 1994). Populations differentiated in allopatry as the result of vicariance, the extent of divergence depending upon the duration of isolation (Endler 1977). When the glaciers receded, species dispersed out of isolation, coming back into contact with other refugial populations. Secondary contact occurs between two or more sympatric species or populations that were formerly geographically separate. Depending on the evolution of reproductive isolating barriers in allopatry, the outcomes of secondary contact range from complete reproductive isolation to the formation of stable hybrid zones and introgressive hybridization. Considerable effort has been expended studying the origin and formation of hybrids zones, particularly in the terrestrial environment, as they represent a series of stages in the process of speciation (e.g., Harrison 1993, Avise 1994, Arnold 1997). In a review of hybrid fitness, Burke and Arnold (2001) observed hybridization may contribute to adaptation and/or speciation by leading to the founding of new lineages (Arnold 1997,

Rieseberg 1997), or may result in the transfer of adaptations from one taxon to another by introgression, perhaps allowing for range expansion of the introgressed form (Lewontin and Birch 1966).

The literature is rich with studies of secondary contact and hybridization in the terrestrial environment (see Harrison 1993, Avise 1994, Arnold 1997, Howard and Berlocher 1998). Glacial refugia, patterns of range expansion and regions of secondary contact have been identified in Europe (Hewitt 1996, 1999, 2000, Taberlet *et al.* 1998) and North America (Pielou 1991). In contrast, secondary contact and hybridization in marine species have received relatively less attention. Only recently has there been an appreciation of the extent and importance of hybridization in the sea (review in Gardner 1997).

As an introduction to my studies of the outcome of secondary contact in a pair of sibling sea stars, I first describe the approaches used to identify secondary contact and summarize reproductive isolation barriers which directly affect the outcome. I illustrate the patterns of reproductive isolation and hybridization arising from secondary contact in terrestrial species using case histories. I then examine secondary contact in marine invertebrates, using selected examples. Finally, I outline the objectives and studies I conducted to examine the outcome of secondary contact in the sea stars *Asterias rubens* and *A. forbesi.*

Identification of secondary contact zones

Hewitt (1996) described two approaches to studying the consequences of Pleistocene glaciations on species. The first was to examine the historical geographic distribution of species in relation to the paleoclimate and topography to form hypotheses about the past spatial structure and dispersal of species. The second was to examine the contemporary population genetic structure and construct phylogenies to infer patterns and processes of divergence and speciation. Studies of hybrid zones, formed as the result of secondary contact, provide insight into the evolution of barriers to reproduction and the possible outcomes of secondary contact.

The historical distribution of species can be estimated from pollen records and fossil histories. The expansion and contraction of plant species in Europe and North America can be tracked using extensive pollen histories, suggesting possible refugia in Europe for species such as the common beech, *Fagus sylvatica* (Huntley and Birks 1983, Huntley 1990). These refugia have been confirmed by phylogenetic analysis of vertebrate species such as the flycatchers, *Ficedula* spp. (Saetre *et al.* 2001), and from examination of extant distributions of species such as the shrew, *Sorex araneus* (Brünner *et al.* 2002). Fossil remains of animals such as beetles (Coope 1977, 1990) and the house mouse, *Mus musculus* (Boursot *et al.* 1993) have also been used to track changes in geographical distribution in response to climatic events such as the Pleistocene glacial/interglacial cycles. Fossil records provide objective evidence of species geographical distributions over geological time (Pielou 1991).

Where fossil records are not abundant, or are of insufficient detail, genetic analyses of population structure and phylogenies can be used to infer biogeography. Studies of population genetic structure provide estimates of gene flow between populations. Genetic divergence among populations is estimated from allele frequency differences as F_{ST}, and is inversely proportional to gene flow, Nm (where N = effective population size and m = migration rate; Wright 1951, Hartl and Clark 1997). High F_{ST} values indicate subdivision of taxa and reduced gene flow. Secondary contact can be inferred from high F_{ST} values across multiple loci that indicate a concordant genetic break between two groups of populations (Hare and Avise 1996). High among-loci variance in F_{ST} values has been predicted (Robertson 1975) and observed (Latta and Mitton 1999) to result from historical separation of taxa. This variance could also reflect among-loci differences in allele frequencies caused by selection (Lewontin and Krakauer 1973, Avise 1994). However, concordance of geographical divisions among loci is indicative of secondary contact, rather than natural selection (Latta and Mitton 1999).

Phylogeographic analyses are used to construct phylogenetic trees (most often from mtDNA haplotype sequences in animals) to examine differentiation among geographic populations or taxa (Avise 1994). Historical differentiation between geographically distinct populations suggests hybrid zones are the result of secondary contact (Avise *et al.* 1984, Taberlet *et al.* 1998). If there is a deep phylogenetic break in the tree (often > 2% sequence divergence, Avise 1989), that separates haplotypes occurring at high frequency on either side of the cline (a gradient in a measurable character, Huxley 1938), then secondary contact is a favored hypothesis. Secondary contact has been inferred from phylogenetic trees most often demonstrating a deep genetic break and reciprocal monophyly between geographically distinct populations or

taxa (review in Avise 1992). Reciprocal monophyly is not a requirement for differentiation in allopatry; monophyly is expected in approximately $4N$ generations of isolation, and prior to that populations are paraphyletic or polyphyletic for some time (Avise *et al.* 1984, Neigel and Avise 1986).

Where hybrid zones are formed by secondary contact, they are characterized by concordant and coincident clines in morphological and genetic traits (Barton and Hewitt 1981). One of the difficulties in interpreting clines, in the absence of fossil or phylogenetic evidence for historical separation and differentiation of taxa in allopatry, is that both primary divergence (clines formed in a more or less continuous population due to natural selection) and secondary contact may yield similar character distributions (Endler 1977, Barton 1983, Barton and Hewitt 1985, Hewitt 1988). As well, some clines may be the result of selection in response to an environmental gradient. For example, allelic variation at the *Lap* locus in mussels (in particular Lap^{94}) has been interpreted as evidence of direct selection in response to salinity differences (Koehn *et al.* 1976, 1980, Hilbish *et al.* 1982, Hilbish 1985). It therefore might be preferable that non-coding markers, such as microsatellites, be used for analysis of contact zones, rather than loci that are more likely to be under selection. The hypothesis of secondary contact following differentiation of populations or taxa in allopatry is best supported by concordance among independent lines of evidence from studies of population structure, phylogeography and geologic records (Hare and Avise 1996).

Reproductive isolation barriers

The outcomes of secondary contact depend upon the degree of differentiation in isolation (Endler 1977). In allopatry, taxa may evolve reproductive isolating barriers (heritable traits that prevent gene flow, Avise 1994) independently through random genetic drift and/or natural selection (Rice and Hostert 1993, Schluter 2001, Tregenza *et al.* 2002), and may continue in sympatry under the influence of selection. Dobzhansky (1937) classified prezygotic isolation barriers into four categories as ecological isolation, seasonal or temporal isolation, ethological isolation and mechanical isolation. Postzygotic barriers were classified into three categories as hybrid inviability, hybrid sterility and hybrid breakdown. The strength of these barriers influences the outcome of secondary contact. For example, closely-related species which have only postzygotic barriers to reproduction can form a hybrid zone that consists of a hybrid swarm (Barton and Hewitt 1989, Jiggins and Mallet 2000).

Prezygotic barriers to reproduction can evolve in response to changes in habitat. In studies of *Drosophila*, lineages raised in different environments evolved some prezygotic reproductive isolation, whereas lineages raised in the same environment did not (Dodd 1989, review in Schluter 2001). If prezygotic barriers successfully prevent cross-fertilization among taxa, then speciation is complete and the taxa are separate.

Postmating, prezygotic reproductive isolation can take the form of female choice or sperm competition within the female reproductive tract in terrestrial animals (Wade *et al.* 1994). Evidence of conspecific sperm outperforming heterospecific sperm has been documented in interspecific studies of the flour beetle *Tribolium* (Robinson *et al.* 1994,

Wade *et al.* 1994), the ground cricket *Allonemobius* (Gregory and Howard 1994), the grasshoppers *Podisma* and *Chorthippus* (Hewitt *et al.* 1989, Bella *et al.* 1992), and *Drosophila* (Harshman and Prout 1994, Price 1997). Conspecific sperm predominance may act as a mechanism of species recognition and contribute to the divergence of the species (Robinson *et al.* 1994). In a review of postcopulatory, prezygotic reproductive barriers, Eady (2001) argues that gametic incompatibility may arise through sexual selection and reduce gene flow among populations.

In the absence of prezygotic barriers, hybrid offspring can be produced. While some hybrids of species such as the sunflowers *Helianthus anuus* and *H. petiolaris* (Rieseberg *et al.* 1996, Gardner *et al.* 2000) may have increased fitness relative to their parents, hybridization often produces offspring with decreased levels of fertility and/or viability (Dobzhansky 1940, Mayr 1963, Barton and Hewitt 1985). This decrease in hybrid fertility and/or viability may also be manifest in F_2 offspring and subsequent generations. Two chromosomal races of the grasshopper *Caledia* produce viable, fertile F_1 hybrids, however the F_2 generation is inviable and only one half of the backcross offspring are viable (Shaw and Wilkinson 1980, review in Shaw *et al.* 1990).

If mechanisms of reproductive isolation are weak, or do not evolve in allopatry, then secondary contact of taxa can result in a hybrid zone. The formation of a unimodal (high frequency of intermediate genotypes or phenotypes) or bimodal (low frequency of intermediate forms, high frequencies of parental forms) hybrid zone depends upon the evolution of reproductive isolation barriers (Jiggins and Mallet 2000). A unimodal hybrid zone forms if there is hybridization and only postzygotic barriers have evolved, or if assortative mating (choice of mates based on phenotypes, Hartl and Clark 1997) is weak and not combined with some selection against hybrid offspring. If there is strong

assortative mating, or prezygotic barriers can maintain isolation between the taxa, a bimodal hybrid zone forms (Jiggins and Mallet 2000). Bimodal hybrid zones in which strong heterozygote deficits and linkage disequilibria are observed suggest taxa are close to completion of speciation (Jiggins and Mallet 2000).

Linkage disequilibrium is the observation of nonrandom associations between alleles at pairs of loci, which are initially strong in secondary contact (Hartl and Clark 1997). These associations can form in allopatry due to drift or can be formed by physical proximity of loci, pleiotropy (allele has more than one function), or linkage due to chromosomal rearrangements (Ortíz-Barrientos *et al.* 2002). In a model comparing primary divergence with secondary contact, Durrett *et al.* (2000) observed hybrid zones with significant linkage disequilibria and clines in neutral loci were often evidence of secondary contact.

Terrestrial studies of secondary contact and its consequences

Studies of secondary contact in the terrestrial environment have focused on species in North America and Europe. These two continents experienced extensive Pleistocene glaciation. The Laurentide ice sheet covered North America south to about 40°N and the Scandinavian ice sheet covered Britain and northwestern Europe to about 52°N (Hewitt 1996). European refugial populations survived the glacial period in southern Iberia, Italy and the Balkans (Hewitt 1996, 1999), while North American populations persisted in Florida and Mexico (Pielou 1991, Hewitt 2000). Postglacial population expansion across the Alps and Pyrenees was slow to impossible and northern

Europe was largely repopulated from Balkan refugia (Hewitt 1996, 1999). Contact zones in the Alps and Pyrenees likely originated from secondary contact between two refugial populations. In North America, Remington (1968) recognized seven major "suture zones", bands of geographic overlap between major biota, including sibling species that hybridize in the zone. Suture-zone hybrids result from secondary contact between two subpopulations, separated by barriers to breeding contact (Remington 1968). The following case histories illustrate the range of outcomes of secondary contact between previously allopatric taxa in the terrestrial environment in which reproductive isolation is incomplete.

Hybridization without introgression, unimodal hybrid zone

The meadow grasshoppers, *Chorthippus parallelus parallelus* and *C. p. erythropus*, form narrow hybrid zones in both the Pyrenees (Butlin and Hewitt 1985) and the Alps (Flanagan *et al.* 1999). These hybrid zones are the result of secondary contact following range expansion from allopatric glacial refugia in southern Iberia, the southern Balkan region, and Italy, as demonstrated by a marked phylogenetic divergence in an anonymous nuclear marker across the Pyrenees (Cooper *et al.* 1995). The two subspecies are differentiated based on morphology, courtship song and mating preference (Butlin and Hewitt 1985, Butlin and Ritchie 1991). Based on a 1% mtDNA sequence divergence, they diverged approximately 0.5 Mya (Lunt *et al.* 1998). Narrow clines (width less than 50 km) in morphology, behaviour, karyotype and molecular markers are broadly coincident in the contact zone (Butlin and Hewitt 1985, Butlin and Ritchie 1991, Hewitt 1993). Both pre- and postzygotic reproductive isolation barriers have been identified,

including isolation by mate choice (Ritchie *et al.* 1989) and postcopulatory, prezygotic sperm competition favoring conspecific fertilization (Bella *et al.* 1992). The two subspecies hybridize in the lab, producing fertile F_1 females, but sterile F_1 males from both reciprocal crosses (Hewitt and Butlin 1997). Backcross males show intermediate sterility, but can produce sperm and viable offspring. No evidence for F_1 male sterility has been found in the field (Ritchie and Hewitt 1995). Despite barriers to reproduction and gene exchange, the subspecies form a unimodal hybrid zone in the areas of secondary contact, with intermediate genotypes and phenotypes predominant in the center of the contact zone (Hewitt 1993). There is, however, no introgression of alleles outside the hybrid zone (Lunt *et al.* 1998).

Hybridization and asymmetric introgression, bimodal hybrid zone

The field crickets, *Gryllus pennsylvania* and *G. firmus* form a narrow hybrid zone, which runs from Connecticut to Virginia (Harrison and Arnold 1982). The hybrid zone in Connecticut is suggested to be the result of secondary contact from post-glacial colonization based on mtDNA and reproductive isolation data (Rand and Harrison 1989). The species have less than 1% mtDNA sequence divergence, suggesting a recent common ancestor (Harrison and Bogdanowicz 1997). Barriers to reproductive isolation include temporal isolation in the southern end of the hybrid zone (Harrison 1985), preferential mating by females of *G. pennsylvania* with conspecific males in the laboratory (Harrison and Rand 1989), and asymmetric production of hybrid offspring: fertile hybrids are formed from crosses between *G. pennsylvania* females and *G. firmus* males, but the reciprocal cross fails to produce offspring (Harrison 1983).

Genotype scores from the secondary contact zone in Connecticut form a bimodal distribution with a high frequency of parental types and few F_1 hybrids (Harrison and Bogdanowicz 1997). An overall deficit of heterozygotes in the contact zone, the bimodal distribution of genotypes and high linkage disequilibria (consistently higher in the center of the hybrid zone) suggest minimal intermixing of the two species in secondary contact (Harrison 1986; Harrison and Rand 1989, Harrison and Bogdanowicz 1997).

There is some introgression of *G. pennsylvania* mtDNA into *G. firmus* populations, but not in the reciprocal direction (Harrison 1983, Harrison *et al.* 1987). There is also some sharing of nuclear alleles, which Harrison and Bogdanowicz (1997) attribute to introgression rather than ancestral polymorphism since allopatric populations are fixed for alternate alleles at a locus. Despite this introgression, the two species remain distinct in sympatry.

Hybridization and symmetric introgression, unimodal hybrid zone

The fire-bellied toads, *Bombina bombina* and *B. variegata*, meet in a long, narrow hybrid zone extending along the mountain ridges from Austria to the Carpathian Mountains (Szymura 1993). The hybrid zone is the result of secondary contact following postglacial range expansion (Arntzen 1978). This interpretation is supported by the glacial history of the area and the presence of a hybrid zone with individuals that are the product of many generations of hybridization (MacCallum *et al.* 1998, Szymura 1993). Fossil evidence and molecular analyses suggest these species diverged in the late Pliocene, approximately 2 – 7 Mya (Szymura 1993); mtDNA sequence divergence between the species is 5.6 – 7 % (Szymura *et al.* 1985).

The two species differ in mating call, warning coloration, life history, habitat, and molecular markers (Szymura *et al.* 1985, Szymura and Barton 1986, Szymura 1993, Nürnberger *et al.* 1995). Hybrid zones in Poland and Croatia are clearly defined by concordant and coincident clines in allozyme loci (Szymura 1993) and quantitative morphological traits (Nürnberger *et al.* 1995). Most *Bombina* spp. hybrid zones have unimodal genotype distributions (Szymura and Barton 1991, MacCallum *et al.* 1998) and exhibit extensive hybridization and some introgression evidenced by introgressed alleles into both populations (Szymura and Barton 1991, Szymura 1993). The two species are not reproductively isolated from one another in sympatry (Szymura 1993).

Studies of secondary contact in marine invertebrates

During the Pleistocene, the cyclical waxing and waning of the ice sheets over North America and Europe caused concurrent oscillations in sea level. At the time of the last glacial maximum, an estimated 50 million km^3 of what is now seawater was bound up in the glaciers, causing the sea level to be 130 m below its current position (Pielou 1979).

The repeated changes in sea level must have affected near-shore marine communities (Palumbi 1994), but the process of allopatric isolation and speciation in these communities is less obvious in the geological and palaeontological record than the effects of glaciers advancing across a continent. Few studies of secondary contact in the marine environment have been as thoroughly conducted as the terrestrial examples described above. While examples of secondary contact have been well studied in North America and in Europe, the oceans have not received the same attention.

There are several potential explanations for this gap in the study of speciation in the ocean. Geographic barriers to gene exchange are less conspicuous in the sea (Lessios *et al.* 2001). Misconceptions about population structure (Palumbi 1994) and hybridization (Gardner 1997) in marine organisms have contributed to the lack of identification of marine hybrid zones and secondary contact. Many marine invertebrates are continuously distributed along coastlines and have widely dispersing larvae with the potential for high gene flow among populations (Palumbi 1992). While many species display little population structure, others have demonstrated surprising amounts of subdivision due to isolation by distance, selection or recent history (Palumbi 1994). As well, taxa that become reproductively isolated in allopatry can go unrecognized, as morphological similarities among sibling species make it difficult to detect cryptic species (Knowlton 1993). Molecular genetic analysis has improved detection of genetic divergence between taxa (Knowlton 2000) and has been a focal technique in studying secondary contact in the sea.

Studies of secondary contact in terrestrial communities combine extensive pollen and fossil records with glacial history in order to set a biogeographic context. In contrast, many marine studies are unable to directly determine the historic distributions of organisms. The marine fossil record generally lacks the resolution to track expansion and contraction of species ranges on the scale of Pleistocene sea level changes (Geary 1992) and is an incomplete representation of the history of the species (Gardner 1997). In his review of the evolutionary history of marine and freshwater species from the southeastern United States, Avise (1992, p. 65) acknowledges that "most historic geologic scenarios are highly speculative" and based mostly upon genetic data rather than historical geography.

Secondary contact in the sea has been indirectly identified by three approaches using morphological and molecular analyses. In the first, similar to terrestrial studies, research is focused on a hybrid zone identified by geographical overlap in the ranges of two morphologically distinct, but closely-related taxa. In the second, phylogenetic analyses of species relationships can reveal historical differentiation between geographically distinct populations that overlap in distribution. In the third, population subdivision is found, often coincidentally, as the result of a population genetic study of a continuously distributed species. In some cases, these genetically differentiated populations are reproductively isolated and exhibit considerable genetic divergence, which can lead to the description of new species (e.g. *Nucella ostrina*, Marko 1998, Marko *et al.* 2003).

The reproductive biology of marine invertebrates has made it difficult to recognize and study secondary contact in the sea. Many marine invertebrates are broadcast spawners with highly dispersive larvae. Often this potential for high dispersal is associated with a lack of genetic differentiation over large spatial scales (Palumbi 1994). Gene flow can be limited, however, by mechanisms such as selection or historical separation. Hybrid zones are also difficult to spatially define in the marine environment, in part due to problems estimating dispersal and the physical scale (Gardner 1997).

As in terrestrial species, mechanisms of prezygotic reproductive isolation in marine species include differences in mate preference, habitat and reproductive periods. In general, broadcast-spawning marine invertebrates lack complex mating behaviour. However, the interactions of the gametes may determine the success of interspecific fertilization in species which are not spatially or temporally isolated (review in Palumbi 1994). Complete fertilization in hybrid crosses is not a common result; more often

hybridization is asymmetrical or completely unsuccessful. Postzygotic reproductive barriers are seldom studied because of the difficulties associated with raising offspring with long generation times through complex life cycles.

There are numerous studies of reproductive isolation and hybridization in marine invertebrates (review in Gardner 1997), however the contact zones formed in many of these examples may be the result of differentiation of populations *in situ* (primary divergence), rather than secondary contact (reviews in Harrison 1993, Avise 1994, Howard and Berlocher 1998). It is important to distinguish between primary divergence and secondary contact because several hypotheses about the concept of species and the process of speciation depend upon the interpretation of hybrid zone origin and formation (Thorpe 1984). To further complicate the situation, as stated by Hare and Avise (1996), the two scenarios may not be mutually exclusive as environmental selection along a gradient can reinforce clinal patterns formed through secondary contact of differentiated populations (Bert and Arnold 1995, Arnold 1997). To illustrate the outcome of secondary contact in marine invertebrates, the examples described below were selected based on a hypothesis of secondary contact by the authors as a possible explanation for observed patterns in molecular analyses (although in most cases, it was not possible to definitively distinguish secondary contact from primary divergence).

Reproductive isolation in secondary contact

Along the coastline of the northeastern Pacific, populations that had been considered one species of prosobranch snail, *Nucella emarginata*, were discovered to be two species whose distributions overlapped just north of Point Conception (Palmer *et al.*

1990); the northern form was subsequently renamed *N. ostrina* (Marko 1998, Marko *et al.* 2003). In an examination of allozyme allele frequencies and morphological traits, Palmer and coworkers (1990) found fixed differences in an allozyme locus concordant with differences in morphological traits between the species. As well, cross-fertilization studies yielded only capsules whose eggs did not develop, indicating reproductive isolation (Palmer *et al.* 1990). In a broader survey across the zone of sympatry, both allozyme and mtDNA trait differences were geographically concordant with the pattern of reproductive isolation. Taken together, these data were interpreted as a range expansion from transient allopatric isolation of *N. emarginata* during the Pleistocene, followed by a northern migration into sympatry with *N. ostrina* in California (Marko 1998).

Reproductive isolation, possible postzygotic barrier

Two species of the sea urchin *Echinometra* are sympatric in the Caribbean and are suggested to be in secondary contact (McCartney *et al.* 2000). Based on mtDNA sequence analyses, *E. lucunter* and *E. viridis* diverged approximately 1.27 – 1.62 Mya, during the first Pleistocene sea level decrease (McCartney *et al.* 2000). McCartney and coworkers (2000) suggested some populations may have become physically isolated during the Pleistocene and agreed with Mayr's (1954) conclusion that the contemporary distribution of the species is best explained by allopatric speciation and subsequent secondary contact.

Although the eggs of *E. viridis* can be fertilized by *E. lucunter* sperm (Lessios and Cunningham 1990, McCartney and Lessios 2002), the annual reproductive cycles overlap (Lessios 1981, 1985), and neither species shows a lunar cycle (Lessios 1991), the species

do not hybridize. Asymmetric gamete incompatibility can partially account for the lack of hybridization. Complete reproductive isolation may be maintained by a postzygotic mechanism not yet discovered (McCartney *et al.* 2000, McCartney and Lessios 2002, but see Rahman *et al.* 2001).

Reproductive isolation, or possible introgression?

Sister species of snapping shrimp, *Alpheus armillatus* and *A. angulatus*, have overlapping ranges on the Atlantic coast of Florida. From phylogenetic analysis of mtDNA sequences, these species likely diverged subsequent to the closure of the Isthmus of Panama (Mathews *et al.* 2002). Lab studies of mating behaviour showed the species were reproductively incompatible, possibly as "the result of strong selection for reproductive isolation resulting from transient allopatry followed by secondary contact" (Mathews *et al.* 2002, p. 1435). This hypothesis was supported by deep genetic divergence between the two species, ranging from 1 – 3% for 16S rRNA and 2 – 5% for COI sequences which corresponded with divergence times of 1 – 3.5 Mya and 1 – 2.5 Mya, respectively. The phylogenetic trees were not, however, reciprocally monophyletic; sequences of one individual of *A. angulatus* placed it in a sister relationship to all of the sequences of *A. armillatus*. Four possible explanations for the paraphyly of the *A. angulatus* sequences were presented: (1) introgression between the two species following secondary contact; (2) sequencing of a pseudogene; (3) persistence of mtDNA lineages that predate the divergence between the two species; and (4) identification of a previously unrecognized taxon. Further investigation is required to determine which of these hypotheses is correct.

Occasional hybridization

In constructing a phylogeny for the sea urchin *Diadema,* Lessios *et al.* (2001) suggested that zones of sympatry in the Indo-Pacific (with specific reference to Okinawa) for *D. savigni* and *D. paucispinum* were more likely to be the result of secondary contact rather than sympatric speciation. Two clades of *D. paucispinum* were found: one predominately in the Indian Ocean, the other restricted to the central and western Pacific; *D. savigni* spans both oceans. Secondary contact between sympatric clades was suggested, based on a divergence time of 1.02 – 1.86 Mya and on the production of viable hybrids in nature (Lessios and Pearse 1996). Since reproductive isolation barriers were weak or absent, Lessios and coworkers (2001) argue this differentiation could only have happened in allopatry and that *D. paucispinum* and *D. savigni* may be one species containing two divergent mtDNA lineages. Further studies have not yet been conducted to determine the extent of hybridization in these two species.

The distribution of *D. savigni* also overlaps with the congeneric *D. setosum* along the western edge of the Pacific and along the Australian and African shores of the Indian Ocean (Pearse 1998). In cross-fertilization studies, gametes of these two species were reciprocally compatible (Uehara *et al.* 1990). Natural hybrids were found in genetic analyses of field-collected individuals with intermediate morphology; however levels of introgression were low with little interspecific gene flow (Lessios and Pearse 1996). Reproductive isolation in sympatric *Diadema* spp. population in Kenya appears to be maintained by temporal reproductive isolation during the lunar spawning period, and reinforced by seasonal differences in reproductive effort (Muthiga 2003).

Extensive hybridization

During the Pleistocene, the Gulf of Mexico was likely isolated from the northwest Atlantic due to decreases in sea level (Pielou 1979). The emergent coastline around the Florida peninsula may have posed a physical barrier to gene exchange between Atlantic and Gulf populations (Reeb and Avise 1990). The stone crabs, *Menippe mercenaria* and *M. adina*, form two hybrid zones, one in the Gulf of Mexico (NW Florida) and the other along the Atlantic coast of Florida. Initially identified as a single species along the Florida coastline, Bert (1986) concluded from allozyme and morphological studies that *M. mercenaria* was composed of two taxa and suggested the two hybrid zones were the result of separate secondary contact events. Williams and Felder (1986) described the two forms as separate species: the western Gulf form is *M. adina* and the peninsular Florida form is *M. mercenaria*. Based on a 2% mtDNA sequence divergence, the species diverged approximately 0.77 – 0.91 Mya (Bert *et al.* 1996). As well, *M. mercenaria* and *M. adina* have similar (but not identical) habitats and overlapping mating seasons (Bert 1985, as cited in Bert 1986).

In the narrow NW Florida hybrid zone, Bert and Harrison (1988) found concordant clines in morphological traits and allozyme allele frequencies across 300 – 460 km. They also detected significant linkage disequilibrium between two diagnostic loci. In the hybrid zone, individuals of pure parental and mixed ancestry co-occur and hybridization is extensive. Classification of individuals using allozymes and mtDNA RFLPs indicated 61% of individuals in the hybrid zone were of mixed ancestry (Bert *et al.* 1996).

The other hybrid zone on the Atlantic coast was found to have a unimodal distribution with a broad range of intermediate phenotypes and genotypes, and few parental forms. As suggested by Bert and Harrison (1988), the zones of hybridization and variation in *Menippe* spp. could reflect selection acting along an environmental gradient or secondary contact of taxa differentiated in allopatry. Based on the geological record and species distributions, they argue for one or more secondary contacts between *M. adina* and *M. mercenaria*, particularly since there are many similarities in changes in allele frequencies and morphological characters despite differences in habitat in the two zones (Bert 1986).

Secondary contact, outcome unknown

Studies of population structure sometimes reveal cryptic structuring within an apparently continuously distributed species and the existence of different Pleistocene refugia. In a study of population genetic structure using mtDNA sequence analysis, Arndt and Smith (1998) uncovered a significant genetic discontinuity among populations of the sea cucumber, *Cucumaria pseudocurata*, a brooding species that lacks a pelagic larval phase. The location of the genetic break corresponds with the contemporary splitting of the Californian and Alaskan currents in the northeast Pacific (as cited in Arndt and Smith 1998). Reproductive patterns and predominant coastal currents may explain the high levels of genetic diversity north and south of this break since species lacking a pelagic larval phase have demonstrated high levels of population differentiation on the Pacific coast (e.g. corals, Hellberg 1994, 1995, 1996). The genetic break may also be the result of Pleistocene glaciations and the genetic diversity on both sides of this break could

suggest survival and isolation in northern and southern refugia. A cline in ossicle structure exists along the Pacific coast, with ossicles of intermediate phenotype found in specimens from northern British Columbia and southeast Alaska (Lambert 1985). Arndt and Smith (1998) recommend further sampling of the area, including analysis with nuclear markers, to determine whether there has been any introgression and/or hybridization between the northern and southern populations.

Conclusions about studies of secondary contact in marine invertebrates

Most research on hybridization and secondary contact has been conducted in the terrestrial environment. There has been little acknowledgement of the potential for hybridization and secondary contact in the marine environment until recently (Gardner 1997). In his review of hybridization in the sea, Gardner (1997) estimates hybridization in the marine environment occurs at the same frequency as hybridization in other environments. While not all hybrid zones are the result of secondary contact between historically differentiated populations, it is one hypothesis to explain observed patterns in morphological and genetic variation.

One of the most notable differences between marine and terrestrial studies of secondary contact is that marine studies are often initially focused on population genetic patterns or phylogenies; historic differentiation and hybrid zones are discovered incidentally to these other goals, whereas terrestrial examples are often identified *a priori* from biogeographic patterns of glacial refugia and range expansion. While clines and genetic discontinuities found within distributions of species such as those in the northeast

Atlantic and Mediterranean Sea (e.g., barnacle *Chthamalus* spp., Pannacciulli *et al.*, 1997; mussel *Mytilus galloprovincialis*, Quesada *et al.* 1995; cuttlefish *Sepia officialis*, Pérez-Losada *et al.* 1999, 2000) have been interpreted as evidence of secondary contact (Pérez-Losada *et al.* 2002), the outcomes are unknown.

Multiple approaches should be used to investigate the outcome of secondary contact in marine invertebrates: (1) field observations to identify spatial and temporal differences in reproduction; (2) mating studies to identify differences in mating behaviour, or in broadcast-spawning invertebrates, cross-fertilization studies to identify gamete incompatibilities; and (3) surveys of allopatric and sympatric populations using molecular markers to detect hybridization and introgression.

Secondary contact of *Asterias* spp. in the northwest Atlantic

In this thesis, I investigate reproductive isolation in a sibling pair of sea stars, *Asterias rubens* L. and *A. forbesi* (Desor), which form a secondary contact zone in the northwest Atlantic. *Asterias rubens* has an amphi-Atlantic distribution (Tortonese 1963), ranging from Portugal to the United Kingdom in the northeast Atlantic and from North Carolina to southern Labrador in the northwest Atlantic (Clark and Downey 1992). North American populations of *A. rubens* were previously described as *A. vulgaris*, a junior synonymy (Tortonese 1963, Clark and Downey 1992). *Asterias forbesi* is restricted to the northwest Atlantic and ranges from the eastern shore of Nova Scotia to the Gulf of Mexico (Clark and Downey 1992). Populations of the two species are sympatric from about Cape Cod to northeastern Nova Scotia.

Using phylogenetic and population genetic analyses of nuclear and mtDNA, Wares (2001) evaluated the origin of the two species and concluded that the zone of sympatry is the result of secondary contact. Prior to his study, two hypotheses had existed to explain the speciation of the two seastars. From allozyme analyses, Schopf and Murphy (1973) suggested the two species were a geminate species pair formed by a late Pleistocene vicariance event near Cape Cod. In contrast, morphological and paleoceanographic data suggested a late Pliocene separation of the genus into a North American and a European species, which was then followed by a recolonization of North America by *A. rubens* during the Holocene (Worley & Franz 1983). Phylogenetic divergence estimates suggest initial vicariance of the North Atlantic *Asterias* populations by the formation of the Labrador Current 3.0 Mya (Wares 2001). *Asterias rubens* is believed to have re-colonized North America recently, possibly since the last glacial maximum, based on low allelic diversity and the lack of haplotypes unique to North America (Wares 2001).

The outcome of secondary contact between *A. forbesi* and *A. rubens* is unclear. Some studies report natural hybrids are frequent (Clark and Downey 1992), while other studies consider hybrids rare (Worley and Franz 1983, Wares 2001). Gametes are apparently compatible (Ernst 1967) and there does not appear to be spatial or temporal reproductive isolation as adults of the two species share habitat (Menge 1986) and spawning periods overlap (Smith 1940, Boolootian 1966, Franz *et al.* 1981, Menge 1986). Despite the lack of apparent reproductive isolation barriers, the phylogenetic analysis of north Atlantic *Asterias* by Wares (2001) did not find any evidence of shared haplotypes between the species (a potential indicator of hybridization and introgression). To clarify

the outcome of secondary contact of *A. rubens* and *A. forbesi* I conducted a series of studies with specific objectives:

(1) To examine pre- and postzygotic reproductive barriers in sympatric *Asterias* populations, specifically to determine whether gametes of the two species are reciprocally compatible, and whether hybrid offspring are viable and fertile. I conducted cross-fertilizations studies *in vitro* across a range of sperm and egg concentrations (Levitan *et al.* 1991, Levitan 2002, McCartney and Lessios 2002) to determine if heterospecific crosses required different gamete concentrations than conspecific crosses for successful fertilization (Chapter Two). I also examined the effects of gamete age on fertilization success to determine whether the specificity and success of the sperm-egg interactions changes over time (Williams and Bentley 2002). I assessed the viability and fertility of laboratory-raised F_1 offspring by conducting backcrosses with lab-cultured, sexually mature hybrids.

(2) To determine whether morphological intermediates exist naturally between *A. forbesi* and *A. rubens*. I conducted an extensive morphological survey of *A. rubens* and *A. forbesi* across their geographic ranges. I scored 857 specimens from allopatric and sympatric populations using five diagnostic characters described by Clark and Downey (1992) and three morphometric traits (Worley and Franz 1983; Chapter Three). I also included in the analysis two F_1 hybrids raised in culture. I then used a quantitative analysis to objectively identify morphological intermediates that were possible hybrids between the two parental phenotypes.

(3) To examine sympatric *Asterias* spp. populations for evidence of hybridization using a molecular marker. I surveyed mtDNA sequence variation among individuals that were identified phenotypically in the morphological analysis as *A. rubens*, *A. forbesi*, and

possible morphological intermediates (Chapter Four). I included specimens from allopatric populations to compare genetic diversity in allopatric and sympatric populations and to provide insight into the genetic history of the contact zone.

(4) To examine gamete compatibility under conditions of sperm competition. I used a nuclear marker (microsatellite) I developed to assign paternity to hybrid offspring produced in sperm competition studies (Chapter Five).

There are relatively few well-studied examples of secondary contact and hybridization in marine invertebrates. Notable exceptions include species complexes of the mussel *Mytilus* (e.g. Gardner 1994, 1996; Bierne *et al.* 2002b), the stone crab *Menippe* (Bert and Harrison 1988, Bert *et al.* 1996), and the hard clam *Mercenaria* (Dillon and Manzi 1989, Bert *et al.* 1993, Bert and Arnold 1995, Foighil *et al.* 1996). The secondary contact zone formed by *A. rubens* and *A. forbesi* in the northwest Atlantic may yield new insight into the outcome of secondary contact following recent glacial retreat and further our understanding of mechanisms of reproductive isolation in broadcast-spawning marine invertebrates.

CHAPTER TWO:

INTERSPECIFIC GAMETE COMPATIBILITY IN *ASTERIAS RUBENS* AND *A. FORBESI*

Introduction

As byproducts of genomic divergence in allopatry, taxa evolve reproductively isolating barriers through natural selection and/or genetic drift (Dobzhansky 1940, Mayr 1963, Templeton 1981, Coyne and Orr 1989, 1997). When closely related species come into secondary contact, the outcome depends in large part upon the evolution of these barriers: the two species may fuse via hybridization, one of the two species may become extinct, or the two species remain distinct (Wilson 1965). Mechanisms of reproductive isolation in marine invertebrates include habitat specialization, spawning asynchrony (on diurnal and seasonal time scales), mate selection and preferential fertilization (Palumbi 1994). Gametic incompatibility is considered one of the most important prezygotic reproductive barriers for closely-related, broadcast-spawning species whose spawning periods overlap in sympatry (e.g., Lessios 1985, Palumbi 1994, Levitan 2002). Cross-fertilization studies conducted *in vitro* enable an examination of sperm-egg interactions and help determine the importance of gametic incompatibility in maintaining the genetic integrity of the species in secondary contact (McCartney and Lessios 2002).

In the northwest Atlantic, populations of the sea stars *Asterias forbesi* and *A. rubens* are sympatric in a secondary contact zone identified using morphological and molecular markers (Worley and Franz 1983, Wares 2001). However, it is unclear whether the species are reproductively isolated in sympatry. Reports of specimens with intermediate morphologies (Clark 1904, Perlmutter and Nigrelli 1960, Ernst 1967,

Walker 1973, Menge 1986) and the apparent ability of the species to cross-fertilize in the lab (Ernst 1967) have been interpreted as evidence of natural hybridization (Schopf and Murphy 1973, Menge 1986, Byrne and Anderson 1994, Palumbi 1994, Gardner 1997). Hybrids are reportedly frequent and do not reach sexual maturity (Clark and Downey 1992); however other studies found hybrids to be rare in nature (Worley and Franz 1983, Wares 2001). Temporal separation of spawning periods and spatial separation of adults with different temperature and habitat preferences are possible mechanisms of prezygotic reproductive isolation in these species (Schopf and Murphy 1973), but may be incomplete barriers to hybridization in the contact zone. Adults of both species share the same habitat and compete for the same resources (Menge 1979). At one site in the contact zone (Bear Cove, NS), a male *A. forbesi* was observed spawning within 30 cm of an *A. rubens* in late July when both species were reproductively active (pers. obs.).

Spawning periods overlap in New England and Atlantic Canada; depending on the year, *A. rubens* reportedly spawn from April to July, whereas *A. forbesi* spawn in July and August (Smith 1940, Boolootian 1966, Menge 1986). *Asterias rubens* spawn when ambient water temperatures are 6 – 15°C, adults grow optimally at 10°C and do not survive at temperatures greater than 25°C (Franz *et al.* 1981, Nichols and Barker 1984, Holanda 1995). *Asterias forbesi* tolerates warmer water temperatures, spawn around 15°C, and reach optimal growth between 18 and 20°C (Franz *et al.* 1981, Holanda 1995). In parts of the secondary contact zone such as the shallow subtidal of the Gulf of Maine, surface sea temperatures vary from 10 to 20°C between May and August (Yoder *et al.* 2002) when spawning seasons of the two species may coincide. Temperature tolerance of gametes has been suggested as a source of gamete incompatibility in broadcast-spawning

marine invertebrates (McClary and Sewell 2003), but clearly the potential for overlap exists in the *Asterias* contact zone.

Gamete incompatibility has not been suggested as a prezygotic barrier to reproduction in *Asterias*. The gametes of the two species are considered compatible, based on a single reference in Ernst (1967, p.22): "the sperm of both species will fertilize the eggs of both". To determine whether gamete incompatibility is involved in prezygotic reproductive isolation in sympatric *A. forbesi* and *A. rubens*, I measured fertilization success in crosses of conspecific and heterospecific gametes over a range of different sperm and egg concentrations at an intermediate temperature (12°C). I also analyzed the effect of gamete age to determine whether the specificity and success of the sperm-egg interactions change over time (Williams and Bentley 2002). As fertilization was successful in both conspecific and heterospecific crosses, I examined the possibility of postzygotic barriers to reproduction (hybrid inviability and sterility). Hybrid offspring were reared for two years to sexual maturity, and then successfully backcrossed with field-collected adults. Results from this study suggest that natural hybridization is possible and may be frequent. Postzygotic barriers to hybridization may be weak because hybrids raised in culture are viable and fertile, and gamete incompatibility does not act as a strong prezygotic barrier to reproduction in no-choice cross-fertilization studies.

Materials and Methods

Sampling of adults and collection of gametes

Mature specimens of *Asterias rubens* and *A. forbesi* were collected from Bear Cove, Nova Scotia (5 – 10 m depth) in July and August 2001. Both species were reproductively active during these months (pers. obs.). Animals were maintained in flow-through seawater at ambient temperature (14 - 17°C) for up to one week and fed mussels (*Mytilus* spp.). Animals were assigned to species using the diagnostic morphological characters described in Clark and Downey (1992).

Gametes were collected by dissection of gonadal tissue from ripe adults. A short incision (1 – 2 cm) was made along the dorsal margin of one side of the proximal part of an arm; approximately the same volume of gonad was removed from each animal. Testes were placed in 20 mL of 0.45 μm filtered seawater (FSW) and ovaries were placed in 20 mL of 10^{-5} M 1-methyl adenine, prepared in FSW. Sperm were extruded immediately from the testes; eggs were incubated for 1 h to allow completion of ovulation and oocyte maturation (Kanatani 1979). Prior to use, sperm and eggs were separated from their respective gonads by decanting the solution into clean beakers. Gamete collection and all experiments were performed at 12°C.

In each experiment, gametes were combined using specified dilutions of initial sperm and egg stocks. Actual gamete concentrations were determined following each experiment from aliquots of sperm and egg stocks preserved in formalin-buffered seawater. Sperm concentrations were estimated from haemocytometer counts of a 10-fold dilution of the initial sperm stock, and eggs were counted using a dissecting microscope.

Experimental design

Conspecific and heterospecific crosses were made for both *Asterias* species. For each heterospecific cross, the same female was used in the conspecific fertilization to serve as a positive control. There were five replicates for each type of cross: FF, RR, FR, and RF, where the female and male parents are indicated in order by the first letter of the specific name (e.g. FR = *A. forbesi* female x *A. rubens* male). Each replicate used a different male and female of each species (five replicates per cross; twenty animals total). Replicate crosses were conducted on different days to reduce potential cross-contamination.

Two independent sets of studies were conducted. In the first, I examined the effect of sperm concentration on fertilization rates of conspecific and heterospecific eggs. I used these results to choose a sperm concentration to yield high fertilization in the second set of studies, in which I measured the effects of egg concentration and gamete age on conspecific and heterospecific fertilization success. Only four replicates of each cross were used in the second set of studies (16 animals total) because the fifth replicate was contaminated by ciliates.

Effect of sperm concentration on fertilization success

To determine whether heterospecific crosses required different sperm concentrations than conspecific crosses for fertilization and cleavage success, a broad range of sperm concentrations was tested.

Sperm stocks were serially diluted (1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) immediately prior to use. Fertilizations were conducted in sterile 25 mL Petri dishes containing 8 mL of

FSW and 1 mL of eggs pipetted from a well-mixed stock suspension. From the freshly prepared sperm solutions, 1 mL of the appropriate sperm dilution was then pipetted over the eggs and mixed gently. Final egg concentrations were 76 to 296 eggs mL^{-1} and final sperm concentrations ranged from about $10^{-3} - 10^{3}$ sperm μL^{-1}.

I scored fertilization success using two criteria: presence of a fertilization envelope, and cell cleavage. After 1 h, fertilization success was scored by examining at least the first 200 undamaged, haphazardly selected eggs and counting the number with fertilization envelopes under a dissecting microscope. Evidence of cleavage in intact eggs was scored after 3 h. In a pilot study, 3 h was sufficient time to allow all fertilized eggs to divide at least once. Both cleavage and fertilization envelopes were used as indices of fertilization to assess the potential for polyspermy at high sperm concentrations and to avoid overestimating effective fertilization success due to inclusion of polyspermic eggs (Styan 1998). Polyspermy results in either mortality or abnormal embryo development and might be reflected in a difference between fertilization rates measured from fertilization envelopes and rates measured from cell cleavage.

To compare the effectiveness of fertilization across replicate crosses, I estimated the F_{50} value, the sperm concentration at which 50% of the eggs were successfully fertilized (Levitan 1996, 1998). To determine the F_{50} for each replicate, data were fit to the nonlinear fertilization kinetics model developed by Vogel *et al.* (1982) for sea urchins. The untransformed proportion of eggs fertilized (P) was fit to the equation:

$$P = 1 - \exp\left((-\beta S_0)/(\beta_0 E_0)*(1 - e(-\beta_0 E_0 t))\right),$$

where S_0 = number of sperm μL^{-1}, E_0 = number of eggs μL^{-1}, t = sperm: egg contact time (in sec.), and β and β_0 are parameters obtained from nonlinear regression of P on S_0 (Vogel *et al.* 1982). β is the rate constant of fertilization, based on sperm-egg contact and

the fertilizability of the egg, and β_0 is the rate constant of sperm-egg contact, based on egg cross-sectional area and sperm swimming velocity (Levitan *et al.* 1991). Both β and β_0 were estimated for each replicate using initial values of 3.8×10^{-6} and 3.3×10^{-4} mm^3 s^{-1} respectively (NLIN command in SAS v8; Vogel *et al.* 1982). Each F_{50} was then determined by solving the resulting nonlinear regression equation for S_0 at $P = 0.5$. F_{50} values were estimated for both indices of fertilization success.

Fertilization success was also analyzed using a logit transformation method which does not assume adherence to a fertilization kinetics model developed for intraspecific crosses (McCartney and Lessios 2002). The proportion of eggs fertilized was transformed to its logit according to the equation: logit $P = \ln(P/1 - P)$. For each replicate, the logit P value was linearly regressed on the log-transformed sperm concentration. The logit method does not account for differences in initial egg concentrations, however these did not vary significantly among the four different types of crosses (one-way ANOVA, $F = 0.012$, df = 3, $p = 0.99$). F_{50} values were estimated from the linear regression by determining the sperm concentration at which logit (0.5) = 0.

Values of F_{50} were analyzed using a one-way ANOVA with cross type (FF, FR, RR, RF) as the independent variable ($\alpha = 0.05$). Separate analyses were conducted for fertilization success measured from counts of fertilization envelopes and counts of cleaved zygotes.

Effect of egg concentration on fertilization success

A series of egg concentration experiments was conducted to examine the effect of egg concentration on fertilization success in heterospecific and conspecific crosses. For

each replicate of the conspecific and heterospecific crosses, four 2-mL Eppendorf tubes were prepared by pipetting 200, 400, 800, and 1600 µL of well-suspended initial egg stock into 1600, 1400, 1000 and 200 µL of FSW respectively. A 10^{-1} dilution of the initial sperm stock was prepared and 200 µL was added immediately to each of the four tubes (final sperm concentration 5 – 63 sperm μL^{-1}). Tubes were incubated for 3 h, and then preserved by adding 1-2 drops of formalin.

Fertilization success was determined for each egg concentration by counting the number of eggs with fertilization envelopes in at least 200 intact eggs. Statistical analyses were performed on the arcsine transformations of these percentages in an ANCOVA with the cross type as the independent variable and egg concentration (eggs mL^{-1}) as a covariate.

Effect of gamete age on fertilization success

To determine whether the specificity of sperm-egg interactions changes over time and to measure the viability of gametes as they age, sperm and eggs were spawned concurrently and then combined at specified intervals after spawning and scored for fertilization success.

Gametes were collected at the same time on each day. For each replicate, five 2-mL Eppendorf tubes were prepared with 1600 µL FSW and 200 µL of well-suspended egg stock (final concentration 60 – 376 eggs mL^{-1}). Gametes were combined at five intervals after collection (1, 3, 6, 9, 12 h). At each interval, 200 µL of a 10^{-1} dilution of the initial sperm stock was added to one of the prepared tubes (final concentration 3 – 44

sperm μL^{-1}). Development was arrested in each tube 3 h after sperm addition by adding 1-2 drops of formalin.

Successful fertilizations were scored by counting the number of eggs with fertilization envelopes in at least 200 intact eggs. The percent fertilization was then arcsine transformed for statistical analyses in a two-way ANOVA with the cross type and the gamete age as independent variables.

F_1 offspring and backcrosses

Larvae from the conspecific and heterospecific crosses in the sperm concentration experiment were transferred after 3 days into 4-L glass jars containing 3 L FSW (initial larval density 1 – 3 larvae mL^{-1}). Cultures were stirred using a rotating paddle system (Strathmann 1987) at a speed of 10 rpm. Larvae were fed a dense algal mixture of *Dunaliella, Isochrysis* and *Rhodomonas* every 2 – 3 days, at which time half the volume of FSW was removed by siphoning and replaced with fresh FSW. A biological film was allowed to grow on the inner surface of the glass jars.

After 8 weeks, brachiolaria larvae with well-developed juvenile rudiments were induced to settle and metamorphose by addition of small cultured mussels (*Mytilus edulis*) and fresh pieces of the macroalgae *Ulva* spp. to the jars (L. Harris, pers. comm.).

Post-settlement juveniles were transferred to 250 mL containers pre-conditioned with the algal mixture to form a biological film. Small pieces of *Ulva* spp. provided additional substrate. Initially, juveniles were fed cultured *Mytilus edulis* spat; larger *Asterias* spp. were fed field-collected mussels (*Mytilus* spp. and *Modiolus modiolus*). Filtered seawater was replaced every 2 – 3 days and cultures were maintained at 12 °C.

After one year, each animal was visually examined for gonadal development by making a small incision along the dorsal margin of one side of the proximal part of an arm. Since gonads were not present in any hybrid or nonhybrid, the animals were all returned to culture. In their second year, juveniles were reared in individual containers in a flow-through tank at ambient seawater temperatures and fed mussels (*Mytilus* spp.) *ad libitum*. Unfortunately, a tank failure resulted in the subsequent loss of most of these juveniles. The two surviving animals, a FR hybrid and a RF hybrid, were reared until they reached sexual maturity at two years of age.

Backcross experiments were conducted with each of the surviving hybrids. Gametes were collected as described above and combined at concentrations of 200 eggs mL^{-1} and 200 sperm μL^{-1} in total volumes of 2 and 25 mL FSW at 12°C. The FR hybrid was male and was crossed separately with an *A. forbesi* female and an *A. rubens* female. The RF hybrid was female and was crossed separately with an *A. forbesi* male and an *A. rubens* male. The development of embryos in the 2 mL containers was arrested after 2 h with 1-2 drops of formalin; fertilization was scored by sampling the first 200 undamaged eggs encountered and counting the number with fertilization envelopes. After 48 h, gastrula embryos in the 25 ml containers were transferred to 125 mL FSW and fed 2 mL of dense *Isochrysis* algae. Larvae were examined daily for evidence of feeding (pigmented stomachs) and subsequently preserved in ethanol after 6 d of development.

Results

Effect of sperm concentration on fertilization success

Gametes of *Asterias forbesi* and *A. rubens* were reciprocally compatible. Eggs of both species were successfully fertilized by conspecific and heterospecific sperm, but there was considerable variation in the compatibility of the heterospecific gametes. The proportion of fertilized eggs increased rapidly as sperm concentration increased. Fertilization rates were low at sperm concentrations less than about 1 sperm μL^{-1} and were typically > 90% successful above 100 sperm μL^{-1}, producing a sigmoid curve when fertilization was plotted against sperm concentration (Fig. 2.1).

To compare fertilization success among the conspecific and heterospecific crosses, F_{50} values were estimated from curves of the non-linear fertilization model (Fig. 2.2) and linear regressions of logit-transformed fertilization data (Fig. 2.3). The model of Vogel *et al.* (1982) fit the fertilization data well for both indices of fertilization success (mean $R^2 = 0.986$ for fertilization envelopes; $R^2 = 0.901$ for cell cleavage; Appendix One). The linear regressions of logit-transformed fertilization success scored on the appearance of fertilization envelopes showed good fit (mean $R^2 = 0.884$), but regressions of fertilization success scored using cell cleavage did not fit well (mean $R^2 = 0.526$, Appendix One).

Both measures of fertilization success (fertilization envelope and cell cleavage) and both methods of analysis produced similar results. F_{50} concentrations estimated from both indices using the nonlinear curves were highly correlated ($r = 0.838$, $p < 0.001$), and analyses of fertilization envelope data estimated using both methods were positively correlated ($r = 0.613$, $p < 0.005$). Mean F_{50} sperm concentrations estimated for the

conspecific *A. forbesi* crosses varied from 32 to 72 sperm μL^{-1} while the conspecific *A. rubens* crosses varied from 16 to 25 sperm μL^{-1} (Table 2.1). In heterospecific crosses, *A. forbesi* eggs required about 3 times more sperm for fertilization with *A. rubens* sperm than with conspecific sperm, and *A. rubens* eggs required about 10 times more *A. forbesi* sperm than with conspecific sperm.

There were no significant differences in the concentrations of sperm required to fertilize 50% of the eggs among conspecific and heterospecific crosses ($p > 0.05$ in all ANOVAs), however the heterogeneity of variances violated the assumption of homoscedasticity required for ANOVA (Sokal and Rohlf 1995). The variance in the estimates of F_{50} for the heterospecific crosses was much higher than the variance in the conspecific crosses (Table 2.1). While some combinations of heterospecific gametes had F_{50} values comparable to conspecific crosses, other combinations required an order of magnitude more heterospecific sperm for successful fertilization of 50% of the eggs. Females of both *A. rubens* and *A. forbesi* differed by an order of magnitude in their receptivity towards heterospecific sperm.

There was evidence of polyspermy in both conspecific and heterospecific crosses when sperm concentrations exceeded about 300 sperm μL^{-1} (Fig. 2.1). While fertilization success did not decline at high sperm concentrations when scored from fertilization envelopes, fertilization decreased in most replicates when cell cleavage was scored. The F_{50} values estimated using the Vogel model were consistently lower when success was scored using cell cleavage than when fertilization envelopes were scored. The cleavage curve fitting may be affected by decreased fertilization at high sperm concentrations.

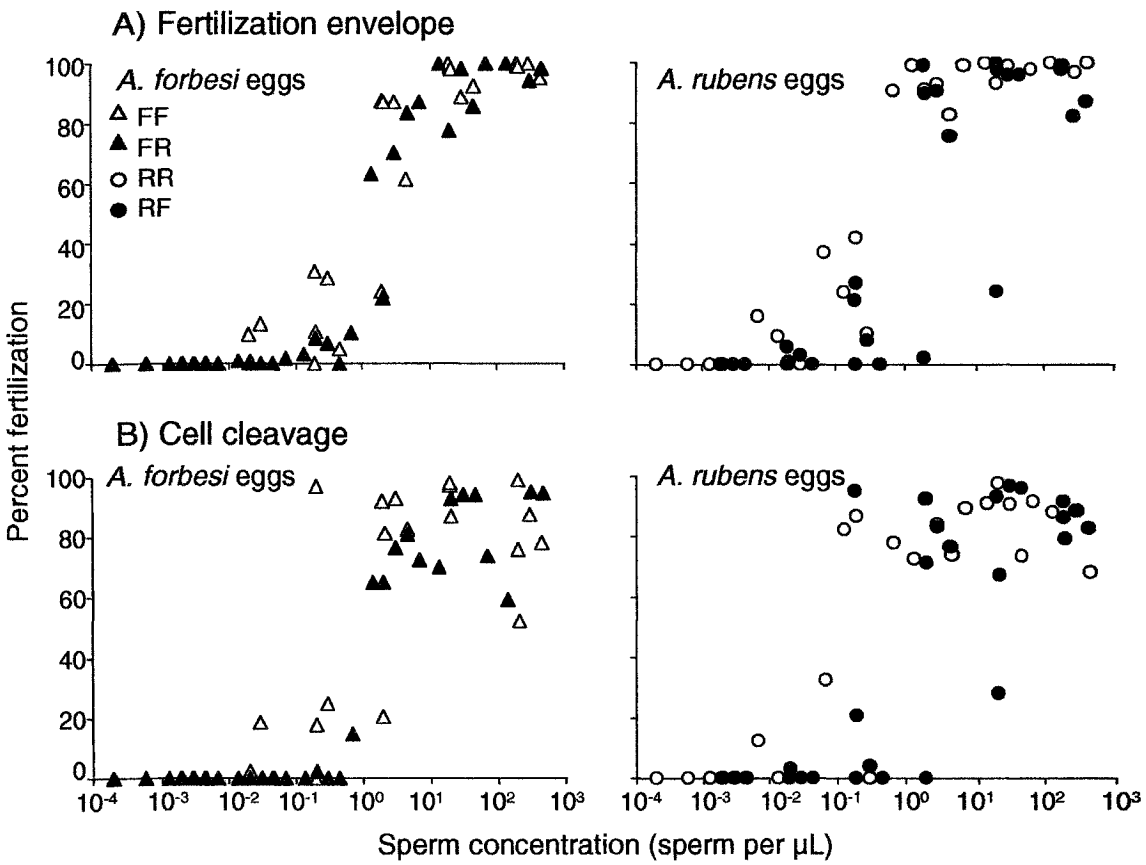

Figure 2.1 Results from conspecific and heterospecific cross-fertilization experiments across a range of sperm concentrations. A) Fertilization scored on appearance of fertilization envelope after 1 h; B) Fertilization scored on cell cleavage after 3 h. In the legend, F refers to *Asterias forbesi*; R refers to *A. rubens*; the female parent is indicated first, then the male parent. Five replicates for each cross FF, FR, RR, RF.

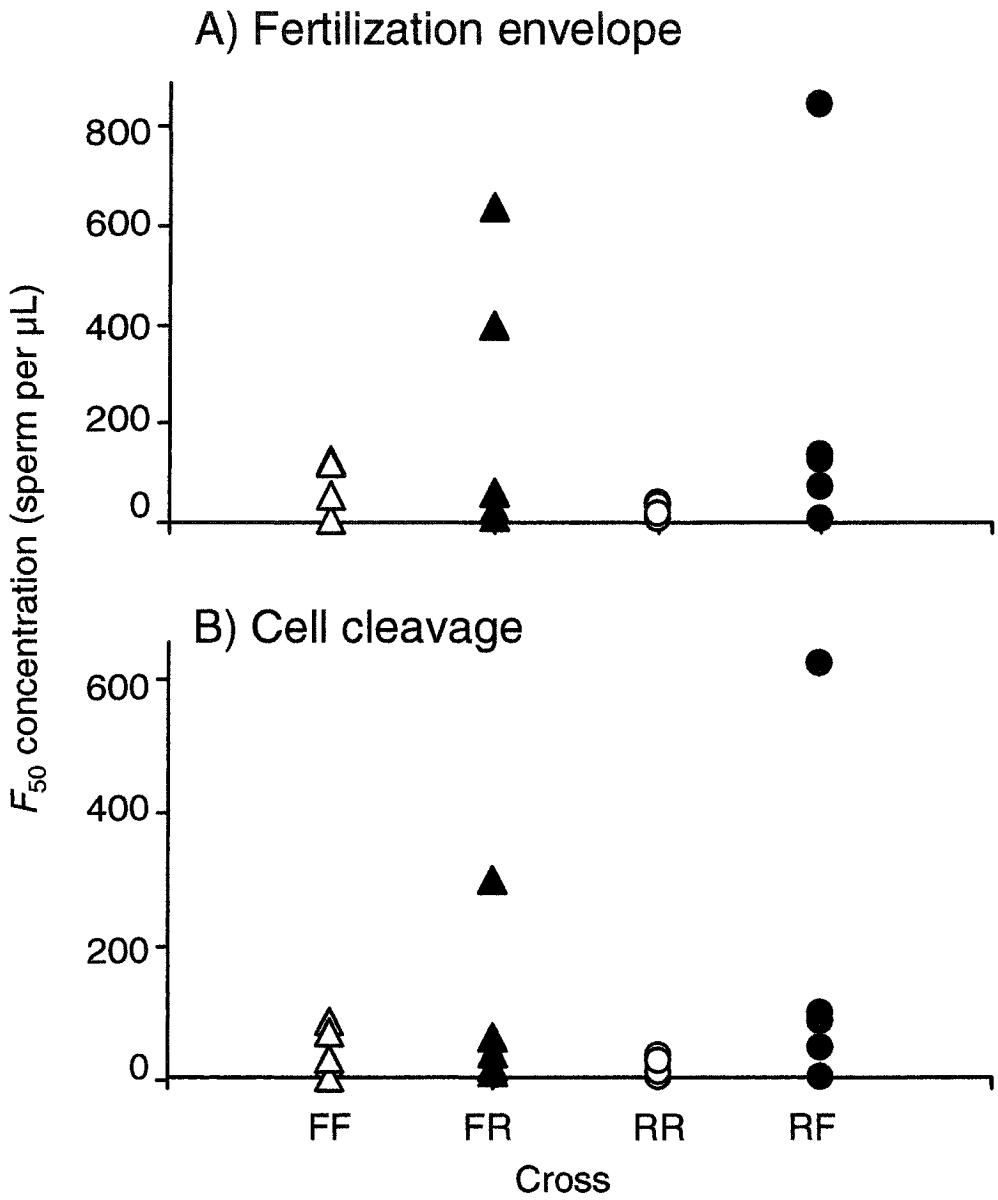

Figure 2.2 Sperm concentrations required to fertilize 50% of conspecific and heterospecific eggs estimated using a nonlinear fertilization kinetics model (Vogel *et al.*1982). A) Fertilization scored on appearance of fertilization envelope after 1 h; B) Fertilization scored on cell cleavage after 3 h. Five replicates for each cross FF, FR, RR, RF.

Table 2.1 Comparison of mean F_{50} values (s.d.) among the conspecific and heterospecific crosses of *Asterias* spp. calculated from non-linear fertilization curves and linear regression. Fertilization data scored using two different indices: the appearance of the fertilization envelope and cell cleavage.

Analysis	Index of fertilization success	Female	Male	n	F_{50} (s.d.)	F_{50} ratio
Non-linear fertilization curves	Fertilization envelope	*A. forbesi*	*A. forbesi*	5	71.50 (49.9)	—
		A. forbesi	*A. rubens*	5	227.64 (279.5)	3.2
		A. rubens	*A. rubens*	5	24.79 (14.5)	—
		A. rubens	*A. forbesi*	5	238.13 (345.5)	9.6
	Cell cleavage	*A. forbesi*	*A. forbesi*	5	39.85 (33.8)	—
		A. forbesi	*A. rubens*	5	82.68 (120.9)	2.1
		A. rubens	*A. rubens*	5	16.04 (12.5)	—
		A. rubens	*A. forbesi*	5	168.25 (254.2)	10.5
Linear regression	Fertilization envelope	*A. forbesi*	*A. forbesi*	5	32.83 (36.25)	—
		A. forbesi	*A. rubens*	5	58.15 (49.69)	1.8
		A. rubens	*A. rubens*	5	24.40 (34.1)	—
		A. rubens	*A. forbesi*	5	94.65 (112.6)	3.9

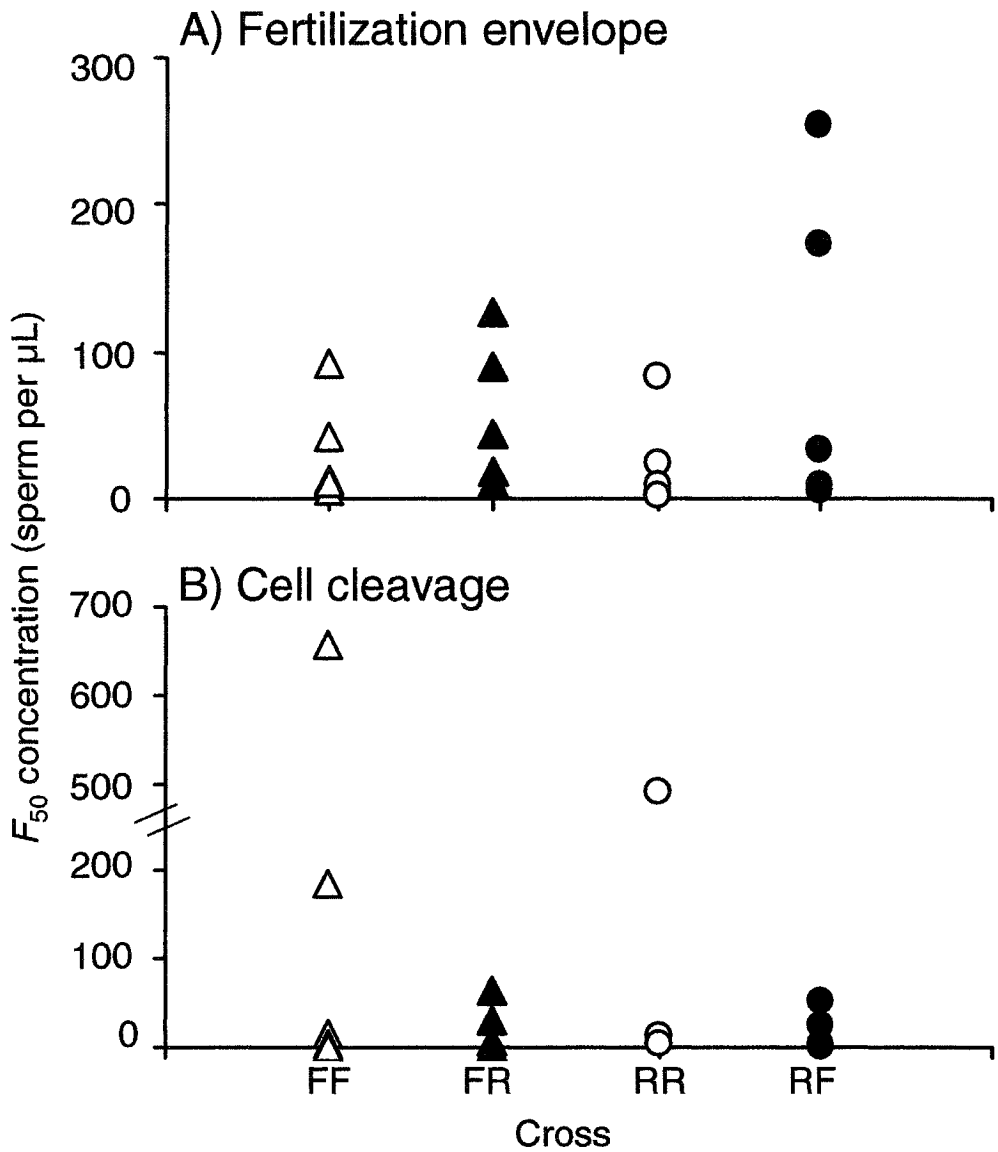

Figure 2.3 Sperm concentrations required to fertilize 50% of conspecific and heterospecific eggs estimated from linear regressions. A) Fertilization success scored based on appearance of fertilization envelope after 1 h; B) Fertilization success scored using cell cleavage after 3 h.

Effect of egg concentration on fertilization success

The proportion of eggs fertilized was significantly negatively correlated with egg concentration in some crosses (ANCOVA, $p = 0.015$ for main effect of cross, $p < 0.001$ for covariate of egg concentration, Fig. 2.4). Linear regression of the proportion of eggs fertilized on egg concentration for each of the cross types showed both the FF and RF crosses differed significantly from zero ($p = 0.043$ and 0.003 respectively). The FF regression was driven primarily by the fertilization involving the highest egg concentration. When this point was removed, the regression was no longer significant ($p > 0.05$).

The slopes of the regressions for *A. rubens* eggs in both conspecific and heterospecific crosses were quite steep compared with the slopes of the regressions for *A. forbesi* eggs (Fig. 2.4). Fertilization specificity was not dependent on egg concentration for *A. forbesi* eggs. The fertilization of *A. rubens* eggs appears to have been affected by egg concentration, particularly when fertilized by *A. forbesi* sperm. At low egg concentrations, heterospecific fertilization of *A. rubens* eggs was generally high, but it is difficult to interpret fertilization at higher egg concentrations since there were few instances when this occurred.

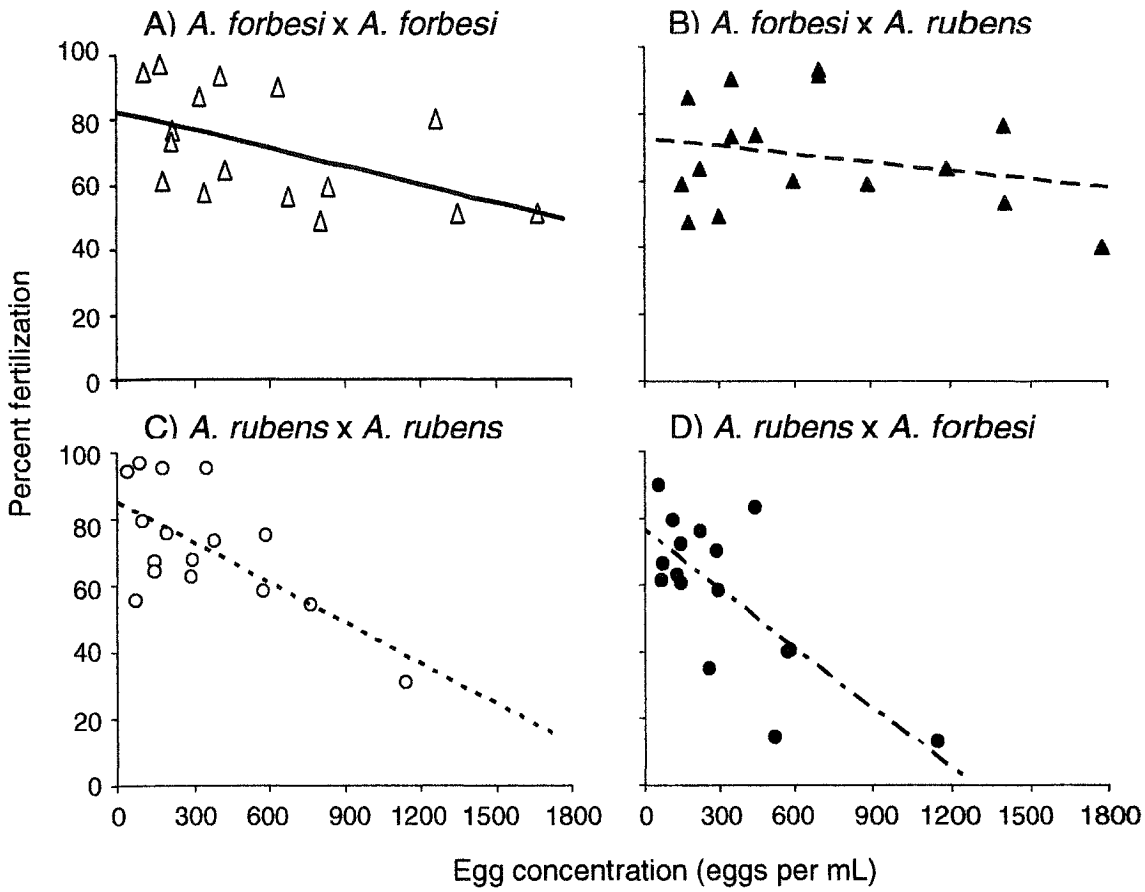

Figure 2.4 Linear regression of egg concentration on fertilization success in conspecific and heterospecific crosses of *Asterias forbesi* and *A. rubens* (maternal species indicated first). Fertilization was scored after 3 h. Four replicates for each cross FF, FR, RR, RF.

Effect of gamete age on fertilization success

Fertilization success declined over time as sperm and eggs aged; few eggs were successfully fertilized at 12 h and there was no fertilization observed after this time (Fig. 2.5). In some replicates, fertilization success was poor after just 1 h in both conspecific and heterospecific crosses, possibly because mean sperm concentration was relatively low (23 sperm μL^{-1}, similar to the estimated F_{50} values calculated in the sperm concentration experiment to yield 50% fertilization). In the two-way ANOVA of cross type and gamete age, both variables were significant main effects in the analysis ($p = 0.014$ for cross type, $p < 0.0001$ for gamete age). However, since there was no significant interaction between the cross type and gamete age, age affected all crosses equally and there were no differences among conspecific and heterospecific crosses.

F_1 offspring and backcrosses

All crosses, both conspecific and heterospecific, produced viable feeding larvae. There was high mortality in all culture jars, but larvae from at least one replicate of each conspecific and heterospecific cross were able to successfully settle and metamorphose. Larvae settled out over an extended period from eight to thirteen weeks after fertilization.

Survival of F_1 juvenile offspring from both heterospecific and conspecific crosses decreased over time. After one year, twelve juveniles remained: three from each of the FF and RR crosses, one from the RF cross and five from the FR cross. The sizes were 30 – 40 mm from the tip of the longest arm to the opposite interradius. The diagnostic characters of the species (Clark and Downey 1992) could not be discerned at this small size.

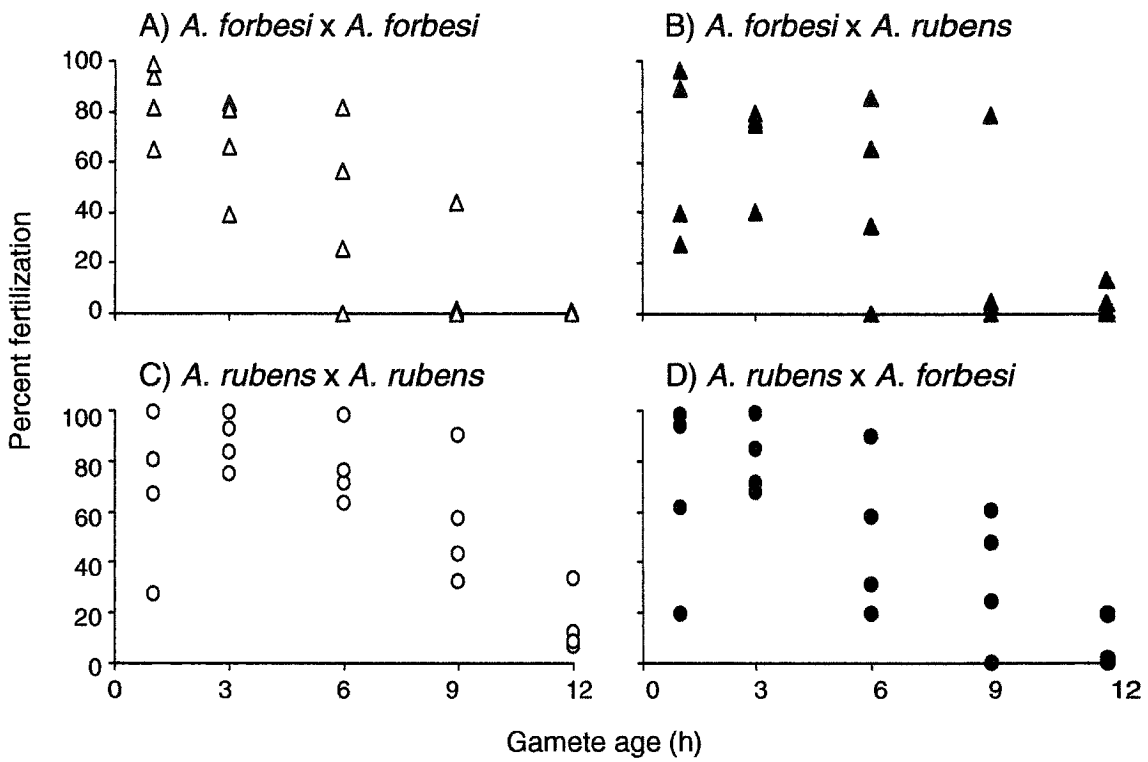

Figure 2.5 Effect of gamete age on fertilization success in conspecific and heterospecific crosses of *Asterias forbesi* and *A. rubens* (maternal species indicated first). Gametes were combined at 1, 3, 6, 9, 12 h and then fertilization was scored after 3 h. Four replicates for each cross FF, FR, RR, RF.

Two hybrids survived to two years of age: one was a female RF hybrid; the other was a male FR hybrid. Both hybrids had the orange madreporite and firm body typical of *A. forbesi*, and long slender abactinal spines with a wreath of minor pedicellariae halfway up these spines typical of *A. rubens*. The only character that differed between the two was the major pedicellariae: the FR hybrid had the short, broad pedicellariae of *A. forbesi* and the RF hybrid had pedicellariae of an intermediate character state, broad and pointed. Both produced viable gametes and were successfully backcrossed to field-collected adult of both species.

Under conditions which yielded 100% conspecific fertilization in studies reported here (egg concentrations of 200 eggs mL^{-1} and relatively high sperm concentrations of 200 sperm μL^{-1}; see Methods), fertilization success for eggs of the hybrid female RF was 19% when backcrossed to a male *A. rubens* and 47% when backcrossed to a male *A. forbesi*. Similarly, the hybrid male FR fertilized 10% of eggs when backcrossed to a female *A. rubens* and 36% of eggs when backcrossed to a female *A. forbesi*. Although the sample of replicate backcrosses is small, in both cases gametes of F_1 hybrid offspring produced higher fertilization rates in backcrosses with *A. forbesi* than with *A. rubens*. All backcrosses produced viable, feeding bipinnaria larvae.

Discussion

Gametic interactions determine reproductive isolation in sympatric broadcast-spawning species with overlapping spawning periods (Levitan 2002). Examples of complete gamete incompatibility and asymmetric compatibility are common (review in Palumbi 1994), but complete gamete compatibility is not often found. In echinoderms, reciprocal gamete compatibility has been reported in laboratory crosses between congeneric sea urchins (*Echinometra:* Lessios and Cunningham 1990, Rahman *et al.* 2001, McCartney and Lessios 2002; *Diadema*: Uehara *et al.* 1990; *Pseudoechinus*: McClary and Sewell 2003; *Strongylocentrotus*: Levitan 2002; *Arbacia* Metz *et al.* 1998) and a sea star (*Patiriella*: Byrne and Anderson 1994). In this study, although heterospecific gamete compatibility of sympatric *Asterias rubens* and *A. forbesi* is highly variable, gamete incompatibility does not appear to be a strong prezygotic barrier to reproduction.

Fertilization success in all conspecific and heterospecific crosses of *Asterias* spp. was affected by the sperm concentration, increasing rapidly from near zero at 0.1 sperm μL^{-1} to greater than 90% in most crosses when sperm concentrations were above 100 sperm μL^{-1}. Mean sperm concentrations required to fertilize 50% of eggs were about 3 times higher for heterospecific crosses with *A. forbesi* eggs and about 10 times higher with *A. rubens* eggs compared with conspecific crosses.

There was considerable variation among replicate sets of parents in the amount of sperm needed for heterospecific fertilization. While the compatibility of some combinations of heterospecific gametes was comparable with conspecific crosses, other combinations required an order of magnitude more heterospecific sperm for successful

fertilization. This variation in compatibility of heterospecific gametes may be the result of intraspecific variation at compatibility loci in females as suggested for the urchins *Echinometra lucunter* (McCartney and Lessios 2002). In a cross-fertilization study, females of *E. lucunter* varied by orders of magnitude in their discrimination against sperm from *E. vanbrunti* and *E. viridis*. However, as was also the case in *Asterias*, females that required more heterospecific sperm did not also require more conspecific sperm, which would have been indicative of differences in gamete quality.

Many studies of heterospecific fertilization compare fertilization success at single sperm and egg concentrations (e.g., Lessios and Cunningham 1990, Uehara *et al.* 1990, Byrne and Anderson 1994). Construction of fertilization curves using serial sperm dilution permits a quantitative assessment of gamete compatibility and increases the sensitivity of the experiments (McCartney and Lessios 2002). The conspecific F_{50} values estimated for *Asterias* spp. were comparable to estimates for other echinoderm species (Table 2.2). Typically, sperm concentrations in the range of 10^1 to10^2 (sperm uL^{-1}) were required to fertilize 50% of the conspecific eggs. One drawback of using F_{50} estimates to compare fertilization success is that a series of data is reduced to a single metric. It is therefore important to ensure sufficient sampling, particularly around the inflection point, to yield reasonably accurate estimates.

In this study I analyzed fertilization data using both the nonlinear kinetics model of Vogel *et al.* (1982) and the linear regression method of McCartney and Lessios (2002). When fertilization success was scored using fertilization envelopes, the estimates of F_{50} from both analytic methods were highly correlated. In contrast, the linear regression analysis of the cell cleavage data did not fit the data well compared with the nonlinear model. Using an empirically-based logit transformation to linearize the sigmoidal curve

has fewer assumptions than fitting the nonlinear fertilization model developed for intraspecific fertilization studies (McCartney and Lessios 2002). However, the theoretically-based Vogel *et al.* (1982) model is sufficiently parameter-rich to fit complex data from experiments that differ in several factors simultaneously (such as sperm concentration and egg concentration).

Two different indices of fertilization success, the appearance of a raised fertilization envelope and cell cleavage, were measured to account for possible influences of polyspermy at high sperm concentrations on the estimated F_{50} values. Some studies have used the presence of a fertilization envelope or further development (cell cleavage) to score fertilization (Vogel *et al.* 1982, Levitan et al. 1991, Levitan 2002), whereas other studies have used only cell cleavage (McCartney and Lessios 2002). In studies of corals, Oliver and Babcock (1992) scored only cleaving embryos and found decreased fertilization at high sperm concentrations, presumably the result of polyspermy . In my study, there was evidence of polyspermy as fertilization success scored using cell cleavage decreased at sperm concentrations above 300 sperm μL^{-1}. While it is unlikely *Asterias* eggs encounter conditions of high sperm concentrations naturally, further analyses should evaluate fertilization data using the polyspermy-adjusted models developed by Styan (1998) to take into account multiple sperm-egg contacts.

Table 2.2 Some estimates of the concentration of sperm required to fertilize 50% of conspecific and heterospecific eggs (F_{50}) in echinoderm species.

Maternal species	Conspecific F_{50} (sperm·μL^{-1})	Heterospecific F_{50} (sperm·μL^{-1})	~F_{50} ratio	Model to estimate F_{50}	Reference
Strongylocentrotus droebachiensis	10^1	3.2 x 10^2	30	Vogel *et al.* (1982)	Levitan 2002
S. franciscanus	10^2	2.5 x 10^6	10^4		
S. purpuratus	10^2	2 x 10^7	10^5		
Echinometra lucunter	94	7 x 10^3 (*E. viridis*)	100	Linear regression & Vogel *et al.* (1982)	McCartney & Lessios 2002
		1.6 x 10^5 (*E. vanbrunti*)	10^4		
E. viridis	62	1.1 x 10^3 (*E. vanbrunti*)	20		
		1.4 x 10^2 (*E. lucunter*)	30		
E. vanbrunti	83	2 x 10^2 (*E. lucunter*)	2		
		81 (*E. viridis*)	1		
Pseudechinus huttoni	50	30 (*P. albocinctus*)	>	Data modeled using 4-parameter logistic equation (Fig. 3)	McClary & Sewell 2003
		30 (*P. novaezealandiae*)	>		
P. albocinctus	80	10 (*P. huttoni*)	>		
		10^3 (*P. novaezealandiae*)	10		
P. novaezealandiae	10^3	> 10^5 (*P. huttoni*)	100		
		> 10^5 (*P. albocinctus*)	100		
Asterias forbesi	72	228 (*A. rubens*)	3	Vogel *et al.* (1982)	This study
A. rubens	25	238 (*A. forbesi*)	10		

Fertilization is typically insensitive to egg concentration (Levitan *et al.* 1991). Fertilization specificity was not dependent on egg concentration for *A. forbesi* eggs. The fertilization of *A. rubens* eggs appears to have been affected by egg concentration, declining at high concentrations of *A. forbesi* sperm. At low egg concentrations, heterospecific fertilization of *A. rubens* eggs was generally high, but it is difficult to interpret fertilization at higher egg concentrations since there were few instances when this occurred.

Gamete lifespan is not likely to be a major factor limiting fertilization success since dilution of the gametes probably reduces effective sperm concentrations below the F_{50} concentration within the first few minutes after spawning, long before gamete viability is affected by age (Levitan *et al.* 1991, Levitan and Young 1995, but see Yund 2000). However, asynchronous spawning (either conspecific or heterospecific) in which gamete encounters occur minutes to hours after the initial spawning event may result in successful fertilization. A reduced respiratory effect (loss of fertilizing capacity of sperm with dilution, Chia and Bickell 1983) and relatively slow aging of sperm may allow gametes to remain competent for a longer time at more dilute concentrations (Benzie and Dixon 1994). In my study, gametes of both *A. forbesi* and *A. rubens* were viable for up to 12 h, but fertilization success of gametes began to decline after 3 h. In a previous study of *A. rubens*, Williams and Bentley (2002) found fertilization success was 100% for the first 4 h after spawning, but decreased to zero by 24 h, and reported that diluted sperm (10^2 sperm μL^{-1}) remained viable for 24 h at 10°C.

Postzygotic barriers to hybridization do not appear to be strong in *A. rubens* and *A. forbesi*. Hybrid offspring of both heterospecific crosses were viable and fertile, and successfully backcrossed with parental species when they reached sexual maturity.

Growth and development of the hybrids in culture was comparable to field studies of *A. rubens* in the northeast Atlantic in which juveniles reached an average diameter of 28.5 mm in the first year, and became sexually mature in their second year at about 50 mm diameter (Nichols and Barker 1984). Hybrid breakdown remains one potential postzygotic barrier between these species, as fertilization success in backcrosses was less than 50% at relatively high sperm concentrations of 200 sperm μL^{-1} that typically produce 80 - 100% fertilization. In addition, hybrid fitness may be reduced relative to conspecific crosses under natural conditions.

Complete gamete compatibility has been reported in several sympatric echinoderm species. Ecological or habitat segregation and temporal separation in breeding have been suggested as reproductive isolating mechanisms in the sea stars *Patiriella calcar* and *P. exigua*, which are reciprocally compatible and produce viable hybrid juveniles in lab studies (Byrne and Anderson 1994). Ecological or habitat differences may reduce gene flow in two sympatric species of the sea urchin *Pseudoechinus*: *P. huttoni* and *P. albocinctus* have reciprocally compatible gametes and hybrids can be reared to sexual maturity and backcrossed in the laboratory (McClary and Sewell 2003). Gametes of the sympatric sea urchins *Diadema setosum* and *D. savigni* are reciprocally compatible (Uehara *et al.* 1990) and natural hybrids have been found using genetic analyses of field-collected animals (Lessios and Pearse 1996), but levels of introgression are low with little interspecific gene flow.

Sperm-egg contact time has been shown to be an important factor influencing fertilization success (Levitan *et al.* 1991). A study examining shorter, ecologically relevant gamete contact times (e.g. 1 – 3 min) may be more sensitive to differences in fertilization success among conspecific and heterospecific gametes (e.g. Pernet 1999). As

well, this study was not designed to detect the effects of sperm competition on fertilization success (review in Howard 1999). Although heterospecific interactions may produce high fertilization rates in the absence of conspecific sperm, this may not be representative of what actually occurs under natural conditions. There may some mechanism of preferential fertilization of conspecific gametes in *Asterias* spp., similar to the gamete recognition protein systems in sea urchins and abalone (review in Palumbi 1994).

Depending upon the compatibility of heterospecific gametes under natural condition, the potential for hybridization of sympatric *Asterias rubens* and *A. forbesi* in the secondary contact zone appears to be high: these species have overlapping reproductive seasons, adults co-occur in the same microhabitats, and both heterospecific crosses show gamete compatibility under a variety of laboratory conditions. Postzygotic barriers appear weak as hybrid offspring are viable and fertile.

CHAPTER THREE:

MORPHOLOGICAL ANALYSIS OF *ASTERIAS FORBESI* AND *A. RUBENS*

Introduction

Evidence of hybridization in marine organisms is often limited to the documentation of morphological variation outside the normal range of phenotypic variation within species (reviewed by Gardner 1997). Hybrids can have morphological characters with character states that are intermediate between the two parental character states (blending inheritance), or can display a set of parental character states from both parents (particulate inheritance of unlinked characters) (Dobzhansky 1937, Mayr 1942, Campton 1987).

Sympatric populations of *Asterias forbesi* and *A. rubens* may hybridize in the northwest Atlantic, but the analysis and interpretation of putative hybrid specimens has been highly variable among previous studies. The morphological similarities between the sibling species have been well described (Verrill 1866, Coe 1912, Aldrich 1956, Downey 1973). One important consequence of these similarities has been disagreement among experts over the identification and frequency of putative hybrids. Some studies included anecdotal accounts of morphological intermediates that were believed to be hybrids (Clark 1904, Perlmutter and Nigrelli 1960, Ernst 1967, Walker 1973). Menge (1986) estimated 1.4% of 295 *Asterias* spp. from Boston Harbor, MA, were morphological intermediates, but did not report which characters were variable. According to Clark and Downey (1992), hybrids were frequently found from Cape Cod to Maine, but were not believed to reach sexual maturity. In contrast, in an extensive survey of skeletal

characters, Worley and Franz (1983) concluded that hybrids were not present and assigned thousands of specimens to one or the other species. They suggested that coastal populations of *forbesi*-like animals from Maine were morphological variants of *A. rubens* or relict populations of *A. forbesi*. The latter hypothesis is plausible in light of the expected oscillations in latitudinal distribution of shallow-water marine organisms in the north Atlantic associated with cyclical Pleistocene climate change (Wares and Cunningham 2001).

A second important consequence of close morphological similarity between *A. rubens* and *A. forbesi* is that few morphological characters have been identified that show diagnostic character state differences between the two species. Some experts consider the shape of the major pedicellariae at the base of the adambulacral spines to be the single most reliable character for species identification (Coe 1912, Aldrich 1956). However, other experts suggest multiple characters are required for identification of species (e.g., Schopf and Murphy 1973). Worley and Franz (1983) used four morphological characters considered to be diagnostic (Coe 1912, Aldrich 1956, Gray *et al.* 1968): the shape of the arms, the size and shape of the madreporite (the external opening to the internal water vascular system), the size and shape of the major pedicellariae, and the structure of the single skeletal ossicles (calcium carbonate crystals articulated with each other in the body wall). The taxonomically informative characters were the size or shape of ventral pedicellariae, inner oral spines, inner and outer adambulacral spines, ossicles, and madreporite. Of these characters, Clark and Downey (1992) included only the shape of the major pedicellariae and body rigidity (associated with ossicle shape) in their key to the Atlantic species of *Asterias*. The colour of the madreporite (Coe 1912, Aldrich 1956, Gray *et al.* 1968), the location of the wreath of minor pedicellariae on the abactinal

spines, and the shape of the abactinal spines were also included in the key as these characters were strongly correlated for more than 85% of *Asterias* collected south of Cape Cod (Clark and Downey 1992).

To determine whether morphological intermediates exist between *A. forbesi* and *A. rubens* and to estimate their frequency, I conducted an extensive survey of the two species in sympatry and allopatry. Specimens were examined and scored for the five qualitative characters (Clark and Downey1992) and three morphometric characters (Worley and Franz 1983). Principal components analysis (PCA) and cluster algorithms were performed on the character scores to determine whether a significant cluster of morphological intermediates was quantitatively supported. This approach provides an objective method for identifying morphological intermediates that are possible hybrids between the two parental phenotypes.

Materials and methods

Asterias samples were obtained from the locations indicated in Table 3.1. Sample sites were selected to cover the range of the species' distributions and to sample extensively within the zone of sympatry from the Gulf of Maine to Nova Scotia. Field collected samples were obtained from depths of 3 – 10 m using SCUBA, with the exception of specimens from Brier Island, NS and Grand Manan, NB, which were collected by dredging conducted by the Department of Fisheries and Oceans. Where possible, a range of different animal sizes was sampled. The two F_1 hybrids raised in culture (Chapter Two) were also included in the analysis. Live animals were scored for two morphological characters that are lost in preservation (colour of madreporite and body rigidity), and then stored in 95% ethanol. Samples on loan from the collections of C.W. Cunningham and the Smithsonian Institution (National Museum of Natural History), as well as the Brier Island samples, were received preserved, and therefore these two character states were scored as unknown.

Morphological traits

Each animal was scored for five qualitative morphological characters, as described in Clark and Downey (1992). The colour of the madreporite, the body rigidity, the shape of the major pedicellariae on the adambulacral spines (referring to Fig. 8 in Coe 1912), the location of the wreath of minor, crossed pedicellariae on the abactinal spines and the shape of the abactinal spines were examined using a stereoscopic zoom microscope (Nikon SMZ1500).

Table 3.1 Sample collection sites and depths. Specimens are preserved in ethanol and, except where noted, have been deposited in the collection of the Nova Scotia Museum of Natural History (NSMNH). Accession numbers for NSMNH samples, samples in the collection of C.W. Cunningham (C.W.C.) at Duke University, and samples in the collection of the Smithsonian Institution, National Museum of Natural History (USNM) are available from the author.

Species & Location	Population	Latitude & Longitude	Depth (m)	Sample size	Collection
A. rubens NE Atlantic	United Kingdom	unknown	10 - 20	2	USNM
	Ireland	53° N 10°E	intertidal	9	C.W.C.
	Norway	64° N 10°E	intertidal	20	C.W.C.
	Iceland	64° N 22°E	intertidal	3	C.W.C.
	France	48° N 3°E	intertidal	5	C.W.C.
	Faroe Islands	62° N 7°W	intertidal	4	C.W.C.
A. rubens NW Atlantic	Bonne Bay, NF	49°31'N 57°33'W	10	94	NSMNH
	Havre-St-Pierre, QC	50°14'N 63°36'W	10	95	NSMNH
A. rubens & *A. forbesi* NW Atlantic	Savage Harbour, PEI	46°42'N 62°85'W	5 - 8	41	NSMNH
	Bras d'Or Lake, NS	45°83'N 60°83'W	10	13	NSMNH
	Bear Cove, NS	44°32'N 63°33'W	3 - 10	219	NSMNH
	Brier Island, NS	44°04'N 66°25'W	67	42	NSMNH
	Grand Manan, NB	44°32'N 63°33'W	26 - 44	95	NSMNH
	Isle of Shoals, ME	42°59'N 70°36'W	3 - 10	193	NSMNH
A. forbesi NW Atlantic	North Carolina	33°31'N 77°24'W	unknown	5	USNM
	South Carolina	32°30' N 79°42'W	unknown	13	USNM
	Florida	26°N 80°W	unknown	4	USNM

For specimens lacking major pedicellariae on the adambulacral spines, I used pedicellariae located on the aboral surface around the perimeter of the madreporite. Character states associated with *A. forbesi* received a score of 1, character states identified as *A. rubens* scored 3 (Table 3.2). Intermediate scores of 2 were given when a character appeared intermediate between the two species: the pedicellariae were broad at the base, but pointed at the tips; pedicellariae were found to form wreaths halfway up and at the base on different spines in the same specimen; abactinal spines were short and slender or long and tuberculate. Body rigidity and the colour of the madreporite were the only two diagnostic characters that did not ever appear intermediate between the species.

Three quantitative traits were measured for each specimen. The distance from the tip of the longest arm to the opposite interradius (*R*), the width of the longest arm at the base (*a*), and the width of the longest arm 1 cm from the tip (*b*) were measured using vernier calipers to the nearest 0.1 mm. Where the longest arm measured less than 1 cm, *b* was designated equal to *a*. The ratio *a/b* is a measure of the shape of the arms (Aldrich 1956) and is associated with differences in skeletal structure between *Asterias* species (Worley and Franz 1983).

Morphological analyses of hybrid zones should be consistent with formal taxonomic designations, robust with respect to the geographic distributions of the potentially hybridizing species, and repeatable across multiple examinations of the same specimens. Unfortunately, the type localities for *A. forbesi* and *A. rubens* are unknown (Clark and Downey 1992). Specimens collected from outside the hybrid zone were examined first to establish the range of character states found among individuals known not to include hybrids. Randomly selected samples were repeatedly examined on different days to ensure consistent scoring of characters.

Table 3.2 Diagnostic adult morphological characteristics for *Asterias forbesi* and *A. rubens* (based on Coe 1912, Clark and Downey 1992)

Character	*Character states (score)*		
Body rigidity	rigid[a] (1)		flaccid[b] (3)
Colour of madreporite	orange – red[a] (1)		pale yellow[b] (3)
Major pedicellariae on adambulacral spines	broad, round[a] (1)	broad at base, slender at tip (2)	slender, pointed[b] (3)
Wreath of pedicellariae around abactinal spines	base of spine[a] (1)	base of some spines, middle of others (2)	midway up spine[b] (3)
Shape of abactinal spines	short, tubercle-like[a] (1)	short and slender or long and tubercle (2)	long, slender[b] (3)

[a] Character states associated with *A. forbesi*
[b] Character states associated with *A. rubens*

Statistical analyses of morphological traits

I used principal components analysis (PCA) of eight characters and 859 seastars (Systat 9.0) to identify compound vectors of character states that explained a large proportion of the phenotypic variation among specimens, and to identify groups of specimens that differed in scores along a few of these compound character axes. PCA is more appropriate than discriminant-function analysis for the exploratory analysis of relationships among character states within and between species and hybrids (Bert *et al.* 1996) because it does not require *a priori* definition of the groups. The PCA was based on a correlation matrix using pairwise deletion for missing values. A covariance matrix was not used because the characters differed in units of measurement (James and McCulloch 1990). Components with a minimum eigenvalue of 0.8 were retained.

I used non-hierarchical cluster analyses with Euclidean distances and the iterative *k*-means algorithm (Systat 9.0) to further explore the clustering of specimens into groups that could be potentially identified as *Asterias* species and their hybrids. Individual character state scores were normalized to values between 0 and 1 by subtracting the minimum value and dividing by the range. Cluster analyses were performed for $k = 2$ and $k = 3$ groups to describe the fit of the data to a clustering algorithm that assumes just two groups (corresponding to *Asterias* species without hybrids) or three groups (including *Asterias* species and an intermediate hybrid cluster). I then compared the fit of the data to the two- or three-cluster algorithm using the Variance Ratio Criterion (VRC; Calinski and Harabasz 1974), analogous to the *F*-statistic in a univariate analysis.

The VRC was calculated as

$$VRC = (SS_{BG}/k\text{-}1)/(SS_{WG}/n\text{-}k),$$

where SS_{BG} is the sum of squares between groups, SS_{WG} is the sum of squares within groups, k is the number of clusters, and n is the number of specimens (n = 859). The best k is selected based on the VRC which has the first local maximum value.

I also compared the fit of the data to the clusters using the overall mean square ratio due to k-partition, a measure of the reduction of within-cluster variance associated with fitting the data to a large number of clusters (Hartigan 1975). The overall mean square ratio due to k-means partitioning was calculated as

$$F = (SS_k/SS_{k+1} - 1)(m - k + 1),$$

where SS_k is the within cluster sum of squares, k is the number of clusters, and m is the number of characters (m = 8). According to Hartigan (1975), the F-distribution is not correct for evaluating k-means because each variable influences the partition and instead he recommends that large values of this ratio (> 10) justify increasing the clusters from k to $k + 1$.

Results

PCA reduced the five qualitative and three quantitative characters to three principal components (PC) with a minimum eigenvalue of 0.8 or greater (Table 3.3). Scores on the first component were most heavily weighted by four of the five qualitative characters: body rigidity, colour of madreporite, pedicellariae shape, and the location of the wreath of pedicellariae on the abactinal spines. The second principal component was described by the three quantitative characters which had high positive loadings. The third component was described by the shape of the abactinal spines. Spine shape was the most difficult character to score. Many specimens had intermediate spine shapes that were short and slender or long and tuberculate. Clark and Downey (1992) are the only authors to describe this trait as diagnostic.

Asterias collected across the north Atlantic were separated into two groups corresponding with the taxonomic species designations. This result is evident in the bivariate plot of first and second principal component scores from the PCA (Fig. 3.1): the group with the low scores on the first PC corresponds to *A. forbesi*; the group with the higher scores on the first PC is *A. rubens*. *Asterias* from allopatric populations were clearly separated, while specimens from sympatric populations in the NW Atlantic were found in both groups and in a smudge between the groups that could be morphological intermediates. Non-hierarchical cluster analyses using $k = 2$ and $k = 3$ were performed to test the plausibility of a third, intermediate group (Fig. 3.2). When specimens were clustered into two groups, VRC = 1166.94; for three groups, VRC = 747.48. Calinski and Harabasz (1974) suggest that the optimal clustering is at the first local maximum of the VRC with respect to k. The overall mean square ratio due to k-partition for the $k = 2$

analysis was F = 1.141. According to Hartigan (1975), values of this ratio > 10 justify increasing the number of clusters from k to $k + 1$. By this criterion, grouping the specimens into three clusters rather than two does not considerably reduce the within-group sum of squares (Fig. 3.3). Based on these analyses, it is more appropriate to group *Asterias* into two clusters rather than three.

In the three-cluster analysis, 84 individuals were assigned to the third, intermediate cluster. Of these, 82 individuals were assigned to *A. rubens* in the two-cluster analysis. In each of three sites within the areas of sympatry, 15% of the individuals grouped within the intermediate cluster (n = 33 from Bear Cove, 30 from Isle of Shoals, 6 from Brier Island, Fig. 3.4). Eight of the thirteen specimens examined from Bras d'Or Lake, NS, grouped within the intermediate cluster, however characters of all the specimens were very difficult to score. The specimens were physically small (mean R = 30.2 mm, ± 13.2 s.d.) and of the thirteen collected, I could locate major pedicellariae in only three. One specimen also lacked the wreath of minor pedicellariae around the spines. Animals less than 40 mm in diameter are considered too small to distinguish characters (D'yakonov 1968). Two *Asterias* spp. from Grand Manan, one from Quebec and four from Newfoundland were also in the intermediate cluster in the $k = 3$ analysis.

Table 3.3 Principle components, eigenvalues, component loadings and amount of total variance explained in a principal components analysis on a correlation matrix of five qualitative and three quantitative morphological traits scored for *Asterias* spp. samples in Table 3.1. The component loadings with the most weight in each PC are indicated in bold.

Component	1	2	3
Eigenvalue	3.599	2.317	0.909
Component loadings			
R	0.507	**0.765**	-0.087
a	0.455	**0.828**	0.055
b	0.142	**0.858**	0.260
Madreporite colour	**0.912**	-0.300	0.104
Body rigidity	**0.875**	-0.306	0.107
Pedicellariae shape	**0.908**	-0.283	0.109
Pedicellariae wreath	**0.717**	-0.188	0.094
Abactinal spines	0.422	0.102	**-0.887**
% Total Variance	44.982	28.959	11.362

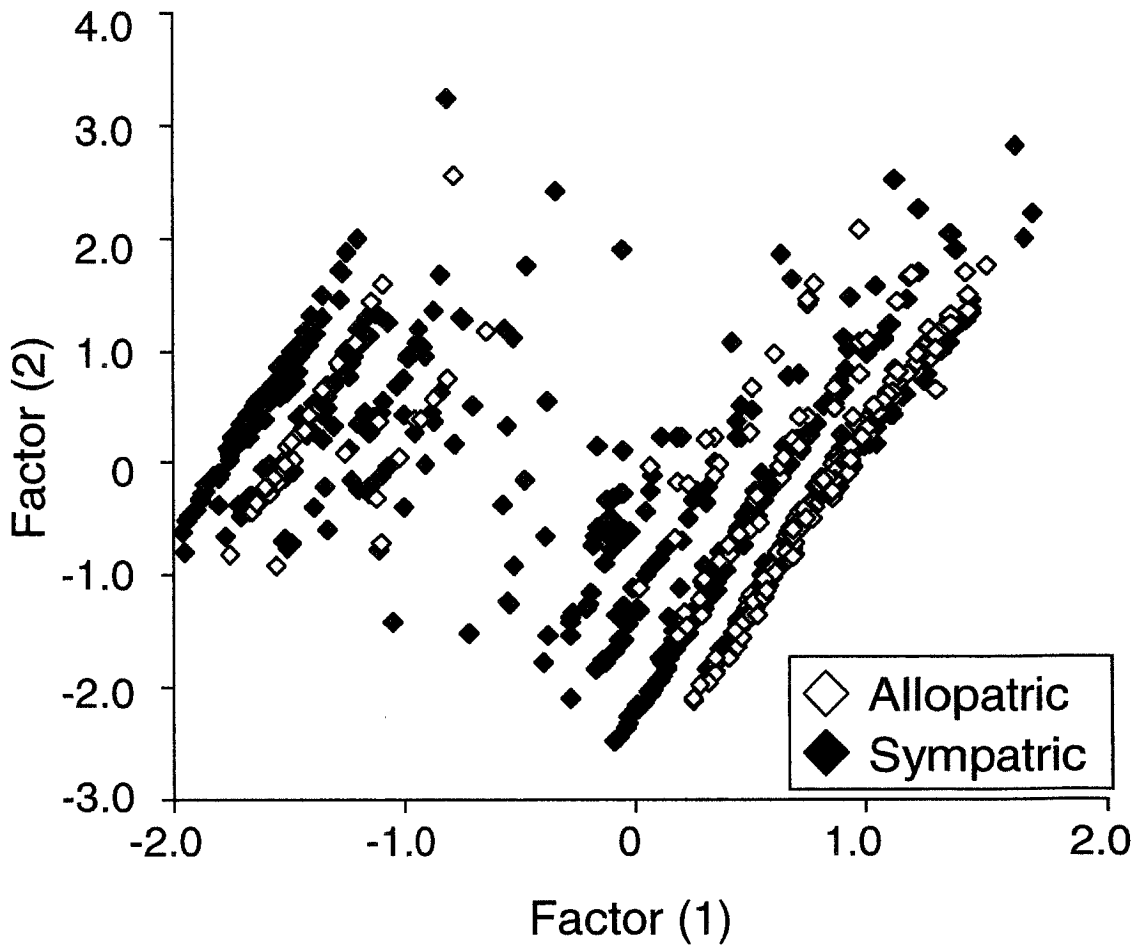

Figure 3.1 Bivariate plot of principal components (PC) for sympatric and allopatric *Asterias* spp. in the north Atlantic (total n = 859). Factor scores generated from a PC analysis on a correlation matrix of 5 morphological and 3 morphometric characters. Samples with low PC1 scores are *A. forbesi* and samples with high PC1 scores are *A. rubens.*

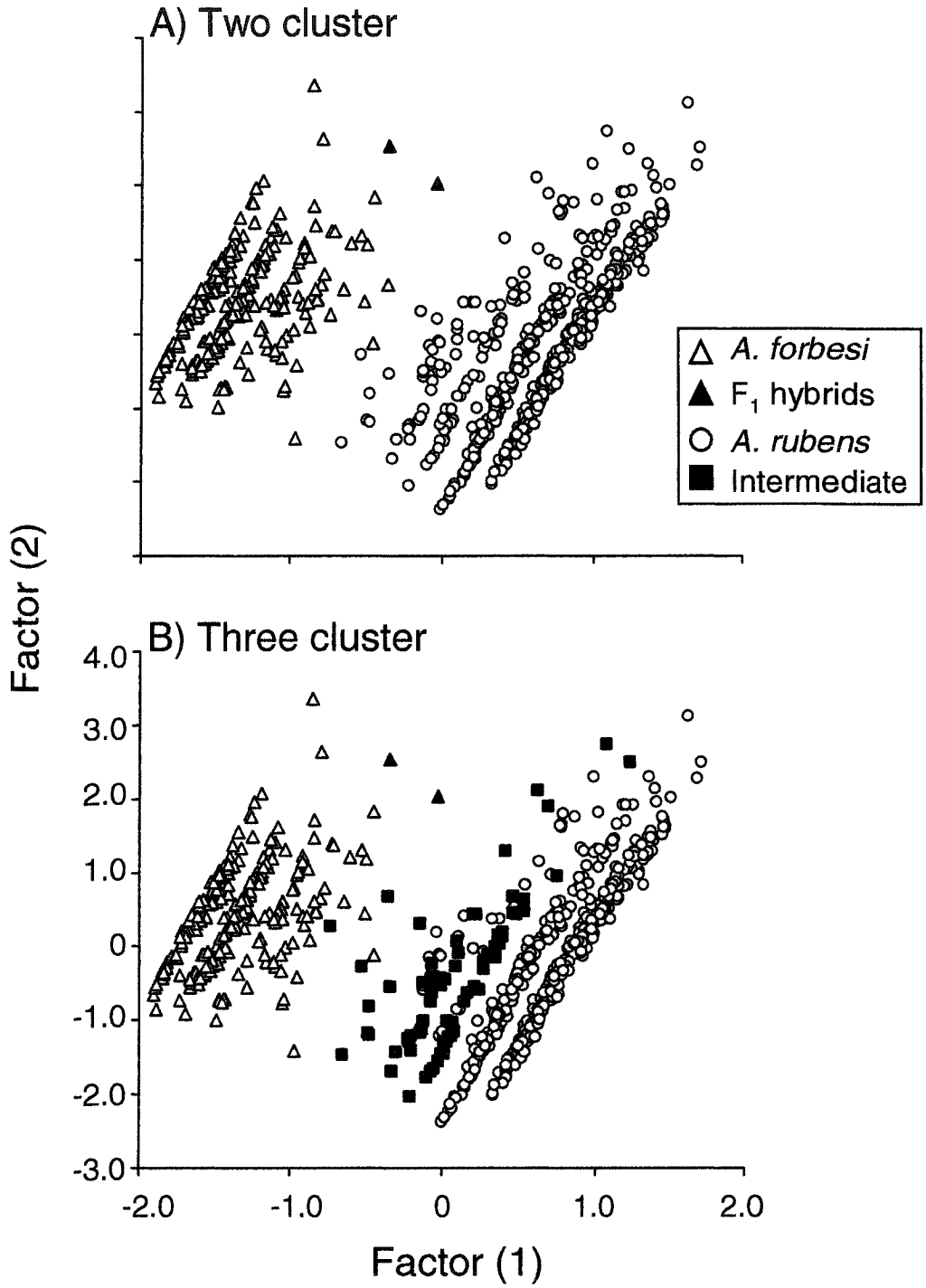

Figure 3.2 Bivariate plots of PC scores showing results of non-hierarchical cluster analyses using Euclidean distances on standardized data. (A) Analysis of $k = 2$ clusters, (B) Analysis of $k = 3$ clusters.

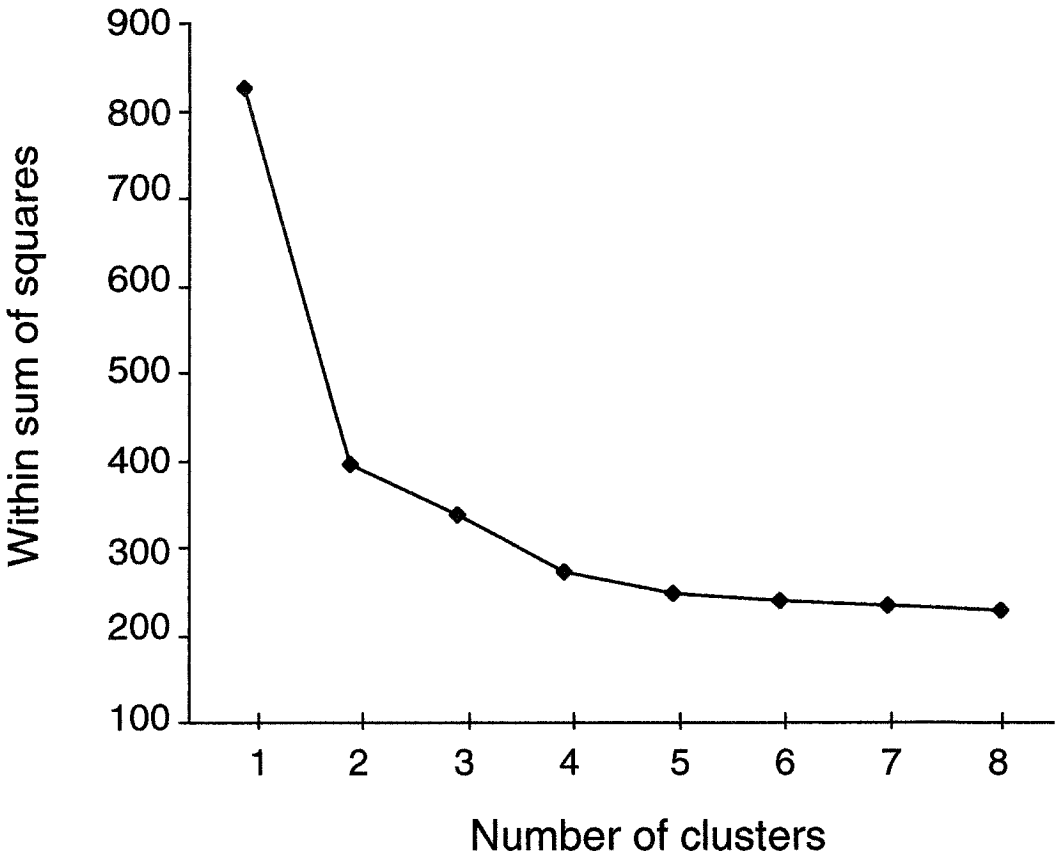

Figure 3.3 Scree diagram of calculated within sum of squares for non-hierarchical cluster analyses of different *k*-means values as in Fig. 3.2.

The two F_1 hybrids raised in culture (Chapter Two) grouped in the *A. forbesi* cluster in both the $k = 2$ and $k = 3$ analyses. In the PC plots (Fig. 3.2), both animals were in the smudge of individuals intermediate to the main *A. forbesi* and *A. rubens* clusters, but did not form part of the third, intermediate cluster. Both specimens had character states for the body rigidity and the madreporite colour characteristic of *A. forbesi* and pedicellariae wreath location and shape of the abactinal spines characteristic of *A. rubens*. The only morphological difference between the two F_1 hybrids was the shape of the pedicellariae which was diagnostic of *A. forbesi* in the RF hybrid and had an intermediate character state in the RF hybrid.

As expected, samples from outside the putative hybrid zone in Quebec, Newfoundland and Europe were comprised entirely of *A. rubens*, while populations sampled south of Cape Hatteras were entirely *A. forbesi*. Although geographically within the range of species overlap, samples collected from Grand Manan, NB, were all *A. rubens*, perhaps because the collections were from depths (67 m) that are not frequented by *A. forbesi* at that latitude (Franz *et al.* 1981). Samples collected from Savage Harbour, PEI, were expected to be *A. rubens,* as *A. forbesi* has not been previously reported in the Gulf of St. Lawrence, however all specimens from this site were assigned to *A. forbesi*.

Different types of morphological intermediates were observed in the survey. Specimens were found with character states intermediate between the two parental character states (n = 85), a set of discrete parental character states from both parents (n = 313), and both intermediate character states and mixed parental character sets (n = 54, Fig. 3.5A). Of the five diagnostic characters described by Clark and Downey (1992), three were found to have intermediate character states (pedicellariae shape, location of pedicellariae wreath, shape of abactinal spines). The character that was most frequently

found with an intermediate character states was the location of the wreath of minor pedicellariae on the spines (Fig. 3.5B). When specimens with intermediate phenotypes were comprised of mixed parental characters, frequently four of the qualitative traits were diagnostic of one parent and the shape of the abactinal spines was diagnostic of the other parent (n = 190, Fig. 3.5C).

In total, 452 specimens exhibited some set of characteristics (either intermediate states, mixed parental characters, or both) that did not fit the taxonomic definition of either *A. rubens* or *A. forbesi*. Of these potential hybrids, the shape of the abactinal spines was the character implicated in most of the specimens, either found with an intermediate character state or as the inconsistent character (n = 244). Removal of this character from the PCA reduced the analysis to two PCs (Table 3.4), and reduced the number of individuals assigned to the third, intermediate cluster from 84 to 25 individuals in the three-cluster analysis (Fig. 3.6; note that only 20 individuals are indicated in this plot, 5 individuals had incomplete data sets and could not be assigned PC co-ordinates). Of these 25 individuals, 11 were specimens from Bear Cove, 8 were from the Isle of Shoals, 5 were from Bras d'Or Lake, and one was from Prince Edward Island. In contrast with the three-cluster analysis using all five qualitative characters (82 *rubens*-like and two *forbesi*-like specimens in the intermediate cluster), of the 25 individuals assigned to the third, intermediate cluster in the analysis without the abactinal spines, 16 were *forbesi*-like and 9 were *rubens*-like in the two-cluster analysis. However, the overall conclusion of separation of the scores into two, rather than three, clusters did not change with removal of the abactinal spines. When specimens were clustered into two groups, VRC = 2172.91; for three groups, VRC = 1167.14. The overall mean square ratio due to *k*-partition for the two-cluster analysis was F = 0.325.

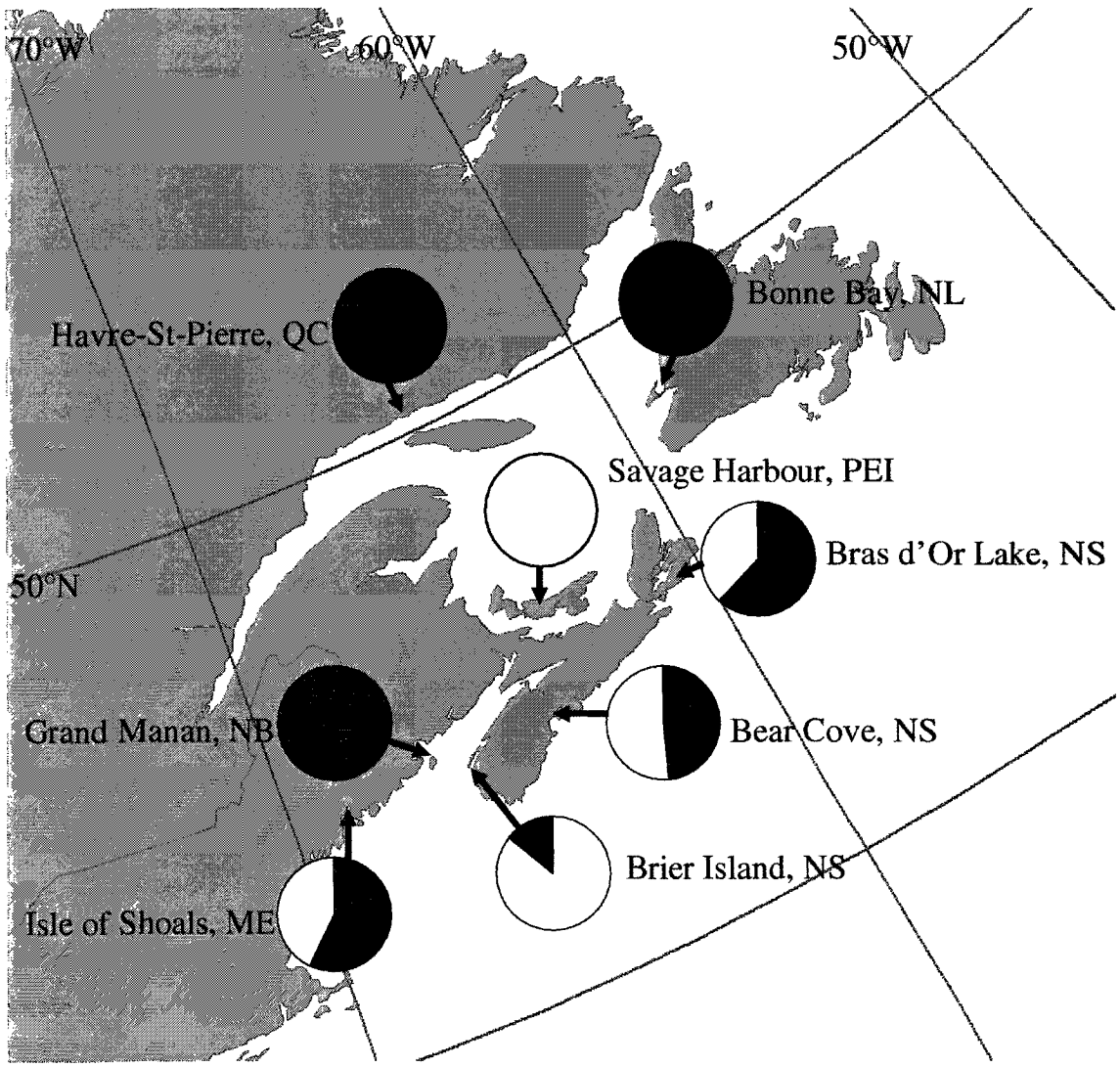

Figure 3.4 Map of the northwest Atlantic showing the relative frequency of *Asterias forbesi* (white) and *A. rubens* (black) at eight sampling sites, determined using principal components analysis of five qualitative and three quantitative characters and $k = 2$ cluster analysis. European samples are all *A. rubens*, samples south of Cape Cod are all *A. forbesi*.

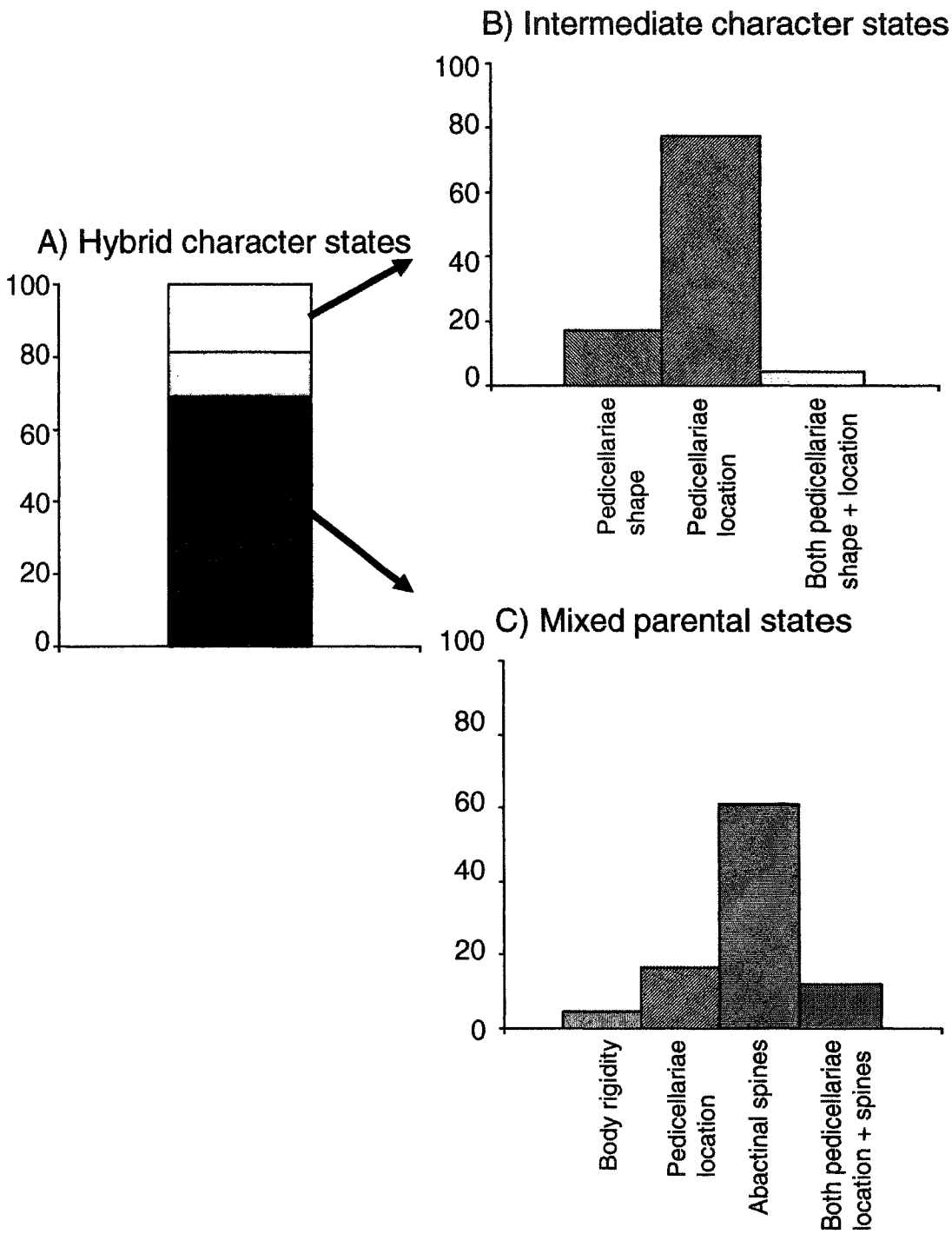

Figure 3.5 Frequency distributions of different types of intermediate morphologies. A) Intermediate character states only (white); mixed parental characters only (black); both intermediate and mixed parental characters (grey). B) Frequency of different characters with intermediate states. C) Frequency of different characters involved in mixed parental type morphologies. Characters (or combinations of characters) with frequencies <5% are not indicated in plots, character descriptions as in Table 3.1.

Table 3.4 Principle components, eigenvalues, component loadings and amount of total variance explained in a principal components analysis on a correlation matrix of four qualitative (no abactinal spines) and three quantitative morphological traits scored for 859 *Asterias* spp. specimens. The component loadings with the most weight in each PC are indicated in bold.

Component	1	2
Eigenvalue	3.464	2.310
Component loadings		
R	0.477	**-0.774**
a	0.436	**-0.840**
b	0.144	**-0.870**
Madreporite colour	**0.925**	0.275
Body rigidity	**0.890**	0.281
Pedicellariae shape	**0.921**	0.257
Pedicellariae wreath	**0.727**	0.167
% Total Variance	49.488	32.993

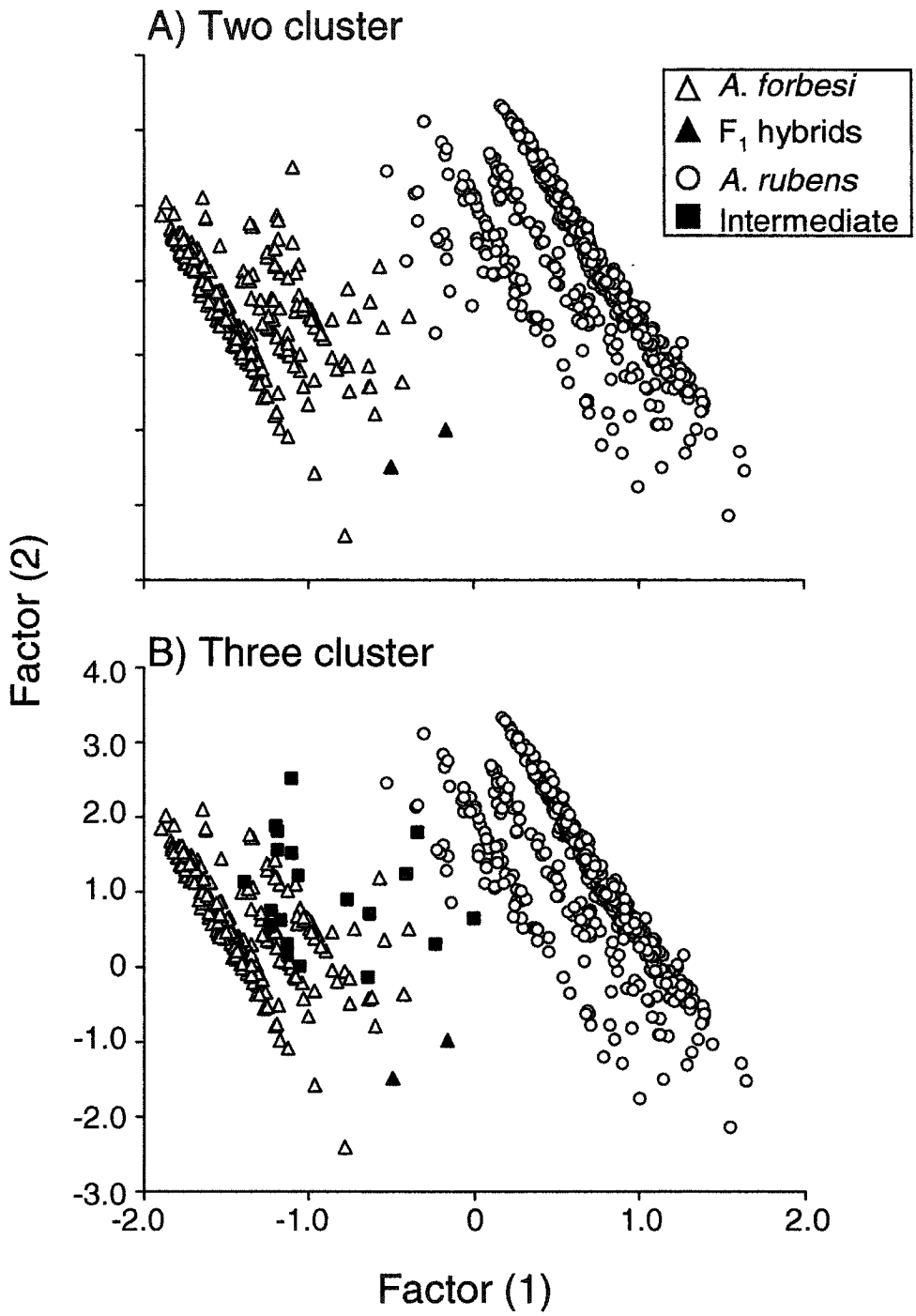

Figure 3.6 Bivariate plots of PC scores showing results of non-hierarchical cluster analyses when the shape of the abactinal spines is removed. Analyses performed using Euclidean distances on standardized data. (A) Analysis of $k = 2$ clusters, (B) Analysis of $k = 3$ clusters.

Discussion

Morphological analyses of sympatric and allopatric populations of *Asterias forbesi* and *A. rubens* do not provide much evidence for a distinct group of intermediate phenotypes that might be F_1 hybrids (or the recent descendants of hybrids). The two animals known to be F_1 hybrids did not form part of the third intermediate cluster. Cluster analyses of PCA scores indicate separation of the specimens into two clusters, corresponding with the taxonomic species. I found no quantitative statistical support for the existence of a third cluster, in spite of the qualitative suggestion of some samples in the morphospace between the major clusters.

In other examinations of hybrid zones using multivariate statistical analyses of morphometric variation, the existence of morphological hybrids is clearly evident, either as overlapping clusters indicative of many intermediate stages between parental types (e.g., *Mytilus edulis* species complex in southwest England, Gardner 1996), or as a discrete, third cluster (e.g., seerfish populations in India, Srinivasa Rao and Lakshmi 1993, as reviewed in Gardner 1997). In my analysis of *Asterias* populations there was no third cluster distinct from the two parental-type clusters, nor was there the overlap of a hybrid cluster with every other cluster as seen in *Mytilus* (Fig. 8, Gardner 1996). As well, a large sample within the putative hybrid zone (Grand Manan) and a large sample supposedly outside the hybrid zone (P.E.I.) were unambiguously assigned to *A. rubens* and *A. forbesi*, respectively.

Depth and temperature tolerance are ecological differences known to separate *A. rubens* and *A. forbesi* (Feder and Christensen 1966, Schopf and Murphy 1973, Franz *et al.* 1981). These differences could account for the absence of *A. forbesi* near Grand Manan,

NB, (26 – 44 m depth) as all 95 specimens from this site were unambiguously assigned to *A. rubens*, a species more tolerant of the cold temperatures at depth (Franz *et al.* 1981). However, differences in thermal tolerance cannot account for the population sampled off Brier Island, NS (67 m depth) as *A. forbesi* was the predominant phenotype found there.

The discovery of *A. forbesi* off the northern coast of P.E.I. in the Gulf of St. Lawrence was unexpected. The northern range limit for *A. forbesi* has previously been reported as Cape Breton, with rare occurrences in the Gulf of St. Lawrence (Towle 1982). As such, this is an area supposedly outside the hybrid zone, however all 41 specimens were unambiguously assigned to *A. forbesi*. Whether my result is indicative of natural northward expansion of this species' geographic range or of anthropogenic introduction is unknown. The congeneric northern Pacific species, *A. amurensis*, has been introduced to Tasmanian waters (Turner 1992), possibly through ballast water discharged from ocean-going vessels from Japan (Ward and Andrew 1995). As generalist omnivores with a long planktonic larval phase, *Asterias* spp. may be excellent candidates for human-mediated introductions and invasions. The relative abundance of *A. forbesi* has increased significantly in the Gulf of Maine in the past three decades (Harris *et al.* 1998).

Within the zone of sympatry, many specimens were found with character states intermediate between the two parental character states, or a set of parental character states from both parents, or both. Three of the five qualitative diagnostic characters were found with character states intermediate between the two parental character states, most frequently the pedicellariae shape and the location of the wreath of pedicellariae on the spines. Intermediate character states were not found for body rigidity and madreporite colour.

When individuals displayed a mixed set of character states from both parents, typically the shape of the abactinal spines was the single character whose state conflicted with all other characters. When specimens were grouped into three clusters, this character defined most of the third, intermediate cluster. Removing this character from the analysis reduced the intermediate cluster from 84 to 19 individuals. I found the shape of the abactinal spines to be highly subjective and difficult to score. Of all previous attempts to diagnose these two species from morphological characters, only Clark and Downey (1992) refer to abactinal spine shape as a diagnostic character. Although Clark and Downey report the five qualitative traits in their key are highly correlated in 85% of *Asterias* spp. from south of Cape Cod, the shape of the abactinal spine was not a good character for identification of *Asterias* spp. collected in sympatry in my study.

Studies of morphological variation in hybrid zones often are able to utilize many traits, both morphometric and qualitative, to discriminate species and detect morphological intermediates that may be hybrids (e.g. Dillon and Manzi 1989, Bert *et al.* 1996). The relatively few traits which discriminate between Atlantic *Asterias* spp. may not be sufficient for detection of morphological intermediates. Although animals known to be F_1 hybrids were part of a smudge of specimens intermediate between the two groups of *Asterias*, they did not form part of the third, intermediate cluster in the $k = 3$ cluster analysis and could not be quantitatively identified as hybrids using morphological characters.

Despite the potential for hybridization between the species suggested by the apparent lack of reproductive isolation barriers (Chapter Two) and reports of morphological intermediate in some field samples (Menge 1986, Clark and Downey 1992), this morphological analysis of sympatric and allopatric *Asterias* does not suggest

that morphological hybrids are common in nature. A phylogenetic and population genetic analysis of DNA sequences from *A. forbesi* and *A. rubens* found no evidence of natural hybridization between the species, including three sample sites within the zone of sympatry (Wares 2001). In contrast to my survey, Wares (2001) reported sampling only *A. forbesi* in Cape Cod, and only *A. rubens* in Maine and Nova Scotia, areas where I have found both species in abundance. In Chapter Four, I present results from a survey of mtDNA sequence variation among individuals that were identified in this morphological survey as *A. rubens, A. forbesi*, and possible morphological intermediates. I use the results of this genetic survey to look for evidence of hybridization and introgression in sympatric *Asterias* populations.

CHAPTER FOUR:

PHYLOGENETIC ANALYSIS OF *ASTERIAS FORBESI* AND *A. RUBENS*

Introduction

Many examples of hybridization in marine invertebrates have been described based on the observation of morphologically intermediate specimens (review in Gardner 1997). However, hybridization can be difficult to detect using morphology alone; some heterospecific crosses produce offspring that more closely resemble the phenotype of one parental species (Lamb and Avise 1987, Byrne and Anderson 1994). Studies of variation in morphological traits alone may limit the description of hybrid zones (Lamb and Avise 1987), inclusion of genetically independent traits such as nuclear and mtDNA markers in the analysis of hybrid zones are necessary for accurate interpretation of hybrid zone interactions and detection of introgression (DePamphilis and Wyatt 1990, Paige and Capman 1993, Bert *et al.* 1996).

Natural hybridization in echinoderms has been discovered, in part, as a result of the expression of intermediate phenotypes (e.g., Chia 1966, Menge 1986, Kwast *et al.* 1990); however, most studies use multiple character sets to examine the extent of hybridization and introgression in sympatric species. Lessios and Pearse (1996) were the first to document natural hybridization between echinoid species using genetic markers. They found specimens morphologically intermediate between two of three sympatric urchins in the genus *Diadema*, developed allozyme loci diagnostic and semi-diagnostic for the species, and assayed individuals of intermediate morphology to determine if they

had hybrid genotypes. F_1 hybrids and backcrosses were identified using these loci, but there was limited evidence of introgression between the species.

Six-rayed asteroid sea stars of the *Leptasterias* species complex in the northeast Pacific have been the focus of morphological and molecular systematic studies by Foltz and his coworkers (Kwast *et al.* 1990, Foltz *et al.* 1996a, b, Hrincevich and Foltz 1996, Foltz 1997, Hrincevich *et al.* 2000, Flowers and Foltz 2001). Morphological intermediates frequently occur in natural populations (Kwast *et al.* 1990), but hybridization (identified using allozyme markers diagnostic for parental species) in nature is rare (Foltz 1997). The situation is further complicated by extensive sharing of mtDNA sequences by two of the nominal species which may be the result of incomplete lineage sorting and/or frequent hybridization (Flowers and Foltz 2001). The conflicting patterns of genetic and morphological variation in *Leptasterias* spp. remain unresolved.

A simpler complex of asteriid species occurs in the northwest Atlantic. Sympatric populations of *Asterias forbesi* and *A. rubens* are the result of secondary contact following the re-colonization of North America by *A. rubens*, possibly since the last glacial maximum (Worley and Franz 1983, Wares 2001). However, the existence of hybrids (and the nature of this area of sympatry as a hybrid zone) has been uncertain. Hybridization has been reported based on specimens of intermediate morphology (Menge 1986, Clark and Downey 1992). In contrast, detailed morphological surveys have tended to classify all individuals into one or the other species (Worley and Franz 1983) or have produced only weak evidence for the occurrence of morphological intermediates that might be F_1 hybrids or recent backcrosses (Chapter Three). These two species have overlapping spawning seasons (Smith 1940, Boolootian 1966, Menge 1986), share similar natural histories (feeding activity and diet, Menge 1979) and habitats (Menge 1986),

produce compatible gametes (Ernst 1967, Chapter Two), and can form viable and fertile hybrid offspring in laboratory crosses (Chapter Two). The potential for natural hybridization seems high in spite of the rarity of obvious morphological intermediates in nature. A recent phylogenetic analysis of speciation in north Atlantic *Asterias* spp. using mtDNA (COI) sequences (Wares 2001) did not find any evidence of shared haplotypes between *A. forbesi* and *A. rubens* (a potential indicator of hybridization and introgression), but the study was not specifically designed to detect hybridization.

In this study I use mtDNA sequences from the highly variable control region to identify hybrids in sympatric populations of *Asterias* spp. and report the first evidence of introgression of mtDNA from *A. rubens* into *A. forbesi*. My sampling design emphasizes specimens within the contact zone, particularly those with morphological characters intermediate between the parental species. I include sequences from specimens outside the contact zone to provide insight into the genetic history of the contact zone and to compare genetic diversity.

Methods

Samples of *Asterias* spp. were collected, scored for diagnostic morphological traits and identified in the morphological analysis described in Chapter Three. Specific samples were selected from across the range of species distribution; most of the samples sequenced were from within the contact zone, including ten individuals of intermediate phenotype (based on the $k = 3$ cluster analysis in Chapter Three, Table 4.1). *Asterias forbesi* collected outside the hybrid zone were museum samples which did not yield amplifiable DNA.

DNA extraction and amplification

Genomic DNA was extracted from ethanol-preserved tube feet using a standard CTAB protocol (Grosberg *et al.* 1996). An approximately 800 bp portion of the mitochondrial genome was amplified by PCR using the 12Sa/16Sa primers of Smith *et al.* (1993). These primers correspond to the highly conserved regions of the 3' end of 12S and 16S rRNA mitochondrial genes and span two transfer RNA genes ($tRNA_{Glu}$ and $tRNA_{Thr}$) and the putative control region (Smith *et al.* 1993). Amplifications were performed in 12.5 µL reactions containing 10-25 ng DNA, 1x polymerase buffer, 0.2 mM of each dNTP (MBI), 2.5 mM $MgCl_2$ (MBI), 0.5 µM each primer, and 0.3 units of *Tsg* polymerase (Biobasic, Toronto). The thermal cycling profile consisted of an initial denaturation at 95°C for 3 min., followed by 37 - 43 cycles of 94°C (30 sec.), 54°C (45 sec.), 72°C (90 sec.). Amplified products were visualized in 1% agarose (1x TBE) using ethidium bromide.

Table 4.1 Phenotype and haplotype distribution of *Asterias rubens* and *A. forbesi* in a survey of the north Atlantic: sampling location (abbreviation), total sample size (n), the number of individuals identified based on phenotype and the number of individuals in each clade (number of haplotypes identified in each location) in 309 bp of mtDNA ($tRNA_{Thr}$ and control region). Phenotypes were determined in a morphological analysis (Chapter Three), specimens of intermediate morphology were identified in a $k = 3$ cluster analysis of all diagnostic characters. The total numbers of unique haplotypes in phylogenetic analyses are indicated in the last row and do not equal the sum of the haplotypes in each population because some haplotypes are shared among populations (see Figs. 4.1 – 4.3).

Population	n	Phenotypes			Clade (# haplotypes)	
		A. rubens	Intermediate	*A. forbesi*	*A. rubens*	*A. forbesi*
Ireland (IRE)	4	4			4 (4)	
Norway (NOR)	2	2			2 (2)	
Faroe Islands (FAR)	4	4			4 (3)	
Iceland (ICE)	1	1			1 (1)	
Newfoundland (NFL)	2	1	1		2 (2)	
Quebec (QUE)	6	6			6 (3)	
Prince Edward Island (PEI)	4	0		4	0	4 (3)
Bras d'Or, NS (BDO)	5	3		2	3 (2)	2 (1)
Bear Cove, NS (BCV)	16	5	1	10	9 (3)	7 (6)
Isle of Shoals, ME (SHO)	21	6	8	7	14 (3)	7 (7)
Total	65	32	10	23	45 (16)	20 (13)

Approximately 75-100 ng of each PCR product was ethanol-precipitated and one strand was sequenced using an internal primer designed from sequence within the $tRNA_{Glu}$ (Glu: 5'-TTTCATGTTATAGGTTTAGG-3'). Sequencing reactions used Li-Cor IRD700 Dye Terminators, following the manufacturer's protocol. The cycle sequencing profile consisted of an initial denaturation at 95°C for 3 min., followed by 30 cycles of 94°C (30 sec.), 50°C (45 sec.), 72°C (60 sec.). Excess dye terminators were removed using Sephadex G-50 fine (Sigma) columns. Products were resolved in 6% (25 cm length, 0.2 mm depth) polyacrylamide gels on a Li-Cor DNA 4200L-2. Sequences were aligned and edited using the image analysis software Align-IR, provided by Li-Cor.

Molecular analyses

Edited sequences were aligned using ClustalX (Thompson *et al.* 1997) and characters were trimmed from the sequence ends to obtain equal sequence lengths. The number of variable and parsimony-informative sites were determined using PAUP* 4.0b10 (Swofford 2002), as was the mean nucleotide base composition. Haplotype and nucleotide diversity were calculated using Arlequin 2.001 (Schneider *et al.* 2000) and compared between species and geographic regions using *t*-tests ($\alpha = 0.05$). Haplotype networks (95% parsimony) were constructed separately for *A. rubens* and *A. forbesi* sequences using TCS Version 1.13 (Clement *et al.* 2000). In separate analyses, gaps in the sequence were excluded and gaps were treated as fifth characters.

Analyses of pairwise genetic distances and of phylogenetic relationships based on maximum-parsimony (MP) and maximum-likelihood (ML) were performed using PAUP*. Mean intra- and interspecific sequence divergences were calculated using the

distance matrix for the unique haplotypes. Heuristic searches were conducted using starting trees obtained by stepwise addition. Tree-bisect-reconnection was used for branch swapping, and branches were collapsed if the maximum branch length was zero; trees were midpoint rooted. The best-fit substitution model for the ML analysis (HKY + G, Tr:Tv = 3.63, gamma-distributed parameter for among-site rate heterogeneity = 0.5619) was determined with Modeltest 3.06 (Posada and Crandall 1998). MP analyses were run with gaps excluded, as well as with gaps treated as a fifth character. Nodal support for all phylogenetic analyses was determined by bootstrap replication (100 replicates, heuristic search, ML analysis using the HKY + G model).

Results

Sequence characteristics and haplotype diversity

The sequence alignment included 309 sites from 63 *Asterias* individuals and 261 sites from two additional individuals (from the Faroe Islands and Bear Cove, NS) for which the last 48 sites could not be reliably sequenced (total of 65 sequences; Table 4.1). Base frequencies were 38.3% A, 20.1% C, 14.8% G, and 26.9% T. There were 85 parsimony-informative sites and 29 unique haplotypes in phylogenetic analyses. Analyses including gapped characters (22 gap sites) produced nearly identical results as analyses excluding gaps and are not presented here.

Mean intraspecific haplotype and nucleotide diversity were significantly higher for *A. forbesi* than for *A. rubens* (Table 4.2, $p < 0.05$). However, when northwest (NW) Atlantic *A. rubens* sequences were excluded from the analysis, there were no significant differences in haplotype and nucleotide diversity between northeast (NE) Atlantic *A. rubens* and NW Atlantic *A. forbesi* ($p > 0.05$). Among *A. rubens* sequences alone, haplotype diversity was higher for NE Atlantic samples than for NW Atlantic samples ($p < 0.05$). Within North American populations of *A. rubens*, haplotype diversity was higher for samples collected from allopatric populations than for *A. rubens* in sympatry with *A. forbesi* ($p < 0.05$).

Two disparate networks were formed for *A. rubens* and *A. forbesi* haplotypes (Figs. 4.1, 4.2). Most haplotypes were defined by a single base-pair substitution from neighbouring haplotypes within the network.

In the *A. rubens* haplotype network, two haplotypes were shared across the north Atlantic and both were in high frequency in North American populations (Fig. 4.1). One

of these haplotypes (14) was shared between Europe and all North American populations, the other haplotype (1) was shared between Europe and two sympatric *A. rubens* populations (Bear Cove, NS, and Isle of Shoals, ME).

A third, missing intermediate haplotype also linked North American and European *A. rubens* populations. An ambiguous loop involving this missing haplotype was resolved using parsimony. Seven European haplotypes were identified in the ten NE Atlantic specimens and six haplotypes unique to North America were identified in NW Atlantic *A. rubens*.

The *A. forbesi* haplotype network was highly diverse (Fig. 4.2): thirteen *A. forbesi* haplotypes were detected in twenty sequences. A common haplotype (9) was found in each of the sympatric *A. forbesi* populations. Allopatric and sympatric *A. forbesi* populations could not be compared because museum samples of allopatric *A. forbesi* did not yield amplifiable DNA. Three specimens identified morphologically as *A. forbesi* were missing from the haplotype network as they shared the same mtDNA haplotype as some *A. rubens* (Fig. 4.3).

Table 4.2 Comparison of intraspecific haplotype diversity (sampling variance) between *A. forbesi* and *A. rubens*, between *A. rubens* from the NE Atlantic and *A. forbesi* from the NW Atlantic, between *A. rubens* from the NW Atlantic and NE Atlantic, and between areas of sympatry and allopatry with *A. forbesi* in the NW Atlantic. Sample size (n) for each calculation is indicated in last row. Significantly higher diversity between species, coasts and areas of sympatry versus allopatry is indicated by * (*t*-tests, $p < 0.05$).

	Between species		NE	NW	*A. rubens*		NW Atlantic *A. rubens*	
	A. rubens	*A. forbesi*	*A. rubens*	*A. forbesi*	NW	NE	Sympatric	Allopatric
Haplotype diversity	0.8889	0.9842*	0.9818	0.9842	0.8182	0.9818*	0.7477	0.9286*
	(0.032)	(0.021)	(0.046)	(0.021)	(0.046)	(0.046)	(0.072)	(0.084)
Nucleotide diversity	0.0105	0.0194*	0.0159	0.0194	0.0084	0.0159*	0.0066	0.0129*
	(0.006)	(0.011)	(0.009)	(0.011)	(0.005)	(0.009)	(0.004)	(0.008)
n	45	20	11	20	34	11	26	8

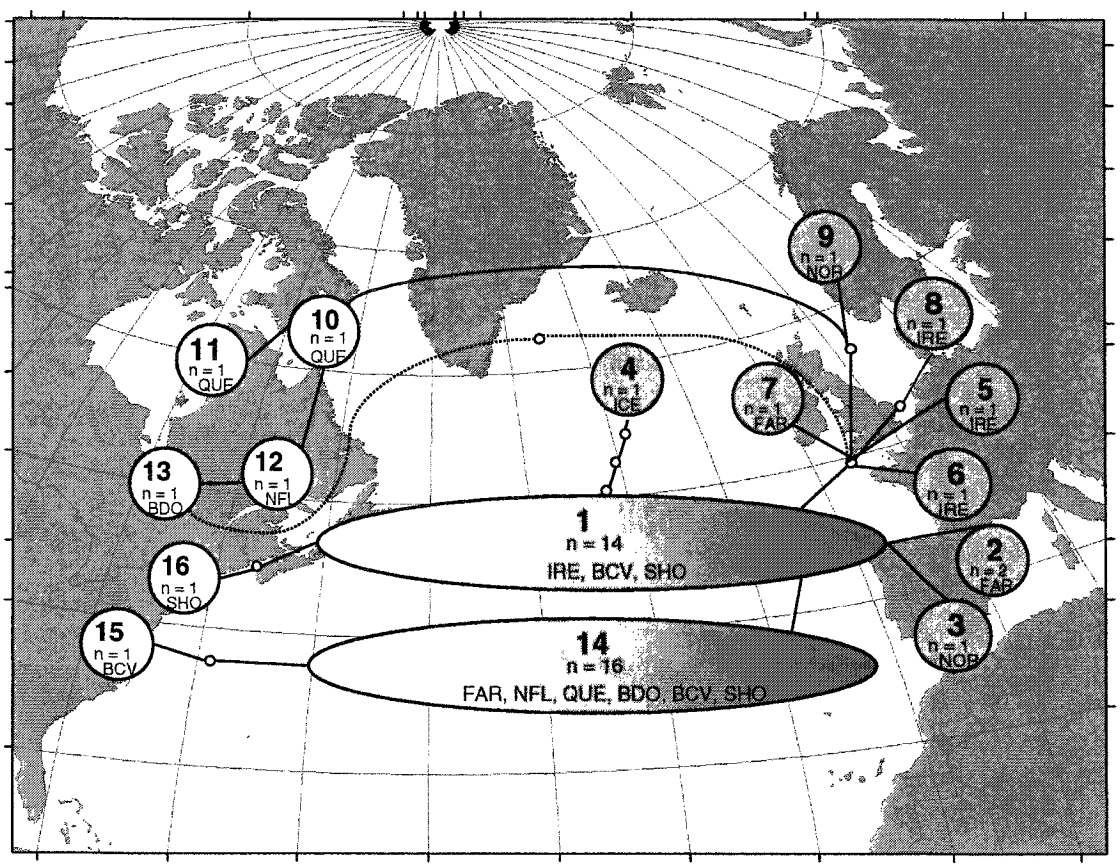

Figure 4.1 95% plausible network for *Asterias rubens* haplotypes (309 bp of $tRNA_{Thr}$ and putative control region mtDNA). Haplotypes found in the NE Atlantic are shaded in grey, haplotypes shared between oceanic regions are represented by ovals. Three specimens identified morphologically as *A. forbesi* are included in this network as they share the same haplotype (14) as *A. rubens*. Haplotype numbers are identified in boldface, n indicates the haplotype sample size, and sample locations are abbreviated as in Table 4.1. Lines connect haplotypes that differ by one mutation; small open circles indicate missing intermediate haplotypes, dotted lines represent an ambiguous loop in the network.

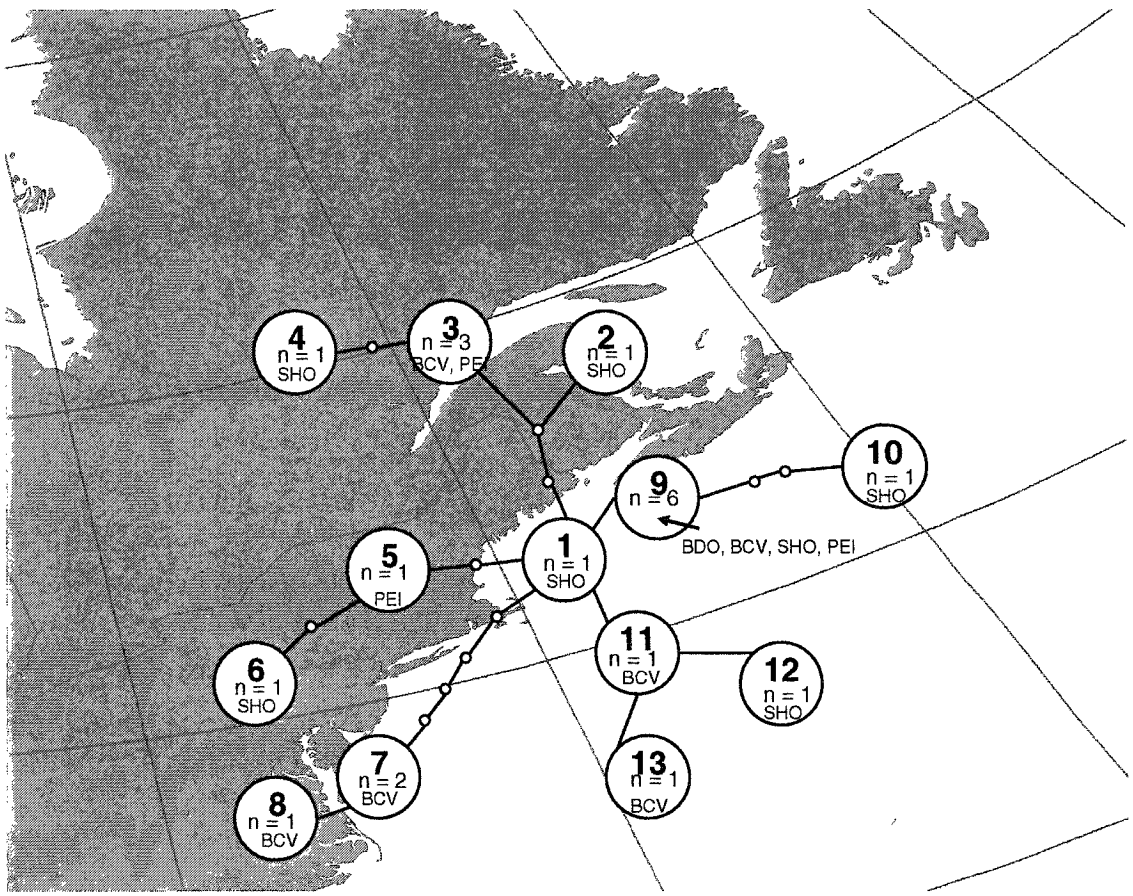

Figure 4.2 95% plausible network for *Asterias forbesi* haplotypes in the northwest Atlantic (309 bp of $tRNA_{Thr}$ and putative control region mtDNA). Three specimens identified morphologically as *A. forbesi* are missing from this network as they have *rubens*-like mtDNA haplotypes. Haplotype numbers are identified in boldface, n is the haplotype sample size and location abbreviations are as in Table 4.1. Lines connect haplotypes that differ by one mutation; small open circles indicate missing intermediate haplotypes.

Phylogenetic analyses

With the exception of the short *A. forbesi* sequence discussed above, the haplotype networks illustrating the stepwise mutational relationships among control region haplotypes were compatible with the results of the phylogenetic analyses. The MP and ML phylogenetic analyses produced congruent topologies with similar levels of bootstrap support (Fig. 4.3). Treating gaps as a fifth character state in the MP analyses did not change the tree topology.

Both ML and MP methods produced two well-supported clades: one from individuals with *A. forbesi* phenotypes, and one that included all *A. rubens* phenotypes plus three sequences obtained from individuals with *A. forbesi* phenotypes. These three individuals were all obtained from Bear Cove, NS, all had *A. forbesi* phenotypes and they all shared a haplotype (14) that also occurred in the Faroe Islands and all of the NW Atlantic populations of *A. rubens* (Figs. 4.1, 4.3). All specimens identified as having phenotypes intermediate to the parental species in the $k = 3$ cluster analysis (Chapter Three) contained *A. rubens* haplotypes (Fig. 4.3). Both the ML and MP phylogenetic analyses clustered sequences into 16 *A. rubens* haplotypes and 13 *A. forbesi* haplotypes (Fig. 4.3).

Sequence divergence was calculated using the pairwise distance matrix for unique haplotypes in the ML analysis. The mean sequence divergence between the *A. forbesi* and *A. rubens* clades was 59.4% (± 4.3 s.d.). Intraspecific sequence divergence for the *A. forbesi* and *A. rubens* clades was 1.1% (± 0.6 s.d.) and 1.9% (± 0.9 s.d.) respectively.

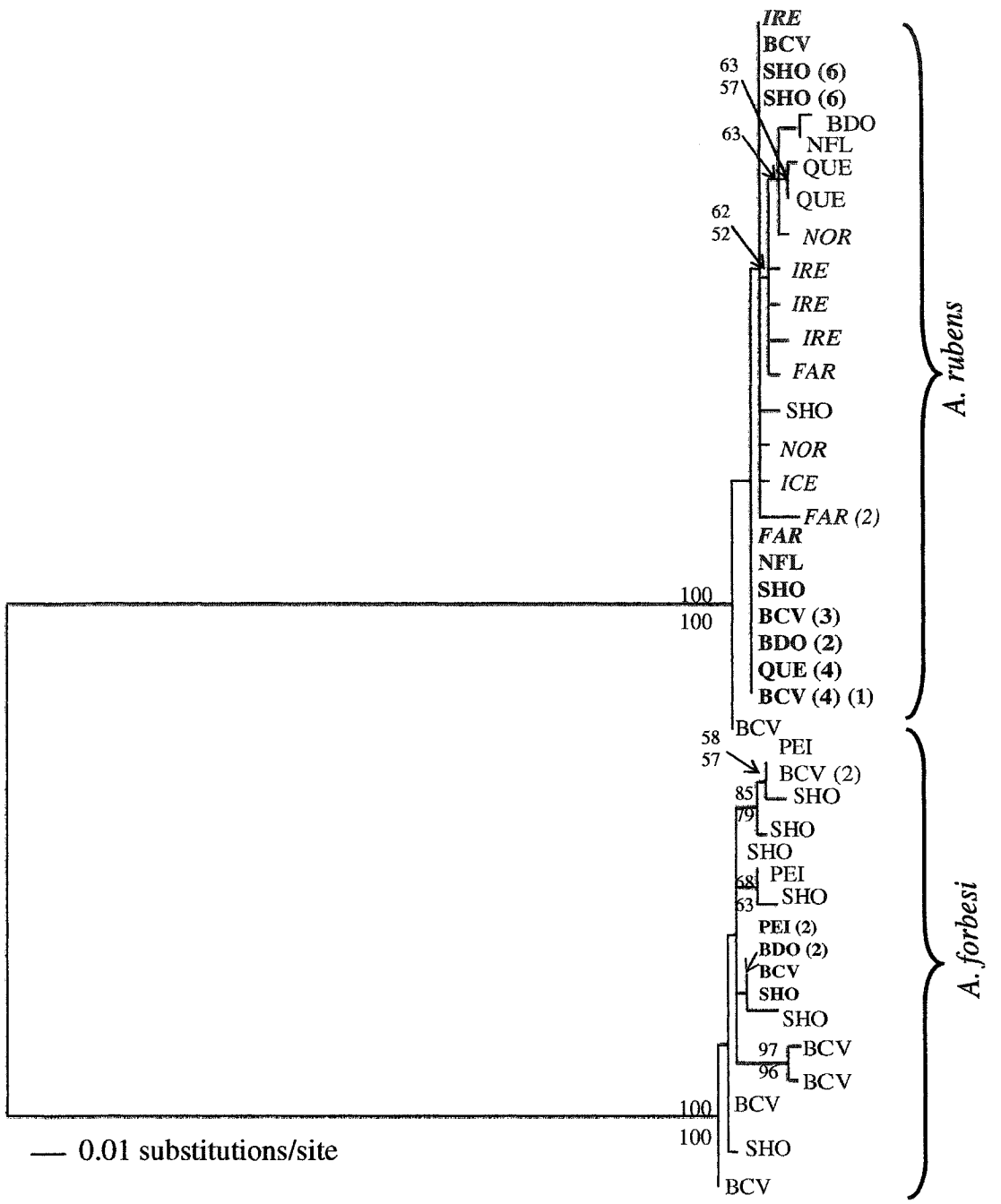

Figure 4.3 Maximum-likelihood midpoint-rooted phylogram of 309 bp mtDNA ($tRNA_{Thr}$ and control region). *Asterias rubens* phenotypes are indicated in red, *A. forbesi* phenotypes are in blue, and intermediate phenotypes ($k = 3$ analysis, Chapter 3) are in purple. Haplotypes are in braces. European haplotypes are italicized, North American haplotypes are in plain font, and haplotypes shared between locations are in boldface. Population abbreviations are as in Table 4.1. Bootstrap values from ML analyses are indicated above the line for nodes with greater than 50% support, bootstrap values from MP analyses are indicated below. Where only one bootstrap value is indicated, it is from the ML analysis.

Discussion

Ancestral polymorphism or hybridization and introgression of mtDNA?

Phylogenetic analyses of mtDNA ($tRNA_{Thr}$ and control region) sequence variation in *Asterias* sibling species in the North Atlantic consistently produced two distinct clades. One clade was comprised exclusively of sequences from *A. forbesi* phenotypes, while *A. rubens* phenotypes clustered into the second clade. This divergence of *A. rubens* and *A. forbesi* is congruent with previous studies of morphology (Worley and Franz 1983, Menge 1986), allozymes (Schopf and Murphy 1973) and mtDNA (Wares 2001). However, the distribution of sequences obtained from phenotypic *A. forbesi* was paraphyletic with respect to the *A. rubens* clade.

Three individuals identified morphologically as *A. forbesi* have the same mtDNA haplotype as some *A. rubens*. These individuals did not exhibit any diagnostic morphological characteristics intermediate between the two species and were scored as "pure" *A. forbesi* in the morphological analysis (Chapter Three). This pattern may be explained by two possible evolutionary processes: (1) incomplete lineage sorting and retention of ancestral polymorphisms; or (2) introgression following secondary contact of two populations which diverged in allopatry (Avise *et al.* 1987).

Asterias forbesi and *A. rubens* are believed to have diverged 3.0 Mya, following the formation of the Labrador Current and have only recently come into secondary contact through westward expansion of the *A. rubens* geographic range into the shallow water off North America (Wares 2001). While there may have been long-term persistence of ancestral mtDNA polymorphisms in *Asterias*, it is not a likely explanation. Complete lineage sorting and reciprocal monophyly of nuclear (ITS) and mtDNA (COI)

gene sequences have been reported in *Asterias* spp. (Wares 2001). As well, the interspecific sequence divergence between the species using the mtDNA control region sequences is very high (~60%), thus the likelihood of the two species retaining the exact same 310 bp of sequence is low. Introgression is a more plausible explanation for the pattern of shared haplotypes seen in *Asterias*.

Sympatric populations of *A. rubens* and *A. forbesi* in the northwest Atlantic are the result of secondary contact (Worley and Franz 1983, Wares 2001). One of the possible consequences of secondary contact between sibling species is introgression of genes from one species into another (Anderson 1949). Although morphologically intermediate hybrids appear rare (Chapter Three), the gametes of *A. forbesi* and *A. rubens* are reciprocally compatible and the species readily hybridize in the lab (Chapter Two). In some cases, using morphological traits alone, hybrids cannot be distinguished from pure parental species (Lamb and Avise 1987). The three individuals with *A. forbesi* phenotypes and *A. rubens* mtDNA haplotypes were collected from a site where reproductively mature individuals of both phenotypes are found together at the same times and in the same microhabitats (pers. obs.). The apparent absence of ecological and reproductive barriers and the molecular evidence presented here suggest that natural hybridization may be relatively common in *A. rubens* and *A. forbesi*. Although my sample of *A. forbesi* phenotypes was relatively small (23 individuals from the contact zone), 13% of that sample appeared to be of hybrid descent.

Genetic history of the secondary contact zone

The secondary contact zone formed by *A. rubens* and *A. forbesi* is believed to be the result of North American colonization by *A. rubens* as recently as the last glacial maximum, about 20 000 years ago (Worley and Franz 1983, Wares 2001). Previous phylogeographic analyses of *A. rubens* found significantly lower haplotype diversity in North America and no unique alleles in North American populations relative to European populations (Wares 2001, Wares and Cunningham 2001). Wares and Cunningham (2001) suggested that these results indicate a recent founding event from Europe rather than persistence of *A. rubens* in North American glacial refugia.

In an analysis of amphi-Atlantic marine invertebrate species, Wares and Cunningham (2001) made three predictions to test evidence of recent colonization of North America from Europe: (1) haplotype diversity will be significantly lower in North American populations; (2) the time to the most recent common ancestor in North American populations will postdate the last glacial maximum; and (3) all North American haplotypes will be shared with Europe or will be descended from European haplotypes involved in the range expansion. The analyses of mtDNA in my study provide evidence that supports the first of these predictions. Haplotype and nucleotide diversities in *A. rubens* were significantly lower in North American populations and there were multiple shared haplotypes between Europe and North America.

In contrast with the third prediction, there were several unique haplotypes in the North America populations: two unique haplotypes (15, 16 in Fig. 4.1) were both derived from haplotypes shared with Europe and four haplotypes (10-13 in Fig. 4.1) were derived from an intermediate haplotype which had a single base-pair difference from a haplotype found in Norway. As well, when NW Atlantic *A. rubens* sequences were excluded from

the diversity analysis, there were no significant differences in haplotype and nucleotide diversity between NE Atlantic *A. rubens* and NW Atlantic *A. forbesi*. These results suggest the contact zone in the NW Atlantic is an important source of diversity for *A. rubens* and may predate the last glacial maximum.

Within North American populations of *A. rubens*, genetic diversity was higher in allopatry than in sympatry. The difference in genetic diversity inside and outside the contact zone may be a signal of recent range expansion (Hewitt 1996, Austerlitz *et al.* 1997), from allopatric populations of *A. rubens* in Newfoundland and Quebec. Based on the haplotype network presented here, the contact zone appears to have been colonized on at least three occasions from European populations (haplotypes 1 and 14).

The two haplotypes shared between Europe and North American populations of *A. rubens* (1, 14 in Fig. 4.1) were also the most common haplotypes in the contact zone. Of these two haplotypes, the one found in all the North American populations was also the haplotype introgressed into *A. forbesi* and is perhaps the oldest in the northwest Atlantic. Older haplotypes are expected to be more widely distributed and more deeply nested within the parsimony network, whereas newer haplotypes should be sampled closer to their geographic point of origin and inferred to be at the periphery of the network (Avise *et al.* 1987, Valliantos *et al.* 2001).

Asterias forbesi is endemic to North America (Clark and Downey 1992). The genetic diversity of *A. forbesi* in sympatry is greater than all of the *A. rubens* in this study. Not including the three individuals with *forbesi*-like phenotypes and *rubens*-like haplotypes, twelve different haplotypes were found in twenty *A. forbesi* from the contact zone. *Asterias forbesi* is believed to have survived south of the last glaciation (Worley and Franz 1983), and its present day distribution ranges as far south as Florida (Franz *et*

al. 1981). It subsequently expanded its range northwards resulting in the present day distribution into the Gulf of Maine and Nova Scotia. This range expansion may be ongoing as *A. forbesi* has been increasing in abundance in the coastal zone around Maine and New Hampshire relative to *A. rubens* (Harris *et al.* 1998). The historic range expansion cannot be analyzed here due to the lack of haplotype sampling from *A. forbesi* outside the contact zone.

Asymmetric introgression of mtDNA

In his review of hybridization in the sea, Gardner (1997) compiled an extensive list of examples of hybridization in marine invertebrates. Based on studies of molecular (allozyme) and morphological markers (Schopf and Murphy 1973, Menge 1986), *A. rubens* and *A. forbesi* were included in the list as hybridizing species, but were suspected to not introgress. If the two species hybridize naturally and there is introgression of genetic material, I expected it would most likely be detected in specimens with diagnostic morphological traits intermediate between the species. This was not the case as all ten specimens with intermediate morphological traits had *A. rubens* haplotypes. Instead, introgression was asymmetrical and consisted only of *rubens*-like haplotypes in individuals with *forbesi*-like phenotypes. Further analysis of the contact zone using biparentally inherited nuclear markers is necessary to further describe the extent and frequency of hybridization.

The combination of *rubens*-like mtDNA haplotypes and *forbesi*-like phenotypes is likely the result of eggs of female hybrids (with *A. rubens* mtDNA, descended from one or more generations of matrilineal backcrossing with *A. forbesi* males) being fertilized by

A. forbesi sperm. Strong asymmetries in the compatibility of *A. rubens* and *A. forbesi* sperm with the eggs of female hybrids could contribute to the asymmetrical introgression of *rubens*-like haplotypes into the *A. forbesi* lineages. Evidence from cross-fertilization studies (Chapter Two) supports this hypothesis. I found substantially higher fertilization success under standard conditions for a hybrid female with *A. rubens* mtDNA backcrossed to a male *A. forbesi* (47%) than to a male *A. rubens* (19%). Although this result was not replicated, it is consistent with the observation of asymmetrical introgression.

Asymmetric introgression in sympatric *Asterias* populations may be due to gamete recognition and fertilization preference (Arnold 1997). Assortative fertilization has been documented in the mussels *Mytilus edulis* and *M. galloprovincialis* (Bierne *et al.* 2002a) and conspecific sperm precedence is well known in terrestrial invertebrates (reviews in Howard and Berlocher 1998, Howard 1999). Heterospecific gamete compatibility was highly variable in crosses between *A. rubens* and *A. forbesi* when a single male was mated with a single female (Chapter Two), the compatibility of some crosses was comparable to conspecific fertilizations while other crosses required an order of magnitude more sperm. There may be sperm competition when eggs are simultaneously presented with conspecific and heterospecific sperm. Asteroids may have some cellular mechanism of conspecific gamete recognition analogous to bindin in echinoids and lysin in mollusks (reviews in Palumbi 1992, 1994). In Chapter Five, I describe the results of paternity analyses of offspring produced in gamete competition studies.

CHAPTER FIVE:

SPERM COMPETITION AFFECTS PATERNITY AND HYBRIDIZATION IN *ASTERIAS* SEA STARS

Introduction

The evolution of barriers to reproduction is essential to the process of speciation (Dobzhansky 1937, Mayr 1942). In broadcast-spawning marine invertebrates, differences in spawning times, habitats and fertilization biology have been implicated in speciation events (review in Palumbi 1994). Among these barriers, gametic interactions which prevent heterospecific fertilization are particularly important in determining reproductive isolation (Lessios and Cunningham 1990; Palumbi and Metz 1991, Levitan 2002).

In several sympatric echinoderm species complexes (i.e., the sea stars *Acanthaster* and *Patiriella*, and the sea urchin *Strongylocentrotus*), genetic divergence and speciation is believed to have occurred without the evolution of gamete incompatibility (Lucas and Jones 1976, Strathmann 1981, Byrne and Anderson 1994). However, these studies were not conducted using multiple males simultaneously (sperm competition, Parker 1970). Instead, fertilization success was measured by mixing the eggs of one species with either conspecific or heterospecific sperm.

In a review of conspecific sperm precedence, Howard (1999) criticized this method of assessing heterospecific gamete compatibility as incapable of detecting differences between conspecific and heterospecific sperm in competition. Conspecific sperm precedence is defined by Howard (1999, p. 110) as the "favored utilized of sperm from conspecific males in fertilization when both conspecific and heterospecific males

have inseminated a female". Conspecific sperm and pollen precedence has been shown to play important roles in isolating closely related terrestrial taxa such as grasshoppers (Hewitt *et al.* 1989, Bella *et al.* 1992), beetles (Wade *et al.* 1994) and ground crickets (Gregory and Howard 1994). Indeed, the only reproductive barrier discovered to date in ground crickets is conspecific sperm precedence (Howard and Gregory 1993, Gregory and Howard 1994, Howard *et al.* 1998, review in Howard 1999). While broadcast-spawning marine invertebrates cannot exhibit sperm precedence (via internal fertilization), gamete recognition and fertilization preference could be mechanisms of reproductive isolation.

The need for gamete competition studies in marine invertebrates has been recognized (Grant *et al.* 1998, McClary and Sewell 2003), but as yet only a few studies have been conducted. In many cases, the offspring of conspecific and heterospecific fertilizations cannot be distinguished based on their early developmental phenotypes. Improvements in molecular analytical techniques have enabled the use of molecular markers to assess paternity in offspring produced in gamete competition studies (e.g., Bierne *et al.* 1998, Gerber *et al.* 2000, Norris *et al.* 2000). Conspecific fertilization preference has been investigated in the oysters *Crassostrea gigas* and *C. angulata* by paternity testing of embryos using microsatellites (Huvet *et al.* 2001). Assortative mating through gamete preference has been demonstrated in the mussels *Mytilus edulis* and *M. galloprovincialis* by genotyping offspring using intron-length polymorphisms (Bierne *et al.* 2002a). These studies suggest that some examples of qualitative heterospecific gametic compatibility are influenced by quantitative differences between conspecific and heterospecific sperm that are only detectable when heterospecific sperm compete with conspecific sires for fertilization of eggs.

In this study I examine conspecific sperm preference in two sympatric sea stars, *Asterias forbesi* and *A. rubens*. There does not appear to be any prezygotic reproduction barriers in the contact zone: spawning periods overlap (Smith 1940, Boolootian 1966, Franz *et al.* 1981, Menge 1986), habitats are shared (Menge 1986), and there is a high degree of gamete compatibility in the absence of sperm competition (Ernst 1967, Chapter Two). Morphological surveys have found no evidence to support the occurrence of hybrids of intermediate morphology (Worley and Franz 1983, Chapter Three); however a phylogenetic analysis of mtDNA sequences found evidence of asymmetric introgression (Chapter Four). Individuals of hybrid origin were found with *A. rubens* mtDNA haplotypes and *A. forbesi* phenotypes, which are most parsimoniously explained as the result of *A. rubens* eggs being fertilized by *A. forbesi* sperm. To determine the paternity of larval offspring produced under conditions of sperm competition, I used a microsatellite marker to genotype offspring from eggs mixed with different concentrations of conspecific and heterospecific sperm. I found evidence of conspecific fertilization preference in some crosses of *A. forbesi* eggs and a suggestion of competitive superiority of *A. forbesi* sperm: in one cross of *A. rubens* eggs, heterospecific sperm were competitively superior even when they were an order of magnitude less abundant than conspecific sperm.

Methods

Sperm competition crosses

Asterias spp. were collected from Bear Cove, NS, in July 2003. Specimens were assigned to either *A. forbesi* or *A. rubens* based on the diagnostic characters of Clark and Downey (1992) and confirmed using mtDNA ($tRNA_{Thr}$ and control region) sequences as described in Chapter Four. Gender was determined by examining a small piece of gonad removed from a small incision in one arm. At the same time, tube feet were removed from a portion of one arm and preserved in 95% ethanol for genetic analyses. Prior to use in the crosses, each adult genotype was determined as described below. Sexually mature specimens were maintained in individual containers in a flow-through seawater system for up to four days. Holding the animals longer than this period of time frequently resulted in spontaneous spawning, possibly due to the handling and surgical procedures.

Gametes were collected and prepared as described in Chapter Two. Gamete concentrations were determined prior to each experiment: sperm concentrations were estimated from haemocytometer counts of the 10^{-1} sperm dilution, and eggs were counted using a dissecting microscope. Sperm from the conspecific and heterospecific males were combined together in 40 mL glass beakers containing FSW; the final volume of sperm and egg suspension was 25 mL. The final sperm concentration for the male in high concentration was 200 sperm μL^{-1}; the sperm concentration for the male in low concentration was 20 sperm μL^{-1}. The sperm suspension was gently mixed immediately before adding the eggs. In no-choice experiments, the high sperm concentration results in 100% fertilization of *Asterias* eggs, and the low concentration yields approximately 50% fertilization (Chapter Two). Egg concentrations were adjusted to 200 eggs mL^{-1}, which

gave sperm: egg ratios of 1000:1 and 100:1 respectively for the two males. Gamete collection, incubation and subsequent larval culture were conducted at 12°C.

Eggs from each female were used in two separate sperm competition experiments. First, eggs were mixed with conspecific sperm in high concentration and heterospecific sperm in low concentration. Second, eggs were mixed with the relative concentration of conspecific and heterospecific sperm reversed. As controls for gamete quality and to ensure all gametes involved in each experiment were capable of fertilization, separate crosses of eggs mixed with only conspecific sperm in high concentration were conducted for *A. rubens* and *A. forbesi*. Only offspring from crosses in which both controls had >90% fertilization success after 2 h (based on the appearance of fertilization envelopes) were cultured and analyzed.

Males and females were chosen for individual replicate experiments such that it would be possible to assign paternity to the offspring using the genotypes of the adults and larvae from a single microsatellite locus. Eight replicate experiments using *A. forbesi* eggs (and eight independent replicates using *A. rubens* eggs) were conducted using different combinations of adults. One replicate experiment was conducted per day to reduce the potential for cross-contamination with unintended sperm. However, inconsistencies in the genotypes of adults for five crosses with *A. forbesi* eggs and three crosses with *A. rubens* eggs combined with poor gamete quality in one cross with *A. rubens* to limit the number of families in which offspring paternity could be reliably interpreted. Here I report results for three replicate experiments using eggs of *A. forbesi* females and two replicates using eggs of *A. rubens* females.

Larvae from each replicate experiment were transferred after two days into 250 mL containers with 125 mL FSW and 2 mL of a dense culture of *Isochrysis galbana*.

Five days after initial fertilization, bipinnaria larvae were gently removed from the culture and preserved in 95% ethanol.

Molecular analyses

The DNA extraction method was suggested by Dr. Jason Addison (Dalhousie University). Genomic DNA was extracted from a single preserved tube foot for each adult. The tube foot was rinsed twice in 100 µL double-distilled, autoclaved water (ddH_2O), then placed in 40 µL ddH_2O with 10 µg Proteinase K (Qiagen). The digestion was incubated for 60 min. at 65°C, then 10 min. at 85°C. Between 0.75 and 1.5 µL of this digest was used as template for each PCR. Genomic DNA from larvae was similarly extracted: larvae were placed individually into wells in a 96-well plate, rinsed twice with ddH_2O and extracted in a volume of 10 µL ddH_2O with 10 µg Proteinase K. Each larval digest (0.75 µL) was used as template in the PCR.

Microsatellite markers were developed for *A. forbesi* and *A. rubens* using the enrichment protocol of Hamilton *et al.* (1999), as described in Appendix Two. Microsatellite locus *Ar50* was selected for use in this study because it could be amplified reliably and was polymorphic in both species (two alleles in *A. forbesi* and six alleles in *A. rubens*). Primer sequences were *Ar50-A*: 5'-AGCCCATGTCGGTCTTAG-3' and *Ar50-B*: 5'-TTTGAAAGGCTCTAATGAG-3'. The 5' end of *Ar50-A* was labeled with an IRD700 dye for visualization. Amplifications were performed in a 5 µL final volume containing 0.2 mM of each dNTP (MBI), 3.0 mM $MgCl_2$ (MBI), 1.5 pmol of each primer, 1x polymerase buffer and 0.1 units of *Tsg* Polymerase (Biobasic). The thermal cycling profile consisted of an initial denaturation at 95°C for 5 min., followed by 36 cycles of

90°C (30 sec.), 49°C (90 sec.), 72°C (60 sec.) and a final extension of 72°C for 1 min. The amplified products were resolved in 6% (25 cm, 0.2 mm thick) denaturing polyacrylamide gels on a Li-Cor 4200 sequencer. Larvae were genotyped once or twice each; adults were genotyped three to five times each.

Statistical analyses

Paternity of each larva was determined using the genotypes. I then compared the ratio of the number of offspring expected for each male (10:1 for high: low sperm concentrations) with the ratio of the number observed using a *G*-test for goodness of fit (also known as the log-likelihood ratio test, Sokal and Rohlf 1995), corrected according to Williams (1976). The adjusted value of *G* was then compared with the χ^2 distribution ($\alpha = 0.05$).

Results

A total of 736 larvae were collected, of which 573 were genotyped (77.8%). The remaining 22.2% yielded no amplifiable *Ar50* alleles from repeated PCR attempts, perhaps due to degradation of some larvae or impurities in the DNA extraction (Huvet *et al.* 2001). No non-parental alleles were observed in the larvae.

In the absence of sperm preference and gametic incompatibility, paternity rates for conspecific and heterospecific males should be proportional to sperm concentrations because the sperm concentrations used in this study fall within the range for successful fertilization of eggs in the absence of sperm competition (Chapter Two). In this case, the relative fertilization success of the two males in competition is not expected to be significantly different from the 10:1 ratio of sperm concentrations.

Paternity rates among offspring from eggs of *A. forbesi* mixed with conspecific sperm in high concentration and heterospecific sperm in low concentration conformed to the 10:1 ratio in two out of three families (Table 5.1). In the third family (1 in Fig. 5.1A), all 63 offspring were sired by conspecific sperm, and abundant *A. forbesi* sperm were more effective than expected in competition with scarce *A. rubens* sperm. When the relative sperm concentrations were reversed, I found in two of three families (1 & 2 in Fig. 5.1B) that *A. forbesi* eggs mixed with high concentrations of heterospecific (and low concentrations of conspecific sperm) were significantly more likely to be fertilized by *A. forbesi*. In a third family (3 in Fig. 5.1B), 89% of *A. forbesi* eggs were fertilized by *A. rubens* sperm and this percentage was not significantly different from the expectation based on relative sperm concentrations.

Table 5.1 Results of paternity analyses of bipinnaria larvae produced in crosses of eggs with combinations of conspecific and heterospecific sperm using a microsatellite marker. Three families with *A. forbesi* females and two families with *A. rubens* females were examined. HF refers to 10:1 *A. forbesi*: *A. rubens* sperm concentration, HR refers to a 10:1 *A. rubens*: *A. forbesi* sperm concentration. N is the number of larvae whose DNA amplified. X refers to offspring of unknown paternity (see Results).
* = $p < 0.02$; ** = $p < 0.001$.

Family	Cross	N	Observed paternity	Expected paternity	G_{adj}
F1	HF	63	F = 63	10/11 * 63 = 57.3	11.8**
			R = 0	1/11 * 63 = 5.73	
	HR	17	R = 3	10/11 * 17 = 15.45	60.99**
			F = 14	1/11 * 17 = 1.55	
F2	HF	74	F = 67	10/11 * 74 = 67.3	0.04
			R = 7	1/11 * 74 = 6.73	
	HR	66	R = 42	10/11 * 66 = 60	36.3**
			F = 24	1/11 * 66 = 6	
F3	HF	57	F = 49	10/11 * 57 = 51.8	1.44
			R = 8	1/11 * 57 = 5.2	
	HR	62	R = 55	10/11 * 62 = 56.4	0.25
			F = 7	1/11 * 62 = 5.64	
R1	HR	74	R = 13	10/11 * 74 = 67.3	224.85**
			F = 61	1/11 * 74 = 6.72	
	HF	49	F = 45	10/11 * 49 = 44.5	0.16
			R = 4	1/11 * 49 = 4.45	
R2	HR	57	R = 45	¾ * 10/11 * 57 = 38.9	5.93*
			F = 4	½ * 1/11 * 57 = 2.6	
			X = 8	3/11 * 57 = 15.5	
	HF	54	F = 14	½ * 10/11 * 54 = 24.5	10.7**
			R = 8	¾ * 1/11 * 54 = 3.68	
			X = 32	21/44 * 54 = 25.7	

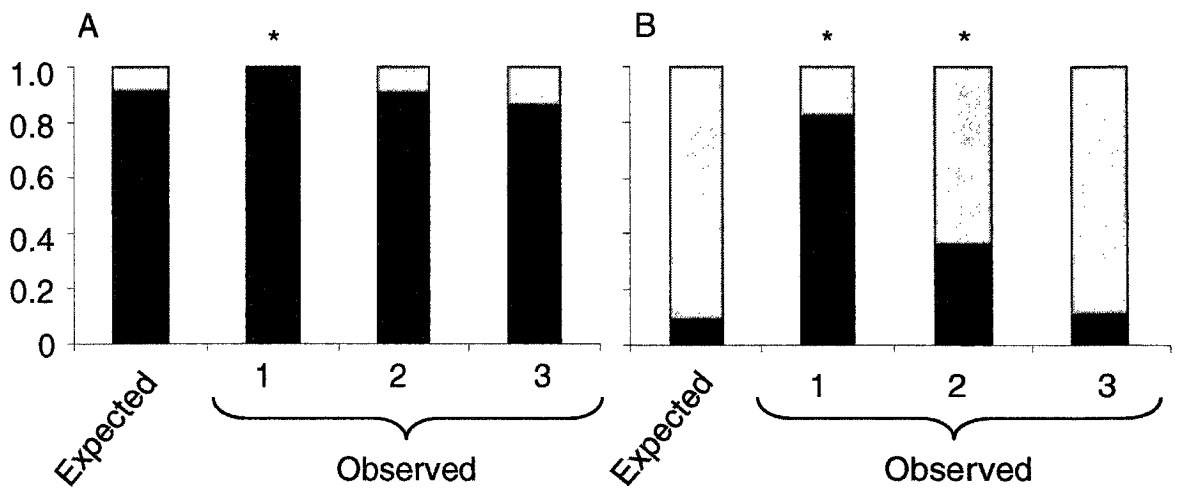

Figure 5.1 Paternity rates of *A. forbesi* eggs in sperm competition studies (based on inheritance of *Ar50* alleles). Offspring sired by *A. forbesi* are shaded in black and offspring sired by *A. rubens* are hatched. (A) Crosses in which conspecific sperm are 10 times more abundant than heterospecific; and (B) crosses in which heterospecific sperm are 10 times more abundant than conspecific. Results for three families of each type (1-3) are shown. Crosses in which observed and expected frequencies were significantly different are indicated by * ($p < 0.001$).

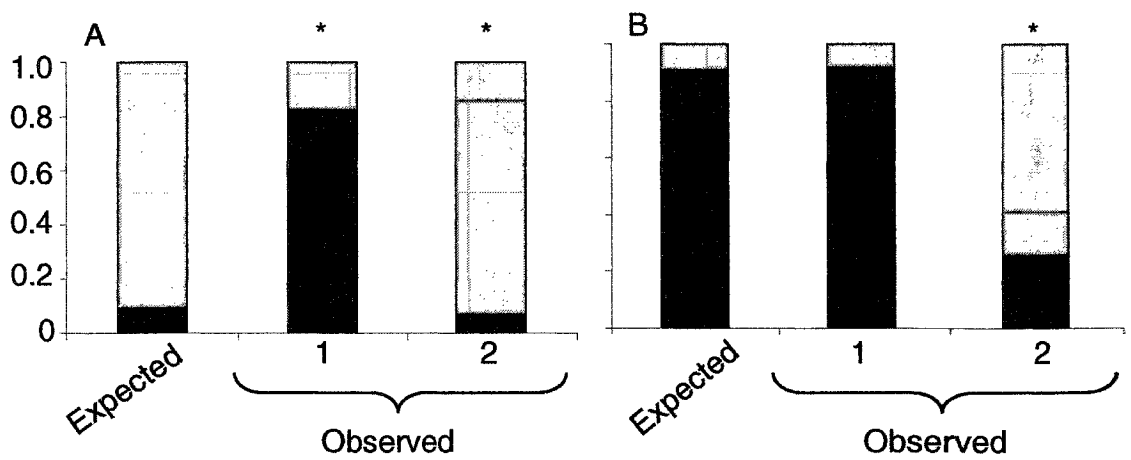

Figure 5.2 Paternity rates of *A. rubens* eggs in sperm competition studies (based on inheritance of *Ar50* alleles). Offspring sired by *A. forbesi* are shaded in black and offspring sired by *A. rubens* are hatched. (A) Crosses in which conspecific sperm are 10 times more abundant than heterospecific; and (B) crosses in which heterospecific sperm are 10 times more abundant than conspecific. Results for two families of each type (1-2) are shown. Paternity of some larvae in family 2 could not be assigned (shaded grey). Crosses in which observed and expected frequencies were significantly different are indicated by * ($p < 0.02$). See results for a discussion of paternity analysis in family two.

These results together suggest that some clutches of eggs from *A. forbesi* are preferentially fertilized by *A. forbesi* sperm, in the presence of low or high concentrations of heterospecific sperm (Fig. 5.1).

I found a corresponding set of results in experiments with *A. rubens* eggs fertilized by conspecific and heterospecific sperm in competition: in some crosses, *A. forbesi* appear to be competitively superior to *A. rubens* sperm. In one replicate experiment with conspecific sperm in high concentration (Table 5.1, family 1 in Fig. 5.2A) approximately 7% of the eggs were expected to be fertilized by heterospecific sperm, but *A. forbesi* sired 82% of the offspring. This same family did conform to the 10:1 expected paternity ratio when *A. rubens* eggs were mixed with high concentrations of heterospecific and low concentrations of conspecific sperm.

The paternity of the offspring from the second family of *A. rubens* in Fig. 5.2 was more difficult to assign because one allele combination was shared between both half-sib families (due to an error in genotyping the parents that was not detected until after the experiment was complete). I include the results from this family here because at least some of the offspring could be assigned paternity whereas in other cases, not included in the analysis, paternity assignment was not possible.

One-half of the offspring from the heterospecific cross and three-quarters of the offspring from the conspecific cross could be assigned paternity with certainty. In the cross with high conspecific sperm concentrations, 45 larvae were sired by *A. rubens,* four larvae were sired by *A. forbesi* and eight larvae had unknown paternity. In the cross with high heterospecific sperm concentrations, eight larvae were sired by *A. rubens*, fourteen larvae were sired by *A. forbesi*, and thirty-two had unknown paternity. In both crosses,

the observed ratios of paternity were significantly different than the expected ratios in *G*-tests (Table 5.1).

The paternity of most of the offspring in the high conspecific sperm concentration cross could be assigned with certainty to *A. rubens* (79%), and only 14% of the offspring were of unknown paternity (family 2, Fig. 5.2A). In contrast, in the high heterospecific sperm concentration cross, 59% of the offspring could not be assigned paternity (Fig. 5.2B). To infer the frequency of *A. forbesi* (and as a consequence *A. rubens*) paternity that best fit the observations, I compared the expected and observed frequencies of *A. forbesi, A. rubens* and unknown sires across a range of possible frequencies of *A. forbesi* paternity ($p = 0.05$ to 1) using *G*-tests. The smallest *G*-test value in this analysis was then interpreted as the frequency of *A. forbesi* paternity that best fit the observations.

In the high conspecific sperm concentration cross the lowest *G*-test value was found when the frequency of *A. forbesi* paternity was 10%; the expected paternity rate was 9.1% based on the sperm concentrations. In the high heterospecific sperm concentration cross, the lowest *G*-test value was found when the frequency of *A. forbesi* paternity was about 80%; the expected paternity rate was 91% based on the sperm concentrations. The results suggest fertilization of the *A. rubens* female in this family was not very different from what would be expected based on the conspecific and heterospecific sperm concentrations nor is there evidence of *A. forbesi* sperm superiority as seen in family 1 (Fig. 5.2). However, these results should be treated with caution because the observed numbers of larvae with unknown paternity were significantly different than expected (Table 5.1).

Discussion

Gametes of *Asterias forbesi* and *A. rubens* were not reciprocally compatible in sperm competition experiments. Significantly more *A. forbesi* eggs were fertilized by *A. forbesi* sperm on two occasions when *A. rubens* sperm were an order of magnitude more abundant in the mixed suspension. In one case when *A. forbesi* sperm were more abundant than *A. rubens*, there was no heterospecific fertilization of *A. forbesi* eggs. In contrast, there was no evidence of preferential conspecific fertilization of *A. rubens* eggs. In three cases, there were no significant deviations from the expected proportions of paternity in offspring based on conspecific and heterospecific sperm concentrations. However, in the fourth instance, there were significantly more *A. rubens* eggs fertilized by *A. forbesi* than by conspecific sperm.

Sperm competition can occur at any of the steps to in prezygotic life from initial release of the sperm to fusion of the sperm and egg membranes. Conspecific sperm may have an advantage over heterospecific sperm by: release in closer proximity (spatially or temporally) to conspecific eggs, activation by species-specific egg-derived chemoattractants, faster binding to and penetration of the egg envelope, and more effective fusion with the egg membrane (Howard 1999). Each of these mechanisms by which conspecific sperm preference can operate has been found in marine invertebrates.

Chemoattractants produced by eggs can play a role in gamete recognition as they can activate conspecific sperm motility and cause chemotaxis towards the egg (Miller 1985a, 1997). Echinoderm sperm chemotaxis specificity has been found in holothurians mainly at the family level and at the genus or species levels in ophiuroids (Miller 1997). Within the asteroids, the families Asteriidae and Solasteridae have shown sperm attractant

cross-reactivity between them, but not with any other asteroid families tested (Miller 1985b). Since sperm chemotaxis is not known to be species-specific within asteroid families (Miller 1985b), it is unlikely that this mechanism is responsible for the differential fertilization success of *A. forbesi* and *A. rubens* in this study.

Several gamete recognition proteins have been identified and recognized for their roles in species recognition (reviews in Vacquier *et al.* 1995, Vacquier 1998, Swanson and Vacquier 2002). In sea urchins, the sperm protein bindin is involved in species-specific sperm-egg attachment and sperm-egg fusion (Glabe and Vacquier 1977, Glabe and Lennarz 1979, Metz *et al.* 1994). Barriers to cross-fertilization have been examined using bindin divergence; in some genera rapid divergence has been observed (*Echinometra*: Metz and Palumbi 1996; *Strongylocentrotus*: Biermann 1998; *Heliocardis*: Zigler *et al.* 2003), while in other sea urchin genera bindin divergence is much lower (*Arbacia*: Metz *et al.* 1998; *Tripneustes*: Zigler and Lessios 2003) In abalone (*Haliotus*) and teguline gastropods (*Tegula*), sperm release a soluble protein called lysin which allows the sperm to penetrate the egg envelope in a species-specific manner (Vacquier *et al.* 1990, Shaw *et al.* 1994, Hellberg and Vacquier 1999). Abalone sperm also release a protein (sp18) that is thought to mediate fusion of sperm and egg (Swanson and Vacquier 1995, Kresge *et al.* 2001). In addition to lysin and sp18, *Tegula* sperm also release a major acrosomal protein (TMAP) whose function is unknown, but is highly divergent (Hellberg *et al.* 2000). As yet, a gamete recognition system has not been found in sea stars, but such sperm-egg molecular interactions could provide one explanation of the conspecific sperm preference demonstrated by *A. forbesi* in this study.

Not all crosses with *A. forbesi* eggs showed evidence of preferential conspecific fertilization. *Asterias forbesi* did not outcompete *A. rubens* in all crosses and *A. rubens*

sperm were capable of fertilizing *A. forbesi* eggs, even when conspecific sperm were more abundant. There are several possible explanations for this partial species-specificity, including differences in gamete quality and nonrandom mating. Variability in compatibility in sperm and egg recognition proteins may differ within species (Palumbi 1999), and the ability to cross-fertilize is influenced by intraspecific processes (Levitan 2002). It is not possible to distinguish among possible explanations of the partial species specificity observed in this study. The evidence suggests some form of prezygotic reproductive isolation barrier, possibly in the form of a gamete recognition system, however further studies are needed to confirm this hypothesis.

While *A. forbesi* exhibit some preferential conspecific fertilization in competition, in at least one cross with *A. rubens* eggs there was significantly more heterospecific fertilization than expected and *A. forbesi* appeared to be competitively superior to *A. rubens* sperm. Differences in interspecific gamete quality such as sperm motility may account for this superiority. Variation in intraspecific sperm motility in multifactorial crosses of the oyster *Crassostrea gigas* has been suggested as an explanation of large variance in parental contributions (Boudry *et al.* 2002). The condition of the male may have also influenced the quality of the sperm: in sperm competition studies of Atlantic cod (*Gadus morhua*), fertilization success was positively correlated with the male's condition (Rakitin *et al.* 1999). My experiment was conducted in July, at the end of the spawning season for *A. rubens* in Nova Scotia and as such *A. rubens* sperm may have been of poorer quality than *A. forbesi*.

Differential viability of hybrid offspring may have been a factor in this study. If hybrid larvae had reduced fitness and viability, this would have resulted in significantly greater conspecific paternity rates. While this explanation is plausible, the observation of

some heterospecific paternity rates in proportion to sperm concentrations suggests hybrid larvae abortion was not extensive. Although no-choice cross-fertilization studies did not examine hybrid fitness (Chapter Two), larvae from all heterospecific crosses were successfully reared through metamorphosis.

Sperm competition may be an important component of speciation mechanisms in marine invertebrates (Bierne *et al.* 2002a). Intraspecific gamete preference has been found in sperm competition studies of the mussels *Mytilus edulis* and *M. galloprovincialis* (Bierne *et al.* 2002a). Despite habitat specialization and asynchronous spawning (Gardner 1994), there is extensive hybridization in these species (Skibinski *et al.* 1983, Daguin *et al.* 2001). Partial prezygotic isolation may be the result of preferential fertilization in competition.

Previous studies of the sea stars *A. forbesi* and *A. rubens* indicate a high potential for cross-fertilization and hybridization between the species (Ernst 1967, Schopf and Murphy 1973, Menge 1986). However, morphological and phylogenetic studies did not detect hybrids in the field (Worley and Franz 1983, Wares 2001). A subsequent phylogenetic analysis focused on specimens collected from the contact zone and discovered evidence of asymmetric introgression of *A. rubens* mtDNA into *A. forbesi* populations (Chapter Four). Possible explanations given for the asymmetry were insufficient sampling, the inability of the marker to detect other hybrids and asymmetric gamete compatibility that had not been detected in interspecific no-choice crosses (Chapter Two). The partial conspecific preference exhibited by *A. forbesi* and the ability of *A. forbesi* sperm to outcompete *A. rubens* sperm in gamete competition studies suggest that if natural hybridization occurs, it is more likely to occur between eggs of *A. rubens* and sperm of *A. forbesi*. If the eggs of female F_1 hybrids are also more likely to be

fertilized by *A. forbesi* sperm, then several generations of backcrossing could result in morphologically unambiguous *A. forbesi* individuals with *A. rubens* mtDNA haplotypes (Chapter Four). In Chapter Two, eggs of a female F_1 hybrid with *A. rubens* mtDNA were more successfully backcrossed to a male *A. forbesi* than to a male *A. rubens*. Such an asymmetrical interaction between closely-related species in sympatry following secondary contact indicates gametic interactions are an important prezygotic reproductive barrier and suggests *Asterias* spp. may have a gamete recognition system similar to bindin in the sea urchins.

CHAPTER SIX:

CONCLUSIONS

In this thesis, I demonstrate that *Asterias forbesi* and *A. rubens* are not in complete reproductive isolation in secondary contact. Although previous studies have suggested the species lack barriers to reproduction (Ernst 1967, Menge 1986, Clark and Downey 1992), recent surveys of morphological and molecular variation have failed to detect hybridization and introgression in the contact zone (Worley and Franz 1983, Wares 2001). In contrast, I found evidence of hybridization and asymmetric introgression using a molecular marker. Gametic interactions are an important determinant of the outcome of secondary contact in these closely-related broadcast-spawning species whose spawning periods overlap in sympatry.

In cross-fertilization studies conducted *in vitro*, eggs of both species were successfully fertilized by conspecific and heterospecific sperm. However, there was high variation in the compatibility of heterospecific crosses between gametes of *A. rubens* and *A. forbesi* (Chapter Two). While the compatibility of some combinations of heterospecific gametes was comparable with conspecific crosses, other combinations required an order of magnitude more heterospecific sperm for successful fertilization. This variation in compatibility of heterospecific gametes may be the result of intraspecific variation at compatibility loci in females.

Differential compatibility of heterospecific gametes was demonstrated in sperm competition studies (Chapter Five). Results of these studies indicated the gametes of *A. rubens* and *A. forbesi* were not reciprocally compatible when sperm of both species were simultaneously in suspension. There was evidence of conspecific fertilization preference

in *A. forbesi*: significantly more *A. forbesi* eggs were fertilized by conspecific sperm on two occasions when *A. rubens* sperm were an order of magnitude more abundant. Sperm of *A. forbesi* were also competitively superior to *A. rubens* in fertilizing *A. forbesi* eggs. In contrast, there was no evidence for conspecific fertilization preference in *A. rubens*. In one instance, there were significantly more *A. rubens* eggs fertilized by *A. forbesi* than by conspecific sperm. If *A. rubens* experiences sperm competition with *A. forbesi* within the contact zone there may be lower rates of fertilization for *A. rubens* males even in spawning groups that include *A. rubens* females.

Prezygotic barriers to reproductive may be more effective in maintaining the genetic integrity of sympatric *Asterias* spp. as postzygotic barriers do not appear to be strong. Hybrid offspring raised in culture were viable and fertile (Chapter Two). An F_1 female with *A. rubens* mtDNA and an F_1 male with *A. forbesi* mtDNA successfully backcrossed with both parental species. Eggs of the female hybrid were more successful in backcrosses with male *A. forbesi* (47%) than with male *A. rubens* (19%). Similarly, the male hybrid more successfully fertilized eggs of *A. forbesi* (36%) than *A. rubens* (10%). Hybrid breakdown may be a potential barrier between these species since the fertilization success of hybrid backcrosses was reduced and hybrid fitness may be reduced under natural conditions.

Evidence of hybridization and asymmetric introgression in the secondary contact zone was detected in the survey of allopatric and sympatric *Asterias* using an mtDNA marker (Chapter Four). *Rubens*-like haplotypes were found in three individuals with A. *forbesi*-like phenotypes. This was probably the result of *A. rubens* eggs being fertilized by *A. forbesi* sperm; however the lack of observable *rubens*-like phenotypic traits in these

individuals suggests they were descended from female hybrids by one or more generations of matrilineal backcrossing with *A. forbesi* (and not *A. rubens*).

Asymmetric gamete compatibility appears to have led to asymmetric introgression of mtDNA from *A. rubens* into *A. forbesi*. There was substantially higher fertilization success of a female F_1 hybrid with *A. rubens* mtDNA backcrossed with an *A. forbesi* male than with an *A. rubens* male, the direction predicted if backcrossing success was biased towards *A. forbesi*. The three specimens with *rubens*-like haplotypes and *forbesi*-like phenotypes were probably the result of similar backcrosses. While *A. forbesi* eggs exhibited conspecific fertilization preference in competition, *A. rubens* did not exhibit any evidence of fertilization preference. In three cases the eggs of *A. rubens* were fertilized by *A. forbesi* and *A. rubens* in proportion to their sperm concentrations in mixed suspension and in the fourth instance, *A. forbesi* was competitively superior. The asymmetrical interaction between sympatric *Asterias* spp. indicates gametic interactions are an important prezygotic reproductive barrier in these taxa and suggests *Asterias* spp. may have a gamete recognition protein system analogous to bindin in the sea urchins.

The phenotypes of *A. forbesi* and *A. rubens* form a bimodal distribution in secondary contact. The morphological survey of sympatric and allopatric *Asterias* populations revealed a high frequency of parental phenotypes and few individuals with intermediate phenotypes (Chapter Three). A distinct group of intermediate phenotypes that might have been F_1 hybrids (or recent descendants of hybrids) was not quantitatively supported. Indeed, the five qualitative diagnostic traits were not capable of detecting individuals of known hybrid pedigree in the analysis.

The bimodal hybrid zone with asymmetric introgression formed by *Asterias* in the northwest Atlantic is similar to the terrestrial hybrid zone between two species of the

cricket genus *Gryllus*. *Gryllus pennsylvania* and *G. firmus* form a narrow hybrid zone which has a deficit of heterozygotes, a bimodal distribution of genotypes, and high linkage disequilibria suggesting minimal intermixing of the two species (Harrison 1986, Harrison and Rand 1989, Harrison and Bogdanowicz 1997). In laboratory studies, female *G. pennsylvania* exhibit conspecific preferential mating (Harrison and Rand 1989). Fertile hybrids are formed only from crosses between *G. pennsylvania* females and *G. firmus* males (Harrison 1983). Asymmetric introgression of *G. pennsylvania* mtDNA into *G. firmus* populations has been detected (Harrison 1983, Harrison *et al.* 1987), yet the two species remain distinct in sympatry. The *Gryllus* hybrid zone has been demonstrated to have the genetic structure matching a mosaic model (Harrison 1986, 1990; Rand and Harrison 1989). This hybrid zone can be used to test hybrid fitness in relation to environment and if there are genotype- environment associations that demonstrate concordant and coincident clinal variation (review in Arnold 1997).

Unlike studies of the *Gryllus* hybrid zone, the *Asterias* contact zone has been only been summarily described using mtDNA and morphological markers. Analyses using biparentally inherited nuclear markers are necessary to determine the extent and frequency of hybridization. Further description of the *Asterias* secondary contact zone in the northwest Atlantic may enable future studies to test theories of speciation and hybrid zone dynamics using these closely-related species.

Other hybrid zones formed by marine invertebrates have provided significant contributions to our understanding of species interactions in secondary contact. For example, the hard-clam hybrid zone in Florida formed by secondary contact between *Mercenaria mercenaria* and *M. campechiensis* has been used as an empirical test of two competing hybrid zone models and demonstrated a complex interaction between the two

types of selective forces involved (Bert and Arnold 1995). The hybrid zones formed by the mussel *Mytilus* species complex are perhaps the best studied in the marine environment and have been used to evaluate mechanisms of hybridization and introgression patterns (e.g., Gardner 1994, 1996; Bierne *et al.* 2002, 2003). Evidence contrary to the predictions of reinforcement has been found in studies of the sea urchin *Echinometra* (Lessios and Cunningham 1990, McCartney and Lessios 2002); gamete incompatibility between allopatric species was higher than between sympatric species. *Asterias forbesi* and *A. rubens* are already model organisms of echinoderm development and biology; further investigation of hybridization and introgression in their contact zone could potentially provide insight into mechanisms of reproductive isolation in asteroid echinoderms.

REFERENCES

Aldrich, A. 1956. A comparative study of the identification characters of *Asterias forbesi* and *A. vulgaris*. *Notulae Natur.* 285: 1-3.

Anderson, E. 1949. *Introgressive Hybridization*. John Wiley and Sons, New York.

Arndt, A., and M.J. Smith. 1998. Genetic diversity and population structure in two species of sea cucumber: differing patterns according to mode of development. *Mol. Ecol.* 7: 1058-1064.

Arnold, M.J. 1997. *Natural hybridization and evolution*. Oxford University Press, New York.

Arntzen, J.W. 1978. Some hypotheses on postglacial migrations of the fire-bellied toad, *Bombina bombina* (Linnaeus) and the yellow-bellied toad, *Bombina variegata* (Linnaeus). *J. Biogeogr.* 5: 339-345.

Austerlitz, F., Jung-Muller, B., Godelle, B., and P.-H. Gouyon. 1997. Evolution of coalescence times, genetic diversity and structure during colonization. *Theor. Popul. Biol.* 51: 148-164.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and K. Struhl, eds. 1999. *Current Protocols in Molecular Biology*. Wiley, New York.

Avise, J.C. 1989. Gene trees and organismal histories: a phylogenetic approach to population biology. *Evolution* 43: 1192-1208.

Avise, J.C. 1992. Molecular population structure and the biogeographic history of a regional fauna: a case history with lessons for conservation biology. *Oikos* 63: 62-76.

Avise, J.C. 1994. *Molecular markers, natural history and evolution*. Chapman & Hall, New York.

Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., Reeb, C.A., and N.C. Saunders. 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annu. Rev. Ecol. Syst.* 18: 489-522.

Avise, J.C., Bermingham, E., Kessler, L.G., and N.C. Saunders. 1984. Characterization of mitochondrial DNA variability in a hybrid swarm between subspecies of bluegill sunfish (*Lepomis mactochirus*). *Evolution* 38: 931-941.

Barton, N.H. 1983. Multilocus clines. *Evolution 37*: 454-471.

Barton, N.H., and G.M. Hewitt. 1981. Hybrid zones and speciation. Pp. 109-145 in *Evolution and Speciation: Essays in Honor of M.J.D. White.* W.R. Atchley and D.S. Woodruff, eds. Cambridge University Press, Cambridge, MA.

Barton, N.H., and G.M. Hewitt. 1985. Analysis of hybrid zones. *Ann. Rev. Ecol. Syst.* 16: 113-148.

Barton, N.H., and G.M. Hewitt. 1989. Adaptation, speciation and hybrid zones. *Nature* 341: 497-503.

Bella, J.L., Butlin, R.K., Ferris, C., and G.M. Hewitt. 1992. Asymmetrical homogamy and unequal sex ratio from reciprocal mating-order crosses between *Chorthippus parallelus* subspecies. *Heredity* 68: 345-352.

Benzie, J.A.H., and P. Dixon. 1994. The effects of sperm concentration, sperm:egg ratio, and gamete age on fertilization success in crown-of-thorns starfish (*Acanthaster planci*) in the laboratory. *Biol. Bull.* 186: 139-152.

Bert, T.M. 1986. Speciation in western Atlantic stone crabs (genus *Menippe*): the role of geological processes and climatic events in the formation and distribution of species. *Mar. Biol.* 93: 157-170.

Bert, T.M., and W.S. Arnold. 1995. An empirical test of predictions of two competing models for the maintenance and fate of hybrid zones: both models are supported in a hard-clam hybrid zone. *Evolution* 49: 276-289.

Bert, T.M., and R.G. Harrison. 1988. Hybridization in western Atlantic stone crabs (genus *Menippe*): evolutionary history and ecological context influence species interactions. *Evolution* 42: 528-544.

Bert, T.M., Hesselman, D.M., Arnold, W.S., Moore, W.S., Cruz-Lopez, H., and D.C. Marelli. 1993. High frequency of gonadal neoplasia in a hard clam (*Mercenaria* sp.) hybrid zone. *Mar. Biol.* 117: 97-104.

Bert, T.M., McCarthy, K.J., Cruz-Lopez, H., and S. Bogdanowicz. 1996. Character discriminatory power, character-set congruence, and the classification of individuals from hybrid zones: an example using stone crabs (*Menippe*). *Evolution* 50: 655-671.

Biermann, C.H. 1998. The molecular evolution of sperm bindin in six species of sea urchins (Echinoidea: Strongylocentrotidae). *Mol. Biol. Evol.* 15: 1761-1771.

Bierne, N., Launey, S., Naciri-Graven, Y., and F. Bonhomme. 1998. Early effect of inbreeding as revealed by microsatellite analysis on *Ostrea edulis* larvae. *Genetics* 55: 190-195.

Bierne, N., Borsa, P., Daguin, C., Jollivet, D., Viard, F., Bonhomme, F., and P. David. 2003. Introgression patterns in the mosaic hybrid zone between *Mytilus edulis* and *M. galloprovincialis*. *Mol. Ecol.* 12: 447-461.

Bierne, N., David, P., Boudry, P., and F. Bonhomme. 2002a. Assortative fertilization and selection at larval stage in the mussels *Mytilus edulis* and *M. galloprovincialis*. *Evolution* 56: 292-298.

Bierne, N., David, P., Langlade, A., and F. Bonhomme. 2002b. Can habitat specialisation maintain a mosaic hybrid zone in marine bivalves? *Mar. Ecol. Prog. Ser.* 245: 157-170.

Boolootian, R.A. 1966. Reproductive physiology. Pp. 561-614 in *Physiology of Echinodermata*. R.A. Boolootian, ed. Interscience, New York.

Boudry, P., Collet, B., Cornette, F., Hervouet, V., and F. Bonhomme 2002. High variance in reproductive success of the Pacific oyster (*Crassostrea gigas*, Thunberg) revealed by microsatellite-based parentage analysis of multifactorial crosses. *Aquaculture* 204: 283-296.

Boursot, P., Auffray, J.-C., Britton-Davidian, J., and F. Bonhomme. 1993. The evolution of house mice. *Annu. Rev. Ecol. Syst.* 24: 119-152.

Brünner, H., Lugon-Moulin, N., and J. Hausser. 2002. Alps, genes and chromosomes: their role in the formation of species in the *Sorex araneus* group (Mammalia, Insectivora), as inferred from two hybrid zones. *Cytogenet. Genome Res.* 96: 85- 96.

Burke, J.M., and M.L. Arnold. 2001. Genetics and the fitness of hybrids. *Annu. Rev. Genet.* 35: 31-52.

Butlin, R.K., and G.M. Hewitt. 1985. A hybrid zone between *Chorthippus parallelus parallelus* and *Chorthippus parallelus erythropus* (Orthoptera: Acrididae): behavioural characters. *Biol. J. Linn. Soc.* 26: 287-299.

Butlin, R.K., and M.G. Ritchie. 1991. Comparisons among morphological characters between localities in the *Chorthippus parallelus* hybrid zone (Orthoptera: Acrididae). *Phil. Trans. R. Soc. B* 334: 297-308.

Byrne, M., and M.J. Anderson. 1994. Hybridization of sympatric *Patiriella* species (Echinodermata: Asteroidea) in New South Wales. *Evolution* 48: 564-576.

Calinski, T., and J. Harabasz. 1974. A dendrite method for cluster analysis. *Comm. Stat.* 3:1-27.

Campton, D.E. 1987. Natural hybridization and introgression in fishes. Methods of detection and genetic interpretations. Pp. 161-192 in *Population Genetics and*

Fishery Management. N. Ryman and F. Utter. University of Washington Press, Seattle.

Chia, F.S. 1966. Systematics of the six-rayed sea star, *Leptasterias*, in the vicinity of San Juan Island, Washington. *Syst. Zool.* 15: 300-306.

Chia, F.S., and L.R. Bickell. 1983. Echinodermata. Pp. 545-620 in *Reproductive Biology of Invertebrates, Vol II: Spermatogenesis and Sperm Function.* K.G. Adiyondi and R.G. Adiyondi, eds. John Wiley and Sons, New York.

Clark, H.L. 1904. The echinoderms of the Woods Hole region. *Bull. U.S. Fish Comm.* 22:547-576.

Clark, A.M., and M.E. Downey. 1992. *Starfishes of the Atlantic.* Chapman & Hall, New York.

Clement, M., Posada, D., and K. A. Crandall. 2000. TCS: a computer program to estimate gene genealogies. *Mol. Ecol.* 9: 1657-1660.

CLIMAP 1976. The surface of the ice-age Earth. *Science* 191: 1131-1137.

Coe, W.R. 1912. Echinoderms of Connecticut. *Conn. State Geol. Natur. Hist. Surv.* 19: 1-152.

Coope, G.R. 1977. Fossil coleoptran assemblages as sensitive indicators of climatic changes during the Devensian (last) Cold Stage. *Phil. Trans. R. Soc. B* 280: 313-340.

Coope, G.R. 1990. The invasion of Northern Europe during the Pleistocene by Mediterranean species of Coleoptera. Pp. 203-215 in *Biological Invasions in Europe and the Mediterranean Basin.* F. di Castri, A.J. Hansen, and M. De Bussche, eds. Kluwer, Dordrecht.

Cooper, S.J.B., Ibrahim, K.M., and G.M. Hewitt. 1995. Postglacial expansion and genome subdivision in the European grasshopper *Chorthippus parallelus. Mol. Ecol.* 4: 49-60.

Coyne, J.A., and H.A. Orr. 1989. Two rules of speciation. Pp. 189-207 in *Speciation and its genetic consequences.* D. Otte and J.A. Endler, eds. Sinauer, Sunderland, MA.

Coyne, J.A., and H.A. Orr. 1997. "Patterns of speciation" revisited. *Evolution* 51: 295-303.

Daguin, C., Bonhomme, F., and P. Borsa. 2001. Mosaicism in the European zone of sympatry and hybridization of *Mytilus edulis* and *M. galloprovincialis* as revealed by intron length polymorphism at locus *mac-1. Heredity* 86: 342-354.

DePamphilis, C.W., and R. Wyatt. 1990. Electrophoretic confirmation of interspecific hybridization in *Aescusus* (hippocastanaceae) and the genetic structure of a broad hybrid zone. *Evolution* 44: 1295-1317.

Dillon Jr., R.T., and J.J. Manzi. 1989. Genetics and shell morphology in a hybrid zone between the hard clams *Mercenaria mercenaria* and *M. campechiensis*. *Mar. Biol.* 100: 217-222.

Dobzhansky, T. 1937. *Genetics and the origin of species*. Columbia University Press, New York.

Dobzhansky, T. 1940. Speciation as a stage in evolutionary divergence. *Amer. Nat.* 74: 312-321.

Dodd, D.M.B. 1989. Reproductive isolation as a consequence of adaptive divergence in *Drosophila pseudoobscura*. *Evolution* 43: 1308-1311.

Downey, M.E. 1973. *Starfishes from the Caribbean and the Gulf of Mexico*. Smithsonian Contr. Zool. 126.

Durrett, R., Buttel, L., and R. Harrison. 2000. Spatial models for hybrid zones. *Heredity* 84: 9-19.

D'yakonov, A.M. 1968. *Sea stars (Asteroids) of the USSR Seas* (translated from Russian). Israel Program for Scientific Translations, Jerusalem.

Eady, P.E. 2001. Postcopulatory, prezygotic reproductive isolation. *J. Zool.* 253: 47-52.

Endler, J.A. 1977. *Geographic variation, speciation, and clines*. Princeton University Press, Princeton.

Ernst, E.J. 1967. *The distribution, ecology, environmental behavior and possible hybridization of the sea stars Asterias forbesi (Desor) and Asterias vulgaris Verrill in the subtidal zone of Long Island.* Ph.D. dissertation, New York University (University Microfilms #68-6060).

Feder, H.M., and A.M. Christensen. 1966. Aspects of asteroid biology. Pp. 88-127 in *Physiology of Echinodermata*. R.A. Boolootian, ed. Interscience, New York.

Flanagan, N.S., Mason, P.L., Gosálvez, J., and G.M. Hewitt. 1999. Chromosomal differentiation through an Alpine hybrid zone in the grasshopper *Chorthippus parallelus*. *J. Evol. Biol.* 12: 577-585.

Flowers, J.M., and D.W. Foltz. 2001. Reconciling molecular systematics and traditional taxonomy in a species-rich clade of sea stars (*Leptasterias* subgenus *Hexasterias*). *Mar. Biol.* 139: 475-483.

Foighil, D.O., Hilbish, T.J., and R.M. Showman. 1996. Mitochondrial gene variation in *Mercenaria* clam sibling species reveals a relict secondary contact zone in the western Gulf of Mexico. *Mar. Biol.* 126: 675-683.

Foltz, D.W. 1997. Hybridization frequency is negatively correlated with divergence time of mitochondrial DNA haplotypes in a sea star (*Leptasterias* spp.) species complex. *Evolution* 51: 283-288.

Foltz, D.W., Breaux, J.P., Campagnaro, E.L., Herke, S.W., Himel, A.E., Hrincevich, A.W., Tamplin, J.W., and W.B. Stickle. 1996b. Limited morphological differences between genetically identified cryptic species within the *Leptasterias* species complex (Echinodermata: Asteroidea). *Can. J. Zool.* 74: 1275-1283.

Foltz, D.W., Stickle, W.B., Campagnaro, E.L., and A.E. Himel. 1996a. Mitochondrial DNA polymorphisms reveal additional genetic heterogeneity within the *Leptasterias hexactis* (Echinodermata: Asteroidea) species complex. *Mar. Biol.* 125: 569-578.

Franz, D.R., Worley, E.K., and S. Merrill. 1981. Distribution patterns of common seastars of the Middle Atlantic continental shelf of the northwest Atlantic (Gulf of Maine to Cape Hatteras). *Biol. Bull.* 160: 394-418.

Gardner, J.P.A. 1994. The structure and dynamics of naturally occurring hybrid *Mytilus edulis* and *M. galloprovincialis* (Bivalvia, Mollusca) populations: review and interpretation. *Arch. Hydrobiol.* 99(Supp.): 37-71.

Gardner, J.P.A. 1996. The *Mytilus edulis* species complex in southwest England: effects of hybridization and introgression upon interlocus associations and morphometric variation. *Mar. Biol.* 125: 385-399.

Gardner, J.P.A. 1997. Hybridization in the sea. *Adv. Mar. Biol.* 31: 1-78.

Gardner, K., Buerkle, A., Whitton, J., and L.H. Rieseberg. 2000. Inferring epistasis in wild sunflower hybrid zones. Pp. 264-279 in *Epistasis and the Evolutionary Process*. J.B. Wolf, E.D. Brodie III, and M.J. Wade, eds. Oxford University Press, Oxford.

Geary, D.H. 1992. An unusual pattern of divergence between two fossil gastropods: ecophenotypy, dimorphism, or hybridization? *Paleobiology* 18: 93-109.

Gerber, S., Mariette, S., Streiff, R., Bodénès, C., and A. Kremer. 2000. Comparison of microsatellites and amplified fragment length polymorphism markers for parentage analysis. *Mol. Ecol.* 9: 1037-1048.

Glabe, C.G., and W.J. Lennarz. 1979. Species-specific sperm adhesion in sea urchins. A quantitative investigation of bindin-mediated egg agglutination. *J. Cell. Biol.* 83: 595-604.

Glabe, C.G., and V.D. Vacquier. 1977. Species specific agglutination of eggs by bindin isolated from sea urchin sperm. *Nature* 267: 822-824.

Grant, C.M., Hooker, S.H., Babcock, R.C., and R.G. Creese. 1998. Synchronous spawning and reproductive incompatibility of two bivalve species: *Paphies subtriangulata* and *Paphies australis*. *Veliger* 41: 148-156.

Gray, I.E., Downey, M., and M.J. Cerame-Vivas. 1968. Sea stars of North Carolina. *Bull. U.S. Fish Wildlife* 87: 127-163.

Gregory, P.G., and D.J. Howard. 1994. A postinsemination barrier to fertilization isolates two closely related ground crickets. *Evolution* 48: 705-710.

Grosberg, R.K., Levitan, D.R., and B.B. Cameron. 1996. Simple extraction and RAPD-PCR protocols. Pp. 470-478 in *Molecular zoology: advances, strategies, and protocols.* J.D. Ferraris and S.R. Palumbi, eds. Wiley-Liss, New York.

Hamilton, M.B., Pincus, E.L., DiFiore, A., and R.C. Fleischer. 1999. Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *Biotechniques* 27: 500-507.

Hare, M.P., and J.C. Avise. 1996. Molecular genetic analysis of a stepped multilocus cline in the American oyster (*Crassostrea virginica*). *Evolution* 50: 2305-2315.

Harris, L.G., Tyrrell, M., and Chester, C.M. 1998. Changing patterns for two sea stars in the Gulf of Maine, 1976-1996. Pp. 243-248 in *Echinoderms: San Francisco.* R. Mooi and M. Telford, eds. Balkema, Rotterdam.

Harrison, R.G. 1983. Barriers to gene exchange between closely related species. I. Laboratory hybridization studies. *Evolution* 37: 245-251.

Harrison, R.G. 1985. Barriers to gene exchange between closely related species. II. Life cycle variation and temporal isolation. *Evolution* 39: 244-259.

Harrison, R.G. 1986. Pattern and process in a narrow hybrid zone. *Heredity* 56: 337-349.

Harrison, R.G. 1990. Hybrid zones: windows on evolutionary process. *Oxford Surveys in Evolutionary Biology* 7: 69-128.

Harrison, R.G. 1993. *Hybrid zones and the Evolutionary Process.* Oxford University Press, New York.

Harrison, R.G., and J. Arnold. 1982. A narrow hybrid zone between closely related cricket species. *Evolution* 36: 535-552.

Harrison, R.G., and S.M. Bogdanowicz. 1997. Patterns of variation and linkage disequilibrium in a field cricket hybrid zone. *Evolution* 51: 493-505.

Harrison, R.G., and D. Rand. 1989. Mosaic hybrid zones and the nature of species boundaries. Pp. 111-133 in *Speciation and its Consequences* D. Otte and J. Endler, eds. Sinauer, Sunderland, MA.

Harrison, R.G., Rand, D., and W.C. Wheeler. 1987. Mitochondrial DNA variation in field crickets across a narrow hybrid zone. *Mol. Biol. Evol.* 4: 144-158.

Harshman, L.G., and T. Prout. 1994. Sperm displacement without sperm transfer in *Drosophila melanogaster. Evolution* 48: 758-766.

Hartigan, J.A. 1975. *Clustering algorithms.* New York: John Wiley & Sons. 351 pp.

Hartl, D.L., and A.G. Clark. 1997. *Principles of population genetics*, 3rd ed. Sinauer, Sunderland, MA.

Hellberg, M. 1994. Relationships between inferred levels of gene flow and geographic distance in a philopatric coral, *Balanophillia elegans. Evolution* 48: 1829-1859.

Hellberg, M. 1995. Stepping-stone gene flow in a solitary coral *Balanophillia elegans*: equilibrium and nonequilibrium at different spatial scales. *Mar. Biol.* 123: 573- 581.

Hellberg, M. 1996. Dependence of gene flow on geographic distance in two solitary corals with different larval dispersal capabilities. *Evolution* 50: 1167-1175.

Hellberg, M.E. and V.D. Vacquier. 1999. Rapid evolution of fertilization selectivity and lysin cDNA sequences in teguline gastropods. *Mol. Biol. Evol.* 16: 839-848.

Hellberg, M.E., Moy, G.W., and V.D. Vacquier. 2000. Positive selection and propeptide repeats promote rapid interspecific divergence of a gastropod sperm protein. *Mol. Biol. Evol.* 17: 458-466.

Hewitt, G.M. 1988. Hybrid zones – natural laboratories for evolutionary studies. *Trends Ecol. Evol.* 3: 158-167.

Hewitt, G.M. 1993. After the ice: *parallelus* meets *erythropus* in the Pyrenees. Pp. 140-164 in *Hybrid Zones and the Evolutionary Process*. R.G. Harrison, ed. Oxford University Press, New York.

Hewitt, G.M. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. *Biol. J. Linn. Soc.* 58: 247-276.

Hewitt, G.M. 1999. Post-glacial recolonization of European Biota. *Biol. J. Linn. Soc.* 68: 87-112.

Hewitt, G.M. 2000. The genetic legacy of the Quaternary ice ages. *Nature* 405-907-913.

Hewitt, G.M., and R.K. Butlin. 1997. Causes and consequences of population structure. Pp. 149-170 in *Behavioural Ecology: An Evolutionary Approach*. J. Krebs and N. Davies, eds. Blackwell, Oxford.

Hewitt, G.M., Mason, P., and R.A. Nichols. 1989. Sperm precedence and homogamy across a hybrid zone in the alpine grasshopper *Podisma pedestris*. *Heredity* 62: 343-353.

Hilbish, T.J. 1985. Demographic and temporal structure of an allele frequency cline in the mussel *Mytilus edulis*. *Mar. Biol.* 86: 163-171.

Hilbish, T.J., Deaton, L.E., and R.K. Koehn. 1982. Effect of an allozyme polymorphism on regulation of cell volume. *Nature* 298: 688-689.

Holanda, P.C. 1995. *Can sea stars Asterias vulgaris and Asterias forbesi be used as indicators of climate change in New England?* M.Sc. dissertation, University of New Hampshire.

Howard, D.J. 1999. Conspecific sperm and pollen precedence and speciation. *Annu. Rev. Ecol. Syst.* 30: 109-132.

Howard, D.J., and S.H. Berlocher. 1998. *Endless Forms, Species and Speciation*. Oxford University Press, New York.

Howard, D.J., and P.G. Gregory. 1993. Postinsemination signaling systems and reinforcement. *Philos. Trans. R. Soc. Lond. B* 340: 231-236.

Howard, D.J., Gregory, P.G., Chu, J., and M.L. Cain. 1998. Conspecific sperm precedence is an effective barrier to hybridization between closely related species. *Evolution* 52: 511-516.

Hrincevich, A.W., and D.W. Foltz. 1996. Mitochondrial DNA sequence variation in a sea star (*Leptasterias* spp.) species complex. *Mol. Phyl. Evol.* 6: 408-415.

Hrincevich, A.W., Rocha-Olivares, A., and D.W. Foltz. 2000. Phylogenetic analysis of molecular lineages in a species-rich subgenus of sea stars (*Leptasterias* Subgenus *Hexasterias*). *Amer. Zool.* 40: 365-374.

Huntley. B. 1990. European vegetation history: palaeovegetation maps from pollen data – 1300 yr BP to present. *J. Quat. Sci.* 5: 103-122.

Huntley, B., and H.J.B. Birks. 1983. *An Atlas of Past and Present Pollen Maps for Europe.* Cambridge University Press, Cambridge.

Huvet, A., Balabaud, K., Bierne, N., and P. Boudry. 2001. Microsatellite analysis of 6-hour-old embryos reveals no preferential intraspecific fertilization between cupped oysters *Crassostrea gigas* and *Crassostrea angulata. Mar. Biotechnol.* 3: 448-453.

Huxley, J.S. 1938. Clines: An auxiliary taxonomic principle. *Nature* 142: 219-220.

James, F.C., and C.E. McCulloch. 1990. Multivariate analysis in ecology and systematics. *Annu. Rev. Ecol. Syst.* 21: 129-166.

Jiggins, C.D., and J. Mallet. 2000. Bimodal hybrid zones and speciation. *Trends in Ecol. Evol.* 15: 250-255.

Kanatani, H. 1979. Hormones in echinoderms. Pp. 273-307 in *Hormones and Evolution.* Vol. 1. E.J.W. Barrington, ed. Academic Press, London.

Knowlton, N. 1993. Sibling species in the sea. *Annu. Rev. Ecol. Syst.* 24: 189-216.

Knowlton, N. 2000. Molecular genetic analyses of species boundaries in the sea. *Hydrobiologia* 420: 73-90.

Koehn, R.K., Milkman, R., and J.B. Mitton. 1976. Population genetics of marine pelecypods. IV. Selection, migration, and genetic differentiation in the blue mussel *Mytilus edulis. Evolution* 30: 2-32.

Koehn, R.K., Newell, R.I.E., and F. Immermann. 1980. Maintenance of an aminopeptidase allele frequency cline by natural selection. Proc. Nat. Acad. Sci. 77: 5385-5389.

Kresge, N., Vacquier, V.D., and C.D. Stout. 2001. Abalone lysin: the dissolving and evolving sperm protein. *Bioessays* 23: 95-103.

Kwast, K.E., Foltz, D.W., and W.B. Stickle. 1990. Population genetics and systematics of the *Leptasterias hexactis* (Echinodermata: Asteroidea) species complex. *Mar. Biol.* 105: 477-489.

Lamb, T., and J.C. Avise. 1987. Morphological variability in genetically defined categories of anuran hybrids. *Evolution* 41: 157-165.

Lambert, P. 1985. Geographic variation of calcareous ossicles and the identification of hree species of sea cucumbers (Echinodermata: Holothuroidea) from the eastern Pacific Ocean. Pp. 437-443 in *Proceedings of the 5th International Echinoderm Conference*. B.F. Keegan and B.D.S. O'Connor, eds. Balkema, Rotterdam.

Latta, R.G., and J.B. Mitton. 1999. Historical separation and present gene flow through a zone of secondary contact in Ponderosa pine. *Evolution* 53: 769-776.

Lessios, H.A. 1981. Reproductive periodicity of the echinoids *Diadema* and *Echinometra* on the two coasts of Panama. *J. Exp. Mar. Biol. Ecol.* 50: 47-61.

Lessios, H.A. 1985. Annual reproductive periodicity in eight species on the Caribbean coast of Panama. Pp. 1-10 in *Echinodermata. Proceedings of the 5th International Echinoderm Conference.* B.F. Keegan and B.D. O'Connor, eds. Balkema, Rotterdam.

Lessios, H.A. 1991. Presence and absence of monthly reproductive rhythms among eight Caribbean echinoids off the coast of Panama. *J. Exp. Mar. Biol. Ecol.* 153: 27-47.

Lessios, H.A. and C.W. Cunningham. 1990. Gametic incompatibility between species of the sea urchin *Echinometra* on the two sides of the Isthmus of Panama. *Evolution* 44: 933-941.

Lessios, H.A., and J.S. Pearse. 1996. Hybridization and introgression between Indo-Pacific species of *Diadema*. *Mar. Biol.* 126: 715-723.

Lessios, H.A., Kessing, B.D., and J.S. Pearse. 2001. Population structure and speciation in tropical seas: global phylogeography of the sea urchin *Diadema*. *Evolution* 55: 955-975.

Levitan, D.R. 1996. Effects of gamete traits on fertilization in the sea and the evolution of sexual dimorphism. *Nature* 382: 153-155.

Levitan, D.R. 1998. Does Bateman's principle apply to broadcast-spawning organisms? Egg traits influence in situ fertilization rates among congeneric sea urchins. *Evolution* 52: 1043-1056.

Levitan, D.R. 2002. The relationship between conspecific fertilization success and reproductive isolation among three congeneric sea urchins. *Evolution* 56: 1599-1609.

Levitan, D.R., and C.M. Young. 1995. Reproductive success in large populations: empirical measures and theoretical predictions of fertilization in the sea biscuit *Clypeaster rosaceus*. *J. Exp. Mar. Biol. Ecol.* 190: 221-241.

Levitan, D.R., Sewell, M.A., and F.-S. Chia. 1991. Kinetics of fertilization in the sea urchin *Strongylocentrotus franciscanus*: interaction of gamete dilution, age, and contact time. *Biol. Bull.* 181: 371-378.

Lewontin, R.C., and L.C. Birch. 1966. Hybridization as a source for adaptation to new environments. *Evolution* 20: 315-336.

Lewontin, R.C., and J. Krakauer. 1973. Distribution of gene frequency as a test of the theory of the selective neutrality of polymorphisms. *Genetics* 74: 175-195.

Lucas, J.S., and M.M. Jones. 1976. Hybrid crown-of-thorns starfish (*Acanthaster planci* x *A. brevispinus*) reared to maturity in the laboratory. *Nature* 263: 409-412.

Lunt, D.H., Ibrahim, K.M, and G.M. Hewitt. 1998. mtDNA phylogeography and postglacial patterns of subdivision in the meadow grasshopper *Chorthippus parallelus*. *Heredity* 80: 633-641.

MacCallum, C.J., Nürnberger, B.D., Barton, N.H., and J.M. Szymura. 1998. Habitat preference in the *Bombina* hybrid zone in Croatia. *Evolution* 52: 227-239.

Marko, P.B. 1998. Historical allopatry and the biogeography of speciation in the prosobranch snail genus *Nucella*. *Evolution* 52: 757-774.

Marko, P.B., Palmer, A.R., and G.J. Vermeij. 2003. Resurrection of *Nucella ostrina* (Gould, 1852), lectotype designation for *N. emarginata* (Deshayes, 1839), and molecular genetic evidence for Pleistocene speciation. *Veliger* 46: 77-85.

Mathews, L.M., Schubart, C.D., Neigel, J.E., and D.L. Felder. 2002. Genetic, ecological, and behavioural divergence between two sibling snapping shrimp species (Crustacea: Decapoda: *Alpheus*). *Mol. Ecol.* 11: 1427-1437.

Mayr, E. 1942. *Systematics and the Origin of Species*, 3rd ed. Columbia University Press, New York.

Mayr, E. 1954. Geographic speciation in tropical echinoids. *Evolution* 8: 1-18.

Mayr, E. 1963. *Animal Species and Evolution*. Harvard University Press, Cambridge, MA.

Mayr, E. 1976. *Evolution and the Diversity of Life*. Harvard University Press, Cambridge, MA.

McCartney, M.A., and H.A. Lessios. 2002. Quantitative analysis of gametic incompatibility between closely related species of neotropical sea urchins. *Biol. Bull.* 202: 166-181.

McCartney, M.A., Keller, G., and H.A. Lessios. 2000. Dispersal barriers in tropical oceans and speciation in Atlantic and eastern Pacific sea urchins of the genus *Echinometra*. *Mol. Ecol.* 9: 1391-1400.

McClary, D.J., and M.A. Sewell. 2003. Hybridization in the sea: gametic and developmental constraints on fertilization in sympatric species of *Pseudoechinus* (Echinodermata: Echinoidea). *J. Exp. Mar. Biol. Ecol.* 284: 51-70.

Menge, B.A. 1979. Coexistence between the seastars *Asterias vulgaris* and *A. forbesi* in a heterogeneous environment: a non-equilibrium explanation. *Oecologia* 41: 245-272.

Menge, B.A. 1986. A preliminary study of the reproductive ecology of the seastars *Asterias vulgaris* and *A. forbesi* in New England. *Bull. Mar. Sci.* 39: 467-476.

Metz, E.C., and S.R. Palumbi. 1996. Positive selection and sequence rearrangements generate extensive polymorphism in the gamete recognition protein bindin. *Mol. Biol. Evol.* 13: 397-406.

Metz, E.C., Kane, R.E., Yanagimachi, H., and S.R. Palumbi. 1994. Fertilization between closely related sea urchins is blocked by incompatibilities during sperm-egg attachment and early stages of fusion. *Biol. Bull.* 187: 23-34.

Metz, E.C., Gomez-Gutierrz, G., and V.D. Vacquier. 1998. Mitochondrial DNA and bindin gene sequence evolution among allopatric species of the sea urchin genus *Arbacia*. *Mol. Biol. Evol.* 15: 185-195.

Miller, R.L. 1985a. Sperm chemo-orientation in the metazoa. Pp. 275-337 in *The Biology of Fertilization*, Vol. 2. C.B. Metz, Jr. and A. Monroy, eds. Academic Press, New York.

Miller, R.L. 1985b. Sperm chemotaxis in echinodermata: Asteroidea, holothuroidea, ophiuroidea. *J. Exp. Zool.* 234: 383-414.

Miller, R.L. 1997. Specificity of sperm chemotaxis among Great Barrier Reef shallow-water holothurians and ophiuroids. *J. Exp. Zool.* 279: 189-200.

Muthiga, N.A. 2003. Coexistence and reproductive isolation of the sympatric echinoids *Diadema savignyi* Michelin and *Diadema setosum* (Leske) on Kenyan coral reefs. *Mar. Biol.* 143: 669-677.

Neigel, J.E., and J.C. Avise. 1986. Phylogenetic relationships of mitochondrial DNA under various demographic models of speciation. Pp. 515-534 in *Evolutionary process and theory.* S. Karlin, ed. Academic Press, New York.

Nichols, D., and M.F. Barker. 1984. Growth of juvenile *Asterias rubens* L. (Echinodermata: Asteroidea) on an intertidal reef in southwestern Britain. *J. Exp. Mar. Biol. Ecol.* 78: 157-165.

Norris, A.T., Bradley, D.G., and E.P. Cunninghan. 2000. Parentage and relatedness determination in farmed Atlantic salmon (*Salmo salar*) using microsatellite markers. *Aquaculture* 182: 73-83.

Nürnberger, B., Barton, N.H., MacCallum, C., Gilchrist, J., and M. Appleby. 1995. Natural selection on quantitative traits in the *Bombina* hybrid zone. *Evolution* 49: 1224-1238.

Oliver, J., and R. Babcock. 1992. Aspects of the fertilization ecology of broadcast spawning corals: sperm dilution effects and in situ measurements of fertilization. *Biol. Bull.* 183: 409-417.

Ortíz-Barrientos, D., Reiland, J., Hey, J., and M.A.F. Noor. 2002. Recombination and the divergence of hybridizing species. *Genetica* 116: 167-178.

Paige, K.N., and W.C. Capman. 1993. The effects of host-plant genotype, hybridization, and environment on gall-aphid attack and survival in cottonwood: the importance of genetic studies and the utility of RFLPs. *Evolution* 47: 36-45.

Palmer, A.R., Gayron, S.D., and D.S. Woodruff. 1990. Reproductive, morphological, and genetic evidence for two cryptic species of northeastern Pacific *Nucella. Veliger* 33: 325-338.

Palumbi, S.R. 1992. Marine speciation on a small planet. *Trends Ecol. Evol.* 7: 114-118.

Palumbi, S.R. 1994. Genetic divergence, reproductive isolation, and marine speciation. *Annu. Rev. Ecol. Syst.* 25: 547-572.

Palumbi, S.R. 1999. All males are not created equal: fertility differences depend on gamete recognition polymorphisms in sea urchins. *Proc. Nat. Acad. Sci.* 96: 12632-12637.

Palumbi, S.R., and E.C. Metz. 1991. Strong reproductive isolation between closely related tropical sea urchins (genus *Echinometra*). *Mol. Biol. Evol.* 8: 227-239.

Pannacciulli, F.G., Bishop, J.D.D., and S.J. Hawkins. 1997. Genetic structure of populations of two species of *Chthamalus* (Crustacea: Cirripedia) in the north-east Atlantic and Mediterranean. *Mar. Biol.* 128: 73-82.

Parker, G.A. 1970. Sperm competition and its evolutionary consequences in insects. *Biol. Rev.* 45: 525-567.

Pearse, J.S. 1998. Distribution of *Diadema savignyi* and *D. setosum* in the tropical Pacific. Pp. 777-782. In *Echinoderms: San Francisco*. R. Mooi and M. Telford, eds. Balkema, Rotterdam.

Pérez-Losada, M., Guerra, A., and A. Sanjuan. 1999. Allozyme differentiation in the cuttlefish *Sepia officialis* (Mollusca: Cephalopoda) from the NE Atlantic and Mediterranean. *Heredity* 83: 280-289.

Pérez-Losada, M., Guerra, A., Carvalho, G.R., Sanjuan, A., and P.W. Shaw. 2002. Extensive population subdivision of the cuttlefish *Sepia officialis* (Mollusca: Cephalopoda) around the Iberian Peninsula indicated by microsatellite DNA variation. *Heredity* 89: 417-424.

Perlmutter, A., and R.F. Nigrelli. 1960. A possible hybrid population of the starfish, *Asterias forbesi* (Desor), in western Long Island Sound. *Proc. Nat. Shellfish Assoc.* 51: 93-96.

Pernet, B. 1999. Gamete interactions and genetic differentiation among three sympatric polychaetes. *Evolution* 53: 435-446.

Pielou, E.C. 1979. *Biogeography*. John Wiley and Sons, London.

Pielou, E.C. 1991. *After the ice age: the return of life to glaciated North America*. Univ. Chicago Press, Chicago.

Price, C.S.C. 1997. Conspecific sperm precedence in *Drosophila*. *Nature* 388: 663-666.

Posada, D., and K.A. Crandall. 2001. Intraspecific gene genealogies: trees grafting into cladograms. *Trends Ecol. Evol.* 16: 37-45.

Quesada, H., Zapata, C., and G. Alvarez. 1995. A multilocus allozyme discontinuity in the mussel *Mytilus galloprovincialis*: the interaction of ecological and life-history factors. *Mar. Ecol. Prog. Ser.* 116: 99-115.

Rahman, M.A., Uehara, T., and J.S. Pearse. 2001. Hybrids of two closely related tropical sea urchins (Genus *Echinometra*): evidence against postzygotic isolating mechanisms. *Biol. Bull.* 200: 97-106.

Rakitin, A., Ferguson, M.M., and E.A. Tripel. 1999. Sperm competition and fertilization success in Atlantic cod (*Gadus morhua*): effect of sire size and condition factor on gamete quality. *Can. J. Fish. Aquat. Sci.* 56: 2315-2323.

Rand, D.M., and R.G. Harrison. 1989. Ecological genetics of a mosaic hybrid zone: mitochondrial, nuclear and reproductive differentiation by soil type in crickets. *Evolution* 43: 432-449.

Reeb, C.A., and J.C. Avise. 1990. A genetic discontinuity in a continuously distributed species: mitochondrial DNA in the American oyster, *Crassostrea virginica*. *Genetics* 124: 397-406.

Rieseberg, L.H. 1997. Hybrid origins of plant species. *Annu. Rev. Ecol. Syst.* 28: 359-389.

Reiseberg, L.H., Sinervo, B., Linder, C.R., Ungerer, M.C., and D.M. Arias. 1996. Role of gene interactions in hybrid speciation: evidence from ancient and experimental hybrids. *Science* 272: 741-745.

Remington, C.L. 1968. Suture-zones of hybrid interactions between recently joined biotas. *Evol. Biol.* 2: 321-428.

Rice, W.R., and E.E. Hostert. 1993. Laboratory experiments on speciation: what have we learned in 40 years? *Evolution* 47: 1637-1653.

Ritchie, M.G., and G.M. Hewitt. 1995. Outcomes of negative heterosis. Pp. 157-174 in *Speciation and the Recognition Concept*. D.M. Lambert and H.G. Spencer, eds. John Hopkins University Press, Baltimore..

Ritchie, M.G., Butlin, R.K., and G.M. Hewitt. 1989. Assortative mating across a hybrid zone in *Chorthippus parallelus* (Orthoptera: Acrididae). *J. Evol. Biol.* 2: 339-352.

Robertson, A. 1975. Gene frequency distributions as a test of selective neutrality. *Genetics* 81: 775-785.

Robinson, T., Johnson, N.A., and M.J. Wade. 1994. Postcopulatory, prezygotic isolation: intraspecific and interspecific sperm precedence in *Tribolium* spp., flour beetles. *Heredity* 73: 155-159.

Saetre, G.-P. Borge, T., Lindell, J., Moum, T., Primmer, C.R., Sheldon, B.C., Haavie, J., Johnsen, A., and H. Ellegren. 2001. Speciation, introgressive hybridization and nonlinear rate of molecular evolution in flycatchers. *Mol. Ecol.* 10: 737-749.

Sambrook, J., Fritsch, E.F., and T. Maniatis. 1989. *Molecular Cloning, a laboratory manual,* 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, NY.

Schluter, D. 2001. Ecology and the origin of species. *Trends Ecol. Evol.* 16: 372-380.

Schneider, S., Roessli, D., and L. Excoffier. 2000. *Arlequin ver. 2.000: a software for population genetic data analysis.* Genetics and Biometry Laboratory, Univ. of Geneva, Switzerland.

Schopf, T.J.M., and L.S. Murphy. 1973. Protein polymorphism of the hybridizing seastars *Asterias forbesi* and *Asterias vulgaris* and implications for their evolution. *Biol. Bull.* 145: 589-597.

Shaw, D.D, and P. Wilkinson. 1980. Chromosomal differentiation, hybrid breakdown and the maintenance of a narrow hybrid zone in *Caledia. Chromosoma* 80; 1-31.

Shaw, A., Lee, Y.-H., Stout, D., and V.D. Vacquier. 1994. The species-specificity and structure of abalone sperm lysin. *Semin. Dev. Biol.* 5: 209-215.

Shaw, D.D., Marchant, A.D., Arnold, M.L., Contreras, N., and B. Kohlmann. 1990. The control of gene flow across a narrow hybrid zone: a selective role for chromosomal rearrangement? *Genome* 68: 1761-1769.

Skibinski, D.O.F., Beardmore, J.A., and T.F. Cross. 1983. Aspects of the population genetics of *Mytilus* (Mytilidae; Mollusca) in the British Isles. *Biol. J. Linn.Soc.* 19: 137-183.

Smith, G.F.M. 1940. Factors limiting distribution and size in the starfish. *J. Fish. Res. Bd. Can.* 5: 84-103.

Smith, M.J., Arndt, A., Gorski, S., and E. Fajber. 1993. The phylogeny of echinoderm classes based on mitochondrial gene arrangements. *J. Mol. Evol.* 36: 545-554.

Sokal, R.R., and Rohlf, F.J. 1995. *Biometry*, 3rd ed. W.H. Freeman and Co., New York.

Srinivasa Rao, K., and K. Lakshmi. 1993. *Scomberomorus lineolatus* (Cuvier), an interspecific natural hybrid (*S. commerson* (Lacépéde) x *S. guttatus* (Bloch and Schneider) off Visakhapatnam, India. *J. Nat. Hist.* 27: 471-491.

Strathmann, R.R. 1981. On barriers to hybridization between *Strongylocentrotus droebachiensis* (O.F. Muller) and *Strongylocentrotus pallidus* (G.O. Sars). *J. Exp. Mar. Biol. Ecol.* 55: 39-47.

Strathmann, R.R. 1987. *Reproduction and Development of Marine Invertebrates of the Northern Pacific Coast.* University of Washington Press.

Styan, C.A. 1998. Polyspermy, egg size, and the fertilization kinetics of free-spawning marine invertebrates. *Amer. Nat.* 152: 290-297.

Swanson, W.J., and V.D. Vacquier. 1995. Liposome fusion induced by a M(r) 18, 000 protein localized to the acrosomal region of acrosome-reacted abalone spermatozoa. *Biochemistry* 34: 14202-14208.

Swanson, W.J., and V.D. Vacquier. 2002. Reproductive protein evolution. *Annu. Rev. Ecol. Syst.* 33: 161-179.

Swofford, D.L. 2002. *PAUP*: Phylogenetic Analysis Using Parsimony (* and Other Methods),* Version 4. Sinauer, Sunderland, MA.

Szymura, J.M. 1993. Analysis of hybrid zones with *Bombina.* pp. 261-289 in *Hybrid zones and the Evolutionary Process.* R.G. Harrison, ed. Oxford University Press, New York.

Szymura, J.M., and N.H. Barton. 1986. Genetic analysis of a hybrid zone between the fire-bellied toads, *Bombina bombina* and *B. variegata,* near Cracow in southern Poland. *Evolution* 40: 1141-1159.

Szymura, J.M., and N.H. Barton. 1991. The genetic structure of the hybrid zone between the fire-bellied toads *Bombina bombina* and B. *variegata*: comparisons between transects and between loci. *Evolution* 45: 237-261.

Szymura, J.M., Spolsky, C., and T. Uzzell. 1985. Concordant change in mitochondrial and nuclear genes in a hybrid zone between two frog species (genus *Bombina*). *Experientia* 41: 1469-1470.

Taberlet, P., Fumagalli, L., Wust-Saucy, A.-G., and J.-F. Cosson. 1998. Comparative phylogeography and postglacial colonization routes in Europe. *Mol. Ecol.* 7: 453-464.

Templeton, A.R. 1981. Mechanisms of speciation – a population genetic approach. *Annu. Rev. Ecol. Syst.* 12: 23-48.

Thompson, J.D., Gibson, T.J., Plewuiack, F., Jeanmovgin, F., and D.G. Higgins. 1997. The ClustalX Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nuc. Acids Res.* 24: 4876-4882.

Thorpe, R.S. 1984. Primary and secondary transition zones in speciation and population differentiation: a phylogenetic analysis of range expansion. *Evolution* 38: 233-243.

Tortonese, E. 1963. Differenziazione geografica e superspecie nel genere *Asterias* (Echinodermata). *Monit. Zool. Ital.* 70-71: 212-221.

Towle, C.H. 1982. *Morphological and isozymal variation in the sea-star Asterias forbesi (Desor).* Ph.D. dissertation, University of New Hampshire.

Tregenza, T., Pritchard, V.L., and R.K. Butlin. 2002. The origins of postmating reproductive isolation: testing hypotheses in the grasshopper *Chorthippus parallelus. Pop. Ecol.* 44: 137-144.

Turner, E. 1992. A northern Pacific seastar, *Asterias amurensis*, in Tasmania. *Aust. Mar. Sci.* 120: 18-19.

Uehara, T., Asakura, H., and Y. Arakaki. 1990. Fertilization blockage and hybridization among species of sea urchins. Pp. 305-310 in *Advances in invertebrate reproduction*. M. Hoshi and O. Yamashita, eds. Elsevier, Amsterdam.

Vacquier, V.D. 1998. Evolution of gamete recognition proteins. *Science* 281: 1995-1998.

Vacquier, V.D., Carner, K.R., and C.D. Stout. 1990. Species-specific sequences of abalone lysin, the sperm protein that creates a hole in the egg envelope. *Proc. Natl. Acad. Sci.* 87: 5792-5796.

Vacquier, V.D., Swanson, W.J., and M.E. Hellberg. 1995. What have we learned about sea urchin sperm bindin? *Dev. Growth Differ.* 37: 1-10.

Valentine, J.W., and D. Jablonski. 1986. Mass extinctions: sensitivity of marine larval types. *Proc. Natl. Acad. Sci.*83: 6912-6914.

Valliantos, M., Lougheed, S.C., and P.T. Boag. 2001. Phylogeography and genetic c haracteristics of a putative secondary-contact zone of the loggerhead shrike in central and eastern North America. *Can. J. Zool.* 79: 2221-2227.

Verrill, A.E. 1866. On the polyps and echinoderms of New England, with description of new species. *Proc. Boston Soc. Natur. Hist.* 10: 333-357.

Vogel, H., Czihak, G., Chang, P., and W. Wolf. 1982. Fertilization kinetics of sea urchin eggs. *Math. Biosci.* 58: 189-216.

Wade, M.J., Patterson, H., Chang, N.W., and N.A. Johnson. 1994. Postcopulatory, prezygotic isolation in flour beetles. *Heredity* 72: 163-167.

Walker, C.W. 1973. *Morphology and histology of the reproductive system of Asterias vulgaris*. M.Sc. dissertation, Cornell University.

Ward, R.D., and J. Andrew. 1995. Population genetics of the northern Pacific seastar *Asterias amurensis* (Echinodermata: Asteriidae): allozyme differentiation among Japanese, Russian, and recently introduced Tasmanian populations. *Mar. Biol.* 124: 99-109.

Wares, J.P. 2001. Biogeography of *Asterias*: north Atlantic climate change and speciation. *Biol. Bull.* 201: 95-103.

Wares, J.P., and C.W. Cunningham. 2001. Phylogeography and historical ecology of the North Atlantic intertidal. *Evolution* 55: 2455-2469.

Wilding, C.S., Grahame, J., and P.J. Mill. 2000. Mitochondrial DNA COI haplotype variation in sibling species of rough periwinkles. *Heredity* 85: 62-74.

Williams, D.A. 1976. Improved likelihood ratio tests for complete contingency tables. *Biometrika* 63: 33-37.

Williams, M.E., and M.G. Bentley. 2002. Fertilization success in marine invertebrates: the influence of gamete age. *Biol. Bull.* 202: 34-42.

Williams, A.B, and D.L. Felder. 1986. Analysis of stone crabs: *Menippe mercenaria* (Say), restricted, and a previously unrecognized species described (Decapoda: Xanthidae). *Proc. Biol. Soc. Wash.* 99: 517-543.

Wilson, E.O. 1965. The challenge from related species. Pp. 7-24 in *The Genetics of Colonizing Species.* Baker, H.G., and G.L. Stebbins, eds. Academic Press, Orlando, FL.

Worley, E.K., and D.R. Franz. 1983. A comparative study of selected skeletal structures in the seastars *Asterias forbesi, A. vulgaris,* and *A. rubens* with a discussion of possible relationships. *Proc. Biol. Soc. Wash.* 96: 524-547.

Wright, S. 1951. The genetical structure of populations. *Ann. Eugen.* 15: 323-354.

Xiang, C., Wange, H., Shiel, P., Berger, P., and D.J. Guerra. 1994. A modified alkaline lysis miniprep protocol using a single microcentrifuge tube. *Biotechniques* 17: 30-32.

Yoder, J.A., Schollaert, S.E., and J.E. O'Reilly. 2002. Climatological phyloplankton chlorophyll and sea surface temperature patterns in continental shelf and slope water off the northeast US coast. *Limnol. Oceanogr.* 47: 672-682.

Yund, P.O. 2000. How severe is sperm limitation in natural populations of marine free-spawners? *Trends Ecol. Evol.* 15: 10-13.

Zigler, K.S., and H.A. Lessios. 2003. Evolution of bindin in the pantropical sea urchin *Tripneustes*: comparisons of bindin of other genera. *Mol. Biol. Evol.* 20: 220-231.

Zigler, K.S., Raff, E.C., Popodi, E., Raff, R.A., and H.A. Lessios. 2003. Adaptive coevolution of bindin in the genus *Heliocardis* is correlated with a shift to direct development. *Evolution* 57: 2293-2302.

APPENDIX ONE:

SUMMARY OF RESULTS FOR SPERM CONCENTRATION STUDIES

Estimates of the sperm concentration at which 50% of the eggs were successfully fertilized (F_{50}) in conspecific and heterospecific fertilization of *Asterias forbesi* (F) and *A. rubens* (R). F_{50} values were estimated using two different models and two different indices of fertilization success (fertilization envelope and cell cleavage).

Table A1.1 Summary of results for sperm concentration studies.

Female	*Male*	*Non-linear regression model*				*Linear regression model*			
		Envelope		Cleavage		Envelope		Cleavage	
		F_{50}	R^2	F_{50}	R^2	F_{50}	R^2	F_{50}	R^2
F2	F2	54	0.991	21	0.933	93	0.934	14	0.575
F4	F3	125	0.997	82	0.997	42	0.855	185	0.534
F5	F4	6	0.989	1	0.903	6	0.927	2	0.265
F6	F5	55	0.969	29	0.952	13	0.903	656	0.552
F7	F6	117	0.996	67	0.674	11	0.968	1	0.273
F2	R2	29	0.995	16	0.981	43	0.899	29	0.856
F4	R3	58	0.963	36	0.979	127	0.834	65	0.864
F5	R4	13	0.997	8	0.585	10	0.959	5	0.535
F6	R5	638	0.980	57	0.987	91	0.878	1	0.074
F7	R6	400	0.995	296	0.898	19	0.957	1	0.110
R3	R2	30	0.991	24	0.961	24	0.878	13	0.810
R4	R3	41	0.982	30	0.760	83	0.849	12	0.648
R5	R4	3	0.995	1	0.876	3	0.864	4	0.780
R6	R5	32	0.986	5	0.974	10	0.851	3	0.879
R7	R6	18	0.980	21	0.932	2	0.879	491	0.665
R3	F2	73	0.970	41	0.956	173	0.786	25	0.598
R4	F3	849	0.998	618	0.988	254	0.834	51	0.144
R5	F4	5	0.996	1	0.950	5	0.841	3	0.569
R6	F5	126	0.960	84	0.977	32	0.820	1	0.351
R7	F6	137	0.982	97	0.763	9	0.957	1	0.405

APPENDIX TWO:

DEVELOPMENT OF MICROSATELLITE MARKERS

Microsatellite markers were developed for *A. forbesi* and *A. rubens* using the enrichment protocol of Hamilton *et al.* (1999). *Asterias forbesi* was obtained from Bear Cove, NS, and *A. rubens* was collected from Havre-St.-Pierre, QC. Tube feet from a single individual were ground in liquid nitrogen and the genomic DNA was extracted using a CTAB buffer and phenol-chloroform extraction (Grosberg *et al.* 1996). Initially, I used a probing and cloning method to find microsatellites. Extracted DNA was digested to completion using three restriction enzymes (*AluI*, *HaeIII*, and *RsaI*) and size fractioned in an agarose gel. Fragments (300 – 750 bp) were excised and purified using a standard phenol-freeze fracture protocol (Ausubel *et al.* 1999), then ligated into the *SmaI* site of pUC 19 (Pharmacia). The library was transformed into competent *Escheria coli* (DH5α Gibco BRL Maximum Efficiency) and screened with ^{32}P-labeled $(GT)_{15}$ and $(GA)_{15}$ oligonucleotides. Thirty-two recombinant clones were isolated and sequenced in both directions using IRD labeled universal M13 primers and resolved in 8% (25 cm, 0.25 mm thick) denaturing polyacrylamide gels on a Li-Cor 4200 automated DNA sequencer. None of these clones contained microsatellite repeats.

A Southern blot was used to confirm the frequency of microsatellites in *A. forbesi* relative to species with a high frequency (cod, *Gadus morhua*) and a species with a low frequency (coral, *Acropora* spp.). Following the Southern hybridization technique described in Sambrook *et al.* (1989), extracted DNA of three species was digested using *RsaI*, and then separated on a 1% TBE agarose gel. The gel was depurinated, denatured, neutralized, washed and blotted onto Hybond N^+ membrane overnight. The membrane

was dried at 80°C for 2 h, then hybridized with a ^{32}P-labeled $(GT)_{15}$ oligonucleotide (56°C). The autoradiograph was then exposed for 68 h. Results of the Southern blot indicated the cod genome contained a high frequency of microsatellites while the frequency of microsatellites in both the coral and the *Asterias* genomes was low.

The universal linker and ligation enrichment procedure of Hamilton *et al.* (1999) was then used to locate microsatellites. Briefly, genomic DNA was extracted then digested using three restriction enzymes (*HaeIII, RsaI* and *NheI*). Double-stranded SNX linkers were then separately ligated to dephosphorylated, cleaned digested genomic DNA. The genomic DNA with attached linkers were hybridized separately to biotin-labeled $(GA)_{15}$ and $(GT)_{15}$ oligonucleotides. Streptavidin-coated iron beads were then added to the hybridization mixture and bound to biotinylated oligonucleotides with attached genomic DNA. The beads and attached DNA were magnetically separated from wash solutions, yielding repeat-enriched genomic DNA which was then amplified using PCR. The SNX linkers were digested and the remaining enriched DNA was ligated into a plasmid. Recombinant plasmids were transformed into competent *E. coli* and colonies were screened for microsatellite inserts. Positive colonies were minipreped for plasmid isolation (Xiang *et al.* 1994). Repeated-enriched DNA was sequenced using IRD labeled universal M13 primers and resolved in 8% denaturing polyacrylamide gels on a Li-Cor 4200 automated DNA sequencer.

Of the 240 colonies which positively screened for microsatellite inserts in *A. rubens*, 97 were sequenced and only 6 actually contained microsatellite repeat motifs. From these six, I designed primers and optimized the reaction conditions for three (*Ar06, Ar50, Ar72*; Table A2.1). For *A. forbesi*, 25 colonies were sequenced; two contained repeat motifs from which I was able to design primers for one (*Af10*; Table A2.1).

Three of the four microsatellite markers were unsuitable for population genetic studies. I tested the amplification and polymorphism of the four microsatellites in allopatric populations of *A. forbesi* (Long Island, NY) and *A. rubens* (Havre-St.-Pierre, QC). *Ar06* rarely amplified: there was no amplification of this microsatellite in 59 *A. forbesi* despite repeated alteration of the reaction conditions and amplified in 3 out of 59 *A. forbesi* with a total of 5 different alleles. *Ar72* amplified more reliably, 28 out of 59 *A. forbesi* amplified, but there were only 2 alleles and one of these alleles was shared with *A. rubens*. *Ar72* amplified in 12 out of 59 *A. rubens*, with a total of 6 alleles. The lack of polymorphism in *Ar72* makes it unsuitable for population genetic studies and it is not diagnostic for the species since one of the two alleles amplified in *A. forbesi* was shared with *A. rubens*. *Ar50* amplified well in both species: 24 out of 50 *A. forbesi* and 46 out of 59 *A. rubens* amplified, however there were only two alleles in *A. forbesi* (both of which were shared with *A. rubens*) and six alleles in *A. rubens*. Primers designed for *Af10* did not amplify either *A. rubens* or *A. forbesi* despite repeated alteration of the reaction conditions and redesigning the primers.

Table A2.1 Microsatellite primer sequences and repeat motifs for *Asterias rubens* and *A. forbesi*

Microsatellite	*Primer sequence*	*Repeat motif*
Ar06	A: 5'-AGCCCATGTCGGTCTTAG-3' B: 5'-TCGCCTCAAGTAGACAATG-3'	$(GA)_9G_2(GA)_4GC(GA)_5$
Ar50	A: 5'-ATTGAATGTTCACTTATTGTG-3' B: 5'-TTTGAAAGGCTCTAATGAG-3'	$(GTT)_{15}$
Ar72	A: 5'-GGCACCCTGGATGGCG-3' B: 5'-TCCGTGTAAATTTGGCGAG-3'	$(GT)_3AT(GT)_4$
Af10	A: 5'-GGGTTCGGAATTTAATCTC-3' B: 5'-TCCACTGCTTTAAAATGC-3'	$(GA)_{29?}$ Unable to sequence entire repeat