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Morphological and phylogenetic evidence for hybridization and introgression in a sea star secondary contact zone

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Abstract. Glacial cycles and other climatic events have been widely invoked as factors promoting divergence, secondary contact, and hybridization between populations of terrestrial organisms, but the origin and fate of secondary contact in the sea is much less well understood. We studied the distribution of morphological and genetic variation in a northwest Atlantic zone of secondary contact between congeneric sea stars of *Asterias* that probably separated after the Pliocene as part of the trans-Arctic interchange. These species have similar reproductive biology and can hybridize in the laboratory. However, multivariate analysis of morphological traits scored from sea stars inside and outside the zone of secondary contact clearly indicated two clusters of phenotypes that corresponded to the two taxonomic species. A quantitative analysis of this clustering pattern did not support the hypothesis of a third grouping that might correspond to intermediate hybrid phenotypes. Known F_1 hybrids from laboratory matings grouped with one of the two taxonomic species. However, a survey of mtDNA sequence variation clearly indicated that ~13% of individuals of one species (*Asterias forbesi*) are descendants of hybridization events that resulted in introgression of haplotypes of *Asterias rubens* into populations of *A. forbesi*. We conclude that morphological phenotypes are inadequate to identify hybrids of *Asterias* and their descendants, and that hybridization and introgression might be common in this secondary contact zone.

Additional key words: phylogeography, *Asterias,* mtDNA, statistical parsimony

The repeated glaciations of the Pleistocene epoch had a significant and well-characterized impact on the phylogeography and genetic differentiation of terrestrial organisms. At the peak of the last glacial maximum 0.02 Mya, the Northern Hemisphere ice complex covered most of North America, northern Eurasia, and the polar seas (CLIMAP 1976). Glacial advances caused extirpation or retreat of terrestrial species into ice-free refuges (Pielou 1991; Hewitt 1996, 1999, 2000; Taberlet et al. 1998). Protracted isolation caused many populations to differentiate in allopatry as a result of such vicariance (Endler 1977). Glacial retreat allowed members of these populations to disperse out of isolation and make secondary contact with members of other refugial populations. Depending on the evolution of reproductive barriers in allopatry, the outcomes of secondary contact range from complete reproductive isolation (with possible selection for reinforcement of this barrier) to the formation of stable hybrid zones and introgressive hybridization (Avise et al. 1987; Harrison 1993; Arnold 1997, 2006; Howard & Berlocher 1998).

The role of Pleistocene glacial cycles in vicariance, differentiation, and secondary contact in the sea is much less well understood, perhaps in large part because the sources of vicariance in the sea (such as changes in the direction and speed of ocean currents; Benzie 1999) are less easily inferred than the large ice sheets that extirpated or divided terrestrial populations. One likely source of marine vicariance associated with these glacial cycles was sea-level decline and local extirpation associated with glacial advances (Ingolfsson 1992): these effects are expected to have been most severe on near-shore marine species (Palumbi 1994). In the northwest Atlantic, the last glaciation is thought to have been particularly difficult for rocky intertidal animals, perhaps forcing many species from this habitat (see Wares & Cunningham 2001). Shallow-water populations and

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communities on rocky substrates in New England and Atlantic Canada are expected to be relatively young (of post-glacial age) and descended from refugial populations in Europe, the southeastern United States, or the north Pacific (e.g., Ingolfsson 1992; Wares & Cunningham 2001; Addison & Hart 2005; Vermeij 2005; P. Rawson & F. Harper, unpubl. data).

A prominent member of this community is the forcipulate sea star genus *Asterias*. One species, *Asterias rubens* Linnaeus 1758, has an amphi-Atlantic distribution (Tortonese 1963) from Portugal to the United Kingdom, Norway, and Iceland in the northeast Atlantic and from North Carolina to southern Labrador in the northwest Atlantic (Clark & Downey 1992). North American populations of *A. rubens* were previously described as *Asterias vulgaris,* a junior synonym (Tortonese 1963; Clark & Downey 1992). *Asterias forbesi* Desor 1848 is restricted to the northwest Atlantic and ranges from the eastern shore of Nova Scotia to the Gulf of Mexico (Clark & Downey 1992). Wares (2001) used mtDNA sequences to estimate the initial vicariance of North Atlantic species of *Asterias* at $\sim$3.0 Mya, following the formation of the Labrador Current. Low allelic diversity and lack of unique haplotypes in northwest Atlantic populations of *A. rubens* suggest that this species recently recolonized North America (Wares 2001). Populations of *A. forbesi* may have survived the Pleistocene glaciations in southern refugia off the southeastern coast of North America, and has subsequently expanded its range northward (Harris et al. 1998). Populations of the two species are now sympatric in a broad zone of secondary contact from about Cape Cod to northeastern Nova Scotia.

What is the typical fate of such secondary contact zones in the sea? Many shallow temperate marine communities include pairs or groups of closely related congeners that appear to be reproductively isolated as good biological species (Miner 1950; Mayr 1954; Palumbi 1994; Kozloff & Price 1996). However, Gardner (1997) suggested that hybridization in the marine environment might be as common as in other environments. Many of Gardner's examples of hybridization in marine invertebrates were based on the observation of morphologically intermediate specimens. Additional examples may be difficult to detect using morphology alone; some heterospecific crosses produce offspring that more closely resemble the phenotype of one parental species (Lamb & Avise 1987; Byrne & Anderson 1994). Analysis of genetic markers independent of morphological traits may often reveal allele or haplotype sharing that is consistent with hybridization and introgression (DePamphilis & Wyatt 1990; Paige & Capman 1993; Bert et al. 1996; Addison & Hart 2005).

Previous morphological, reproductive, and genetic evidence for hybridization between North Atlantic species of *Asterias* is mixed. 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 over the identification and frequency of putative hybrids. Some studies included anecdotal accounts of morphological intermediates that were considered to be hybrids (Clark 1904; Perlmutter & Nigrelli 1960; Ernst 1967; Walker 1973). Menge (1986) estimated that 1.4% of 295 *Asterias* species from Boston Harbor, MA, were morphological intermediates, but did not report which characters were variable. Clark & Downey (1992) suggested that hybrids were common from Cape Cod to Maine but that these hybrids did not reach sexual maturity. In contrast, in an extensive survey of skeletal characters, Worley & 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* that had been isolated from southern populations by periodic climate changes. The latter hypothesis is plausible in light of the expected oscillations in the latitudinal distribution of shallow-water marine organisms in the North Atlantic associated with cyclical Pleistocene climate change (Wares & Cunningham 2001).

Laboratory studies suggest that these two species are potentially reproductively compatible. They have overlapping spawning seasons in the zone of secondary contact (Smith 1940; Boolootian 1966; Menge 1986), and share similar natural histories (feeding activity and diet, Menge 1979) and habitats (Menge 1986). Natural hybridization and introgression are likely to occur because adults of the two species release their gametes in close proximity to each other, hybrid fertilization rates in the laboratory are often high (Ernst 1967; Harper & Hart 2005), and hybrid zygotes can mature into viable and fertile F_1 adults (Harper & Hart 2005).

However, despite this mixed morphological and reproductive evidence for hybridization potential, there is no genetic evidence that hybridization in *Asterias* leads to introgression in natural populations. A recent phylogenetic analysis of speciation in North Atlantic *Asterias* species using mtDNA (COI) and nuclear ITS sequences (Wares 2001) did not find any

evidence of shared haplotypes between *A. forbesi* and *A. rubens* (a potential indicator of hybridization and introgression). Although Wares' study was not specifically designed to detect introgression, its results (combined with the morphological interpretation of Worley & Franz 1983) suggest that the *Asterias* zone of secondary contact may not be a hybrid zone.

Here, we use morphological and genetic analyses of sympatric and allopatric *Asterias* populations to reconsider the potential for hybridization and introgression in this zone of secondary contact. In the morphological analysis, specimens were examined and scored for the five qualitative characters (Clark & Downey 1992) and three morphometric characters (Worley & Franz 1983) analyzed in previous studies. We used principal components analysis (PCA) and clustering algorithms to determine whether a significant cluster of morphological intermediates was quantitatively supported. This approach provides an objective method for identifying morphological intermediates between the two parental phenotypes that are possible hybrids. We then obtained mtDNA sequences of the highly variable control region from a subset of animals identified in the morphological survey to look for introgression in sympatric populations. Our sampling design emphasized specimens within the contact zone, including some with morphological characters intermediate between the parental species. The results suggest that introgression between *Asterias* species may be frequent and asymmetrical without leaving an easily detected signature of morphological intermediates.

Methods

Adult sea stars (longest arm length from tip to opposite interradius $R = 28$–90 mm) were collected over the range of the species' distributions in the North Atlantic and within the known zone of sympatry from the Gulf of Maine to Nova Scotia (Table 1). 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 Canada Department of Fisheries and Oceans. Live animals were scored for two morphological characters (madreporite color and body rigidity) that are lost with preservation and were 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.

Table 1. Sample collection sites, depth, and sample size (N = sample size for morphological analysis; S = number of individuals for mtDNA sequence analysis). 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 in the collection of the Smithsonian Institution, National Museum of Natural History (USNM) are available from the authors.

Species and Location	Population	Latitude and longitude	Depth (m)	*N*	S	Collection
A. rubens	United Kingdom	Unknown	10–20	2	0	USNM
NE Atlantic	Ireland	53°N 10°E	Intertidal	9	4	C.W.C.
	Norway	64°N 10°E	Intertidal	20	2	C.W.C.
	Iceland	64°N 22°E	Intertidal	3	1	C.W.C.
	France	48°N 3°E	Intertidal	5	0	C.W.C.
	Faröe Islands	62°N 7°W	Intertidal	4	3	C.W.C.
A. rubens	Bonne Bay, NF	49°31′N 57°33′W	10	94	2	NSMNH
NW Atlantic	Havre-St-Pierre, QC	50°14′N 63°36′W	10	95	6	NSMNH
A. rubens and *A. forbesi*	Savage Harbour, PEI	46°42′N 62°85′W	5–8	41	4	NSMNH
NW Atlantic	Bras d'Or Lake, NS	45°83′N 60°83′W	10	13	5	NSMNH
	Bear Cove, NS	44°32′N 63°33′W	3–10	219	15	NSMNH
	Brier Island, NS	44°04′N 66°25′W	67	42	0	NSMNH
	Grand Manan, NB	44°32′N 63°33′W	26–44	95	0	NSMNH
	Isle of Shoals, ME	42°59′N 70°36′W	3–10	193	21	NSMNH
A. forbesi	North Carolina	33°31′N 77°24′W	Unknown	5	0	USNM
NW Atlantic	South Carolina	32°30′ N 79°42′W	Unknown	13	0	USNM
	Florida	26°N 80°W	Unknown	4	0	USNM

Table 2. *Asterias* species. Diagnostic adult morphological characteristics for *Asterias forbesi* and *Asterias rubens* (based on Coe 1912; Clark & Downey 1992).

Character	*A. forbesi*	Character states (score)	
		Intermediate	*A. rubens*
Body rigidity	Rigid (1)		Flaccid (3)
Color of madreporite	Orange-red (1)		Pale yellow (3)
Major pedicellariae on adambulacral spines	Broad, round (1)	Broad at base, slender at tip (2)	Slender, pointed (3)
Wreath of pedicellariae around abactinal spines	Base of spine (1)	Base of some spines, middle of others (2)	Midway up spine (3)
Shape of abactinal spines	Short, tubercle-like (1)	Short and slender or long and tubercle (2)	Long, slender (3)

Morphological traits

Each animal was examined using a stereoscopic zoom microscope (Nikon SMZ 1500, Nikon, Melville, NY, USA) and scored for the five qualitative morphological characters described as diagnostic by Clark & Downey (1992) (Table 2). Specimens lacking major pedicellariae on the adambulacral spines were scored using pedicellariae located on the aboral surface around the perimeter of the madreporite. Character states associated with *Asterias forbesi* received a score of 1, and character states associated with *Asterias rubens* were scored as 3. Character states that appeared to be intermediate between the two species received a score of 2: 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; and abactinal spines were short and slender or long and tuberculate. Body rigidity and the color of the madreporite did not ever appear intermediate between the two species and were always scored as 1 or 3 in live specimens or as missing in preserved specimens.

Three quantitative traits were measured for each specimen. The longest arm length (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. The ratio a/b is a measure of the shape of the arms (Aldrich 1956) and is associated with differences in skeletal structure among *Asterias* species (Worley & Franz 1983).

In addition to the 857 animals sampled from the North Atlantic, two F_1 hybrids reared in culture were included in the morphological analysis. These hybrids were the result of cross-fertilization studies conducted to examine *in vitro* hybridization between *A. forbesi* and *A. rubens* (Harper & Hart 2005). Although hybrid zygotes were easy to produce and rear as larvae (Harper & Hart 2005), most juveniles (of hybrid or nonhybrid origin) died due to failures of the seawater system. One surviving F_1 hybrid was the result of a cross between a female of *A. rubens* and a male of *A. forbesi;* the other hybrid was from a cross between a female of *A. forbesi* and a male of *A. rubens*. Both hybrids had the orange madreporite and firm body typical of *A. forbesi,* and the long, slender abactinal spines with a wreath of minor pedicellariae halfway up the spines characteristic of *A. rubens*. The only character that differed between the two was the major pedicellariae: the hybrid from the female of *A. forbesi* had the short, broad pedicellariae typical of *A. forbesi,* while the other hybrid had pedicellariae of an intermediate character state: broad and pointed.

The type localities for *A. forbesi* and *A. rubens* are unknown (Clark & Downey 1992); therefore, animals 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.

Statistical analyses of morphological traits

We used PCA of eight characters and 859 sea stars (Systat 9.0, Systat, San Jose, CA, USA) to identify compound vectors of character states that explained a large proportion of the phenotypic variation among specimens. PCA is more appropriate than discriminant-function analysis for the exploratory analysis of relationships among character states within and between species and hybrids because it does not require an *a priori* definition of the groups (Bert et al. 1996). The PCA was based on a correlation

matrix using pairwise deletion for missing values. Components with a minimum eigenvalue of 0.8 were retained.

Nonhierarchical cluster analyses with Euclidean distances and the iterative *k*-means algorithm (Systat 9.0) were used 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). The fit of the data was then compared with the two- or three-cluster algorithm using the variance ratio criterion (VRC; Calinski & Harabasz 1974), analogous to the F-statistic in a univariate analysis. We also compared the fit of the data with the clusters using the overall mean square ratio due to *k*-partition, a measure of the reduction of variance within *k* clusters associated with fitting the data to $k+1$ clusters (Hartigan 1975).

mtDNA sequencing and analysis

We selected a subset of *Asterias* species identified in the morphological analysis from across the range of the species' distributions (Table 1). Of the 63 individuals sequenced, 41 were haphazardly selected from sympatric populations (including nine individuals of "intermediate" morphological phenotype from the Bear Cove and Isle of Shoals locations), ten were from allopatric *A. rubens* populations in Europe, and 12 were from allopatric populations of *A. rubens* in North America (including one individual of "intermediate" phenotype from the Bonne Bay location). We tried to obtain sequences of *A. forbesi* from allopatric populations (from museum samples) but these did not yield amplifiable DNA.

Genomic DNA was extracted from ethanol-preserved tube feet using a standard CTAB protocol (Grosberg et al. 1996). An $\sim$800 bp portion of the mitochondrial genome was amplified by PCR using the 12Sa/16Sa primers of Smith et al. (1993). These primers correspond to the highly conserved regions of the 3′ end of 12S and 16S rRNA mitochondrial genes; the PCR product includes two transfer RNA genes ($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, 1 × polymerase buffer, 0.2 mM dNTPs (MBI, Burlington, Ontario, Canada), 2.5 mmol L^{-1} $MgCl_2$ (MBI), 0.5 μmol L^{-1} primers, and 0.3 units of *Tsg* polymerase (Biobasic, Toronto, Canada). The thermal cycling profile consisted of an initial denaturation at 95°C for 3 min, followed by 37–43 cycles of 94°C (30 s), 54°C (45 s), 72°C (90 s), and a final extension of 72°C for 1 min.

Approximately 75–100 ng of each PCR product was ethanol-precipitated and one strand was sequenced using an internal primer (5′-TTTCATGTTATAGGTTTAGG-3′) designed from preliminary sequence data for $tRNA^{Glu}$. Sequencing reactions used Li-Cor IRD700 Dye Terminators (Li-Cor, Lincoln, NE USA) 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 s), 50°C (45 s), and 72°C (60 s). Excess dye terminators were removed using Sephadex G-50 fine (Sigma, St. Louis, MO, USA) columns. Sequence reaction products were resolved in 6% (25 cm length, 0.2 mm depth) polyacrylamide gels on a Li-Cor DNA 4200L-2 instrument. Individual sequences were edited using the Li-Cor image analysis software Align-IR, aligned to each other using default parameter values in ClustalX (Thompson et al. 1997), trimmed to standard length (309 bp), and submitted as two population sets to GenBank (accession numbers EF179717–EF179779). We visualized similarities among sequences (and introgression between species) by estimating 95% statistical parsimony networks in TCS 1.21 (Clement et al. 2000; Posada & Crandall 2001) with gaps coded as a fifth state.

Results

Morphological analyses

PCA reduced the five qualitative and three quantitative characters to three principal components (PC) with a minimum eigenvalue of $\geq$0.8 or greater (Table 3). Scores on the first component were most heavily weighted by four of the five qualitative characters: body rigidity, madreporite color, pedicellariae shape, and the wreath of pedicellariae on the abactinal spines. Scores on the second principal component emphasized the three quantitative characters, which had high positive loadings. The third component was mainly 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. While Clark & Downey (1992) described this character difference as diagnostic for the two North Atlantic species of *Asterias,* other workers have not used this character.

Table 3. *Asterias* species. Principle components, eigenvalues, component loadings, and amount of total variance explained in a principal components analysis on a correlation matrix of five qualitative (Table 2) and three quantitative morphological traits scored for *Asterias* spp. samples in Table 1.

Component	1	2	3
Eigenvalue	3.614	2.303	0.911
Component loadings			
R	0.531	0.765	−0.027
a	0.491	0.805	0.077
b	0.233	0.856	0.208
Madreporite color	0.908	−0.314	0.083
Body rigidity	0.862	−0.348	0.080
Pedicellariae shape	0.889	−0.344	0.088
Pedicellariae wreath	0.701	−0.236	0.125
Abactinal spines	0.430	0.119	−0.888
% total variance	45.178	28.793	11.382

Based on the PCA of morphological traits, *Asterias* species collected across the North Atlantic were divided 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. 1A): the group with the low scores on the first PC corresponds to *Asterias forbesi;* the group with the higher scores on the first PC is *Asterias rubens*. *Asterias* species from allopatric populations were clearly separated, while specimens from sympatric populations in the northwest Atlantic were found in both groups and in a smudge between the groups.

Nonhierarchical cluster analyses using $k = 2$ and 3 were performed to test the plausibility of a third, intermediate group (Fig. 1B). When specimens were clustered into two groups, VRC = 1166.94; for three groups, VRC = 747.48. Calinski & Harabasz (1974) suggest that the optimal clustering is at the first local maximum of the VRC with respect to k, which in this study occurs in the two-cluster analysis. The overall mean square ratio due to k-partition for the $k = 2$ analysis was F = 1.141. Hartigan (1975) argued that $F > 10$ justified increasing the number of clusters from k to $k+1$. By this criterion, grouping the specimens into three clusters rather than two does not significantly reduce the within-group sum of squares, and our samples of *Asterias* are most appropriately grouped into two morphological clusters (which we refer to below by the species names) rather than three morphological clusters that would include an objectively identified group of intermediates, potentially of hybrid origin.

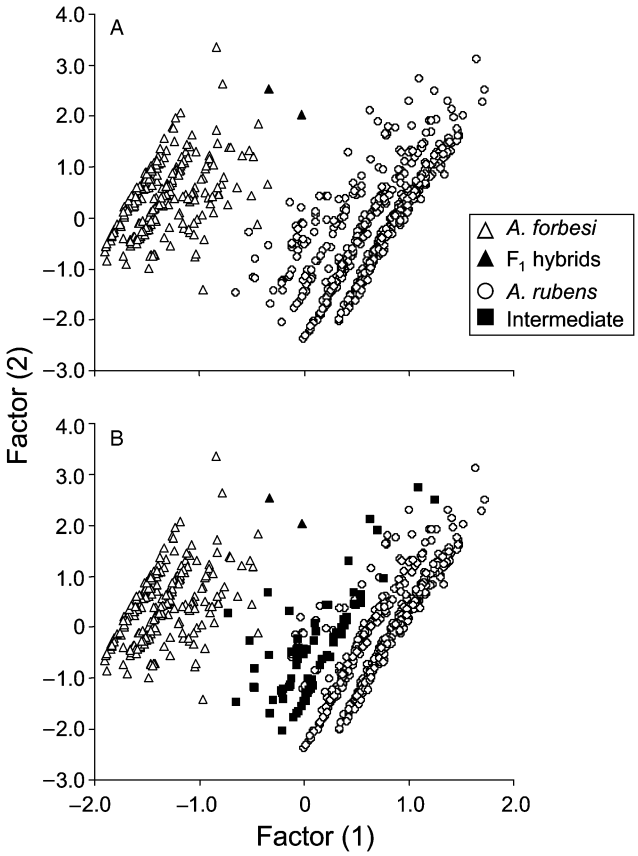

Fig. 1. Bivariate plots of principle components scores showing results of nonhierarchical cluster analyses of standardized morphological character scores of *Asterias forbesi* and *Asterias rubens,* using Euclidean distances. Group assignments (symbol shapes) were based on the outcome of nonhierarchical cluster analyses. **A.** Analysis of $k = 2$ clusters. **B.** Analysis of $k = 3$ clusters.

As expected, samples from outside the putative hybrid zone in Quebec, Newfoundland, and Europe were comprised entirely of phenotypes of *A. rubens,* and populations sampled south of Cape Hatteras were entirely *A. forbesi* (Fig. 2). 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 were expected to be *A. rubens* because *A. forbesi* has not been reported previously in the Gulf of St. Lawrence; however, all specimens from this site had phenotypes of *A. forbesi.*

The two F_1 hybrids raised in culture grouped in the *A. forbesi* cluster in both the $k = 2$ and 3 analyses. In the PC plots, both animals were in the smudge of

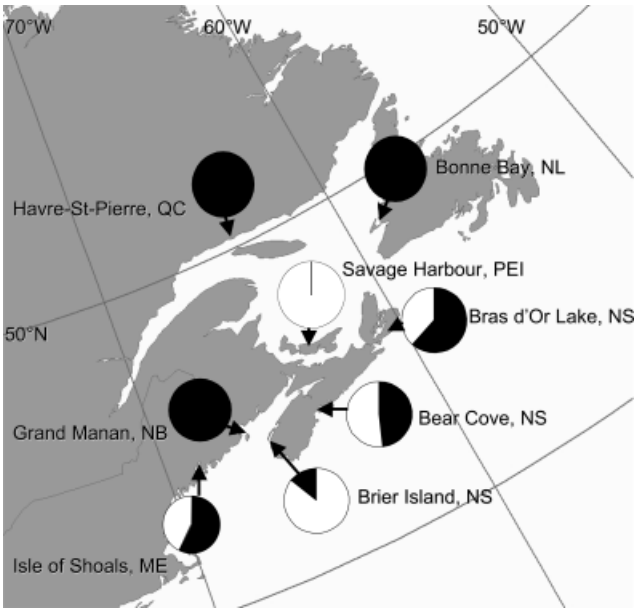

Fig. 2. Map of the northwest Atlantic showing the relative frequency of *Asterias forbesi* (white) and *Asterias rubens* (black) phenotypes at eight sampling sites. Frequencies were determined using principal components analysis of five qualitative and three quantitative characters and $k = 2$ cluster analysis. Northeast Atlantic samples are all *A. rubens;* western samples from south of Cape Cod are all *A. forbesi.*

individuals intermediate to the main clusters, but did not form part of the third intermediate cluster (Fig. 1). Both specimens had character states for body rigidity and madreporite color 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 hybrids was the shape of the pedicellariae, which was diagnostic of *A. forbesi* in one animal and had an intermediate character state in the other.

In total, 452 specimens exhibited some set of characteristics (either intermediate states, mixed parental character states, 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 with a character state inconsistent with the diagnostic states of other characters (n = 244). Removal of this character from the PCA did not alter the overall conclusion of separation of the scores into two clusters. 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. This difference is consistent with the overall utility of the eight morphological characters: some characters (e.g., madreporite color; pedicellaria shape) were associated with high component loadings on PCs with large eigenvalues, and contributed to the separation of samples into major clusters (or species); in contrast, abactinal spine shape was associated with a high component loading only on the PC with the lowest eigenvalue and thus contributed little to distinguishing major axes of morphological variation among samples.

mtDNA network analysis

We found 37 unique haplotypes among control region sequences from 63 *Asterias* individuals (Table 1). These haplotypes formed two subnetworks separated from each other by more than the 95% parsimony connection limit (more than seven missing intermediate haplotypes). One subnetwork included 19 of the 22 individuals with *A. forbesi* morphological phenotypes and 16 haplotypes that differed from each other by 1–14 mutations (including up to six missing intermediate haplotypes). The second subnetwork included all 41 individuals with *A. rubens* phenotypes and 21 haplotypes that differed by 1–10 mutations (and up to four missing intermediate haplotypes). Members of the two subnetworks differed by 89–101 mutations (mean 94.1).

Nine of the 21 haplotypes in the *A. rubens* subnetwork were found among ten individuals from northeast Atlantic populations; the remaining 12 haplotypes were found among 31 individuals from northwest Atlantic populations. TCS inferred the root haplotype to be among northwest Atlantic individuals, probably due to our more intensive sampling of sequences from the zone of secondary contact that included the three most frequent haplotypes (N = 12, 7, 6). Nonetheless, the real phylogenetic root among *A. rubens* lineages is almost certainly among older northeast Atlantic populations (Wares 2001; Vermeij 2005).

The relationships among haplotypes in the second subnetwork imply several independent introductions of haplotypes into the northwest Atlantic. At least four such events (examples shown as bold lines in Fig. 3) are required to connect the four disparate sets of northeast Atlantic haplotypes to other parts of the subnetwork. Although we found no cases of single haplotypes shared across the north Atlantic (e.g., Wares 2001), some trans-Atlantic pairs differed by just one or two steps. In contrast, haplotypes within each of these regions differed by as many as nine (northeast) or ten (northwest) steps.

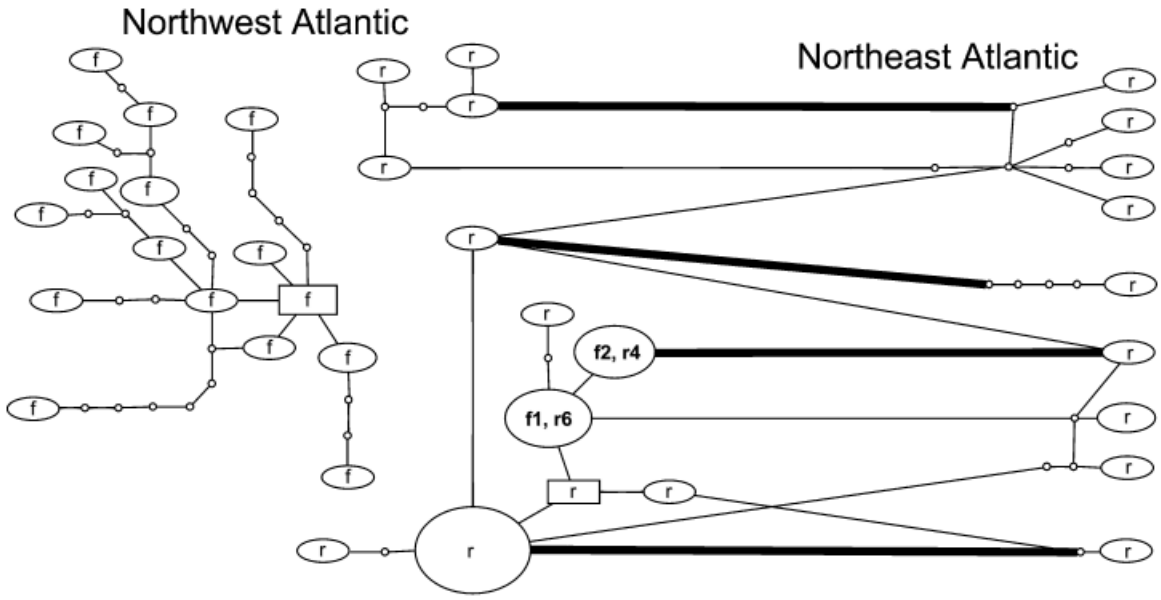

Fig. 3. Statistical parsimony networks for *Asterias* control region mtDNA sequences. Large symbols represent unique haplotypes (37 total); size is proportional to haplotype frequency (maximum N = 12); lines represent single mutational differences (substitutions or indels); small open circles represent missing intermediate haplotypes. Sequences in the two subnetworks differ by more than seven missing intermediate haplotypes (the 95% connection limit; maximum difference = 101 steps). The single square symbol in each subnetwork represents the inferred root haplotype (based on haplotype frequency and number of steps from other haplotypes of high frequency). Letters indicate *Asterias forbesi* (f) and *Asterias rubens* (r) morphological phenotypes; numbers indicate the frequency in each species of two shared haplotypes (bold type face). Nine haplotypes from northeast Atlantic *A. rubens* are shown to the right; all other haplotypes were collected from northwest Atlantic individuals. Four bold lines show plausible cases of trans-Atlantic gene flow between *A. rubens* populations that could connect disparate northeast Atlantic haplotypes to other parts of the subnetwork.

We found specific evidence of asymmetrical introgression in the form of three individuals of *A. forbesi* (all from the Bear Cove population sample) with one of two common haplotypes that were characteristic of northwest Atlantic populations of *A. rubens*. These haplotypes differed by one indel from each other and by an additional substitution from a northeast Atlantic haplotype. The northeast Atlantic origin of northwest Atlantic populations of *A. rubens,* the occurrence of only western *rubens*-like haplotypes in individuals of *A. forbesi,* and the otherwise very large differences ($\leq$101 steps) between sequences in the *A. rubens* and *A. forbesi* subnetworks argue in favor of trans-Atlantic gene flow, followed by introgression—rather than shared ancestral polymorphism—as the cause of haplotype sharing between species in this subnetwork.

We found no evidence of introgression in the other direction. Ten individuals of ''intermediate'' morphology (based on examination of the PCA described above) all had *A. rubens* haplotypes (not shown in Fig. 3). No individuals in our relatively large sample of fully *rubens*-like phenotypes had *forbesi*-like haplotypes.

Discussion

The phenotypes of *Asterias forbesi* and *Asterias rubens* form two clusters of individuals both inside and outside the zone of secondary contact. The morphological survey of sympatric and allopatric populations of *Asterias* revealed a high frequency of these individuals belonging to these two discrete phenotypes and few individuals with intermediate phenotypes. A distinct group of intermediate phenotypes that might have been hybrids was not quantitatively supported. Indeed, the five qualitative diagnostic characters were not capable of detecting individuals of known hybrid pedigree. However, evidence of hybridization and asymmetric introgression in the secondary contact zone was detected in the survey using mtDNA sequences: *rubens*-like haplotypes were found in three individuals with

forbesi-like phenotypes. This apparent asymmetric introgression may be the result of asymmetric gamete compatibility.

Existence of intermediate phenotypes?

Morphological analyses of sympatric and allopatric populations of *A. forbesi* and *A. rubens* do not support the inference of 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. We 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* species complex in southwest England; Gardner 1996), or as a discrete, third cluster (e.g., seerfish populations in India; see Srinivasa Rao & Lakshmi 1993; Gardner 1997). In our analysis of populations of *Asterias,* 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* (Gardner 1996:fig. 8). Also, individuals from a large sample within the putative hybrid zone (Grand Manan) and a large sample supposedly outside the hybrid zone (Savage Harbour) were unambiguously assigned to *A. rubens* and *A. forbesi,* respectively.

Of the five qualitative morphological characters examined, the shape of the abactinal spines was the most subjective and difficult to score. 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. Of all previous attempts to diagnose these two species from morphological characters, only Clark & Downey (1992) refer to abactinal spine shape as a diagnostic character. Although they describe the five qualitative traits in their key as highly correlated in 85% of *Asterias* species from south of Cape Cod, in our study the shape of the abactinal spine was not a good character for identification of sympatric *Asterias* species.

Studies of morphological variation in hybrid zones often are able to use many traits, both morphometric and qualitative, to discriminate species and detect morphological intermediates that may be hybrids (e.g., Dillon & Manzi 1989; Bert et al. 1996). The relatively few traits that discriminate between Atlantic *Asterias* species 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 the traditional morphological characters.

Where is the zone of secondary contact?

The northern range limit for *A. forbesi* (and for the zone of secondary contact) has been reported previously as Cape Breton, with rare occurrences in the Gulf of St. Lawrence (Towle 1982). As such, the population of *A. forbesi* off the northern coast of Prince Edward Island in the Gulf of St. Lawrence was not expected. This is an area supposedly outside the hybrid zone; however, all 41 specimens were unambiguously assigned to *A. forbesi.* Whether this result is indicative of a natural northward expansion of this species' geographic range or of anthropogenic introduction is unknown. The congeneric northern Pacific species, *Asterias amurensis* Lutken 1871, has been introduced into Tasmanian waters (Turner 1992), possibly through ballast water discharged from ocean-going vessels from Japan (Ward & Andrew 1995). As generalist omnivores with a long planktonic larval phase, *Asterias* species 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). Whatever the mechanism responsible for this range extension, it suggests that the size of the zone of secondary contact and the opportunities for hybridization between these two species might be much greater than expected previously.

Ancestral polymorphism or introgression of mtDNA?

Phylogenetic analyses of mtDNA sequence variation in sibling species of *Asterias* in the North Atlantic produced two distinct subnetworks or clades. One subnetwork was comprised exclusively of sequences from *A. forbesi* phenotypes, while all *A. rubens* phe-

notypes clustered into the second subnetwork. The two subnetworks were separated by a large number of nucleotide substitutions, deletions, or insertions. A parallel analysis based on maximum likelihood inference of sister group (rather than ancestor–descendant) relationships produced qualitatively identical results (not shown), with a long internal branch separating a clade of exclusively *A. forbesi* phenotypes from a second clade containing all *A. rubens* phenotypes. This divergence of *A. rubens* and *A. forbesi* is congruent with previous studies of morphology (Worley & Franz 1983; Menge 1986), allozymes (Schopf & Murphy 1973), and DNA (Wares 2001).

Three individuals identified morphologically as *A. forbesi* had 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. One possible explanation for this pattern is incomplete lineage sorting and retention of ancestral haplotype polymorphisms in *A. forbesi* (but not *A. rubens*) populations (Avise et al. 1987). However, the large between-clade sequence divergence (relative to small within-clade sequence differences) suggests a history of lineage sorting or of selection, followed by modest accumulation of new mutations. In addition, complete lineage sorting and reciprocal monophyly of nuclear (ITS) and mtDNA (COI) gene sequences have been reported in *Asterias* species (Wares 2001).

A more plausible explanation of this pattern is hybridization and introgression. 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, the gametes of *A. forbesi* and *A. rubens* are reciprocally compatible and the species readily hybridize in the lab (Harper & Hart 2005). 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 (F. Harper, unpubl. data). 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 our sample of *A. forbesi* phenotypes was relatively small (22 individuals from within the zone of secondary contact), 13% of that sample appeared to be of hybrid descent. This result is similar to other studies in which morphological traits alone could not distinguish hybrids from parental species (Lamb & Avise 1987).

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 & Murphy 1973; Menge 1986), *A. rubens* and *A. forbesi* were included in the list as hybridizing species, but were suspected not to show introgression. If the two species hybridize naturally and there is introgression of genetic markers, we expected it would most likely be detected in specimens with diagnostic morphological traits intermediate between the species. However, we found only *rubens*-like haplotypes among individuals with intermediate morphological traits. Instead, introgression appeared to be asymmetrical and consisted only of *rubens*-like haplotypes in individuals with *forbesi*-like phenotypes. Further analysis of the contact zone using multiple biparentally inherited nuclear markers (e.g., Kronforst et al. 2006) 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 *A. rubens* eggs being fertilized by *A. forbesi* sperm. However, the lack of observable *rubens*-like phenotypic traits in the three hybrid individuals (and the absence of observable introgression in the other direction) suggests that they were descended from female hybrids by one or more generations of matrilineal backcrossing with *A. forbesi* males (and not *A. rubens*). The strong dominance of the *forbesi*-like phenotypic character states could also contribute to this asymmetry and mask a mixture of underlying *forbesi*- and *rubens*-like nuclear DNA markers. This latter mechanism seems unlikely, though, because we did not observe such dominance across all phenotypic characters in a small sample of known F_1 hybrids.

Asymmetric introgression in sympatric populations of *Asterias* may be due to unexpected but parallel asymmetries in gamete recognition and fertilization preference (Arnold 1997). Assortative fertilization has been documented among *Mytilus* species (Bierne et al. 2002) and conspecific sperm precedence is well known in terrestrial invertebrates (reviews in Howard & Berlocher 1998; Howard 1999). In sperm competition studies using a nuclear DNA marker to identify paternity of offspring in hybrid crosses, we found evidence of conspecific fertilization preference in some crosses using eggs of *A. forbesi,* and in one cross using eggs of *A. rubens,* we found that sperm of *A. forbesi* were competitively superior even when they were an order of magnitude less abundant than conspecific sperm (Harper & Hart 2005). Similar studies in other groups of broadcast-spawning marine invertebrates are not yet common,

but have also found conspecific sperm preference (Geyer & Palumbi 2005). Preferential hybridization between males of *A. forbesi* and females of *A. rubens* (and their female hybrids) could contribute to the pattern of asymmetrical introgression of maternal mtDNA markers of *A. rubens* into a background of phenotypes of *A. forbesi*.

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