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Sperm Polymorphism Within the Sea Urchin *Strongylocentrotus droebachiensis*: Divergence Between Pacific and Atlantic Oceans

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Abstract. The rapid evolution of traits related to fertilization such as sperm morphology may be pivotal in the evolution of reproductive barriers and speciation. The sea urchin *Strongylocentrotus droebachiensis* has a circumarctic distribution and shows substantial genetic subdivision between northeastern Atlantic populations and northwestern Atlantic and Pacific populations. Using transmission electron microscopy, we show here that sperm shape, size, and ultrastructure differ markedly among populations of *S. droebachiensis* from different oceans and reflect patterns of genetic divergence. Sperm nuclei from northwestern Atlantic and Pacific populations were longer and narrower than those from the northeastern Atlantic. We additionally demonstrate population-level differences in the amount and location of filamentous actin (F-actin) prior to the occurrence of the acrosome reaction. Sperm from Pacific and northwest Atlantic populations differed from that of all other echinoids examined in that intact sperm contains a partly preformed acrosomal process, a structure more closely resembling the acrosomal rod seen in some molluscs. Immunofluorescent studies using anti-bindin antibodies and the F-actin-specific stain phalloidin confirmed these findings. Divergence of reproductive traits such as sperm morphology may be related to divergence in gamete compatibility and genetic divergence, and could represent the first stages of speciation in free-spawning marine invertebrates.

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Introduction

Current theory on variation in sperm morphology focuses on male-female co-evolution and male-male competition. Sperm competition has emerged as an important selective force in shaping sperm quality in snakes (Tourmente *et al.*, 2006), mammals (Gomendio *et al.*, 2006), and insects (Birkhead and Pizzari, 2002; Hunter and Birkhead, 2002; Pattarini *et al.*, 2006). Sperm morphology has been correlated with mating system (Rouse and Jamieson, 1987), fertilization biology (polychaetes, Franzén, 1956; molluscs, Ribes *et al.*, 2002; Oppliger *et al.*, 2003; mammals, Gomendio *et al.*, 2006), egg size, and developmental mode (bivalves, Franzén, 1983). Species-level variation in sperm traits is thus well recognized, and has wide phylogenetic and systematic applications (*e.g.,* Buckland-Nicks and Scheltema, 1995; Jamieson *et al.*, 1995). Conversely, although variation among individuals has previously been described (Hodgson *et al.*, 1996; Ward, 1998; Buckland-Nicks, 1998), the possible ubiquity of within-species variation in sperm size and structure has only recently started to gain attention (Hellriegel and Blanckenhorn, 2002; Till-Bottraud *et al.*, 2005). Variation among males in morphological sperm characters has been described in several groups, including molluscs, birds, and mammals. A special case of intraspecific sperm-size variation occurs in heteromorphic species where males produce several distinct sperm morphotypes—often only one of which is fertile—including centipedes, spiders, various insects, and some molluscs and chordates (Nishiwaki and Tochimoto, 1969; Buckland-Nicks, 1998; Swallow and

Wilkinson, 2002; Oppliger *et al.*, 2003; Bernasconi and Hellriegel, 2005; Holman and Snook, 2007).

Within the Echinoidea, gross sperm morphology has been examined for more than 100 species (about 15 ultrastructurally to date), and general sperm structure appears to be highly conserved (Summers *et al.*, 1975; Chia *et al.*, 1975; Chia and Bickell, 1983; Eckelbarger *et al.*, 1989b). Exceptions are mainly found in deep-sea species, including aberrant sperm in the concentricycloid *Xyloplax turnerae* and dimorphic sperm in the abyssal *Phryssocystis multispina* (Eckelbarger *et al.*, 1989c). A high incidence of elongated sperm heads has also been described in bathyal echinoids compared to shallow-water species (Eckelbarger *et al.*, 1989a). Intraspecific variation in sperm head length has previously been reported between western and central Pacific populations of *Echinometra oblonga* (Landry *et al.*, 2003). It is unknown whether this geographic variation reflects ultrastructural differences.

In marine invertebrates, positive selection on reproductive traits is thought to drive the rapid divergence of sperm and egg proteins (Swanson and Vacquier, 2002) and of carbohydrates in the egg coat (Biermann *et al.*, 2004; Mah *et al.*, 2005). Likewise, sexual selection has been implicated in the evolution of egg size, anisogamy, and egg accessory coats (Levitan, 1998, 2004). Similarly, sperm morphology may be under positive selection (Landry *et al.*, 2003), but while basic sperm morphology has been described for many marine invertebrates, few data exist on intraspecific variation in sperm traits.

Here we characterize intraspecific variation in sperm morphology and ultrastructure in *Strongylocentrotus droebachiensis* in four populations from two oceans. Unreacted sea urchin sperm contain a membrane-bounded acrosomal vesicle at the apex, which sits atop a nuclear fossa (a depression in the nucleus—see below) containing a granular mass of unpolymerized globular actin (G-actin) surrounding the actomere. The actomere—usually a short actin filament bundle—functions as a nucleating site that seeds the polymerization of actin filaments. In sea urchins, the acrosome reaction is induced by sulfated fucans in the egg jelly, often in a species-specific manner (Foltz and Lennarz, 1990; Vacquier and Moy, 1997; Biermann *et al.*, 2004). During the acrosome reaction, the extension of the acrosomal process occurs through rapid, localized polymerization of G-actin into filamentous actin (F-actin). At the same time, the acrosomal vesicle releases the protein bindin. We used fluorescently labeled anti-bindin antibodies and phalloidin, which selectively binds to F-actin, to examine the distribution of filamentous actin in intact and acrosome-reacted sperm. We also demonstrate striking differences in sperm head shape and ultrastructure using transmission electron microscopy.

Additionally, we examine the amount of genetic divergence among populations with nuclear and mitochondrial DNA sequences to assess whether sperm structure is correlated with genetic affiliation.

Materials and Methods

Strongylocentrotus droebachiensis (O. F. Müller, 1776) and *S. pallidus* (G. O. Sars, 1871) were collected using scuba from subtidal habitats near Bergen, Norway (Straumen; 62°N), the Arctic archipelago Svalbard (Isfjorden, Spitzbergen; 78°N), and Washington State, USA (San Juan Islands, 48.5°N). *Strongylocentrotus purpuratus* were collected intertidally in Washington State. *Strongylocentrotus droebachiensis* sperm samples from New Hampshire, USA (Portsmouth; 43°N) were collected and fixed for transmission electron microscopy by M. Russell, Villanova University. Sperm for these studies were either fixed directly (*i.e.,* "dry"), or treated prior to fixation with filtered seawater (FSW) as a control or with egg-jelly to induce the acrosome reaction. Because the acrosomal process in *S. droebachiensis* is too small to see using conventional microscopy, we used transmission electron microscopy on a subset of the phalloidin-stained sperm samples to confirm that we were indeed scoring intact *versus* acrosome-reacted sperm in all populations, and to further elucidate ultrastructure. Copious F-actin has previously been observed only in sea urchin sperm that have undergone the acrosome reaction. To show that only egg-jelly treated sperm undergo the acrosome reaction in significant numbers, and thus control our observations that a preformed acrosomal filament was indeed present in intact sperm in some populations, we additionally used an immunocytochemical assay (anti-bindin antibodies). Sperm that have undergone the acrosome reaction (acrosomal exocytosis) show bright red staining on the bindin-coated acrosomal process (also see Biermann *et al.*, 2004).

Ripe Atlantic animals were transported to the University of Washington Friday Harbor Laboratories; all work with live animals was performed in quarantine facilities. Spawning was induced by intracoelomic injection of 0.55 mol l^{-1} KCl. Sperm were collected "dry" and fixed immediately or stored on ice before use. Samples for transmission electron microscopy of intact sperm were either fixed by adding them directly to aqueous 1.5% 0.1 mol l^{-1} osmium tetraoxide (OsO_4), or added to FSW for 30 s followed by OsO_4 fixation. For samples of acrosome-reacted sperm, dry sperm were added to egg-jelly water and allowed to react for 30 s prior to fixation in OsO_4. All samples were fixed for 1 h at room temperature, centrifuged (2000 rpm, 2 min), the supernatant poured off, and the remaining pellet resuspended in distilled water. Material was dehydrated by gradually adding 70% acetone to the samples, followed by a series of ascending acetone concentrations (70%, 90%, and 100%). After dehydration, the samples were embedded in Epon. Ultrathin sections (50 nm) were obtained and mounted on 250-mesh copper grids and stained in uranyl acetate (1 h)

and lead citrate (10 min). The specimens were examined on a Phillips EM-300 and a JEOL 1011 transmission electron microscope.

Sperm for fluorescent staining were diluted 1:10 in HEPES-buffered (10 mm, pH 7.9, 9 °C) 0.2-μm FSW. Fifty microliters of the sperm suspension was gently mixed into 100 μl of either egg-jelly water or FSW. After 5 min at 9 °C, sperm were fixed by the addition of 750 μl of ice-cold hyperosmotic 3% paraformaldehyde in FSW. All subsequent washing and staining steps were done at room temperature; sperm were gently centrifuged and resuspended by pipetting with wide-bore tips or vortexing. Postfixation (30 min), sperm were washed in 500 μl of phosphate-buffered saline (PBS; Sigma, St. Louis, MO) and stained with agitation for at least 30 min with 1 unit Alexa488-labeled phalloidin (Molecular Probes, Eugene, OR) in 100 μl of PBS blocker (0.1 mol l^{-1} glycine, 1 mg/ml bovine serum albumin, 0.02% sodium azide in PBS, pH 7.4). Twenty microliters of rabbit anti-bindin was then added, and agitation was continued for 2 h. Sperm were washed twice in 500 μl of PBS and resuspended in 100 μl of secondary antibody stain (PBS, pH 7.4, with 10 mg/ml BSA and 1:400 Texas Red-labeled goat anti-rabbit, Jackson Immunoresearch, West Grove, PA). After 3–12 h, the cells were washed twice in 500 μl of PBS, resuspended in 20–50 μl PBS or 25% glycerol, and mounted in a thin layer. The sperm and the resulting red and green fluorescence were photographed using a Radiance 2000 confocal microscope (60× lens; BioRad, Hercules, CA).

To examine genetic variation among populations, we sequenced 16 alleles (from 14 individuals) of the nuclear gene for sperm bindin (the protein that attaches activated sperm to the egg, 1839 nucleotides) from various circumarctic locations, plus a section (312 nucleotides) of the mitochondrial gene for ATPase subunit 6 for 11 of those individuals. We used PAUP* 4.0b10 (Swofford, 2003) to construct maximum likelihood, maximum parsimony, and minimum evolution genealogies from the DNA sequences. Molecular and phylogenetic methods are detailed in the Appendix. We used MEGA ver. 4.0 (Tamura *et al.*, 2007) to draw linearized trees (Takezaki *et al.*, 1995; see Fig. 4.). Genetic distances within and between the two major clades (maximum composite likelihood distance with pairwise deletion of gaps, standard errors by bootstrap) were calculated in MEGA (Tamura *et al.*, 2007).

Results

Sperm from *Strongylocentrotus droebachiensis* were polymorphic for size, shape, and ultrastructure (Table 1,

Table 1

Sperm and egg properties of three species of Strongylocentrotus *by location*

Species	Size (μm)	Sperm head		F-actin pre acrosomal reaction	Egg size (μm)
		Shape	Fossa		
S. droebachiensis					
USA					
Friday Harbor	6.4 × 1.4 (7.0 × 1.5)[2]	Long, pointy	Deep invagination	Preformed filaments in intact sperm	148–163 (155-160)[4]
New Hampshire	5.0 × 1.7	Long, pointy	Deep invagination	Preformed filaments in intact sperm	—
Norway					
Bergen	3.5 × 1.0*	Short, conical	Shallow invagination	Actomere only	145–155§ (146)[5]
Svalbard	4.9 × 1.5*	Intermediate, variable	Shallow invagination	Actomere only	165–210
Sweden					
Kristineberg[1]	(3.9 × 1.2)[1]	Short, conical	Shallow invagination	Actomere only	(136)[5]
S. pallidus					
Friday Harbor	3.8 × 2.2 (3.5 × 1.5)[2]	Very short, wide conical	Spherical invagination	Actomere only	155–165 (155–170)[4]
S. purpuratus					
Friday Harbor	(3.6 × 1.3)[3] (5.0 × 2.0)[2]	Very short, wide conical	Spherical invagination	Actomere only	(78–80)[4]

Sperm size is length × widest width of head of intact sperm. Egg size is diameter. Values in parentheses are from the literature: [1]Afzelius (1955); [2]Chia *et al.* (1975); [3]Rothschild and Tyler (1955); [4]Strathmann (1987); [5]Hagström and Lønning (1967). F-actin pre acrosomal reaction denotes the amount of filamentous actin present in unreacted sperm visualized by fluorescent staining and electron microscopy.

* Asterisk indicates measurements made from transmission electron micrographs.

§ Egg sizes ranging 133–173 are infrequently recorded from Bergen.

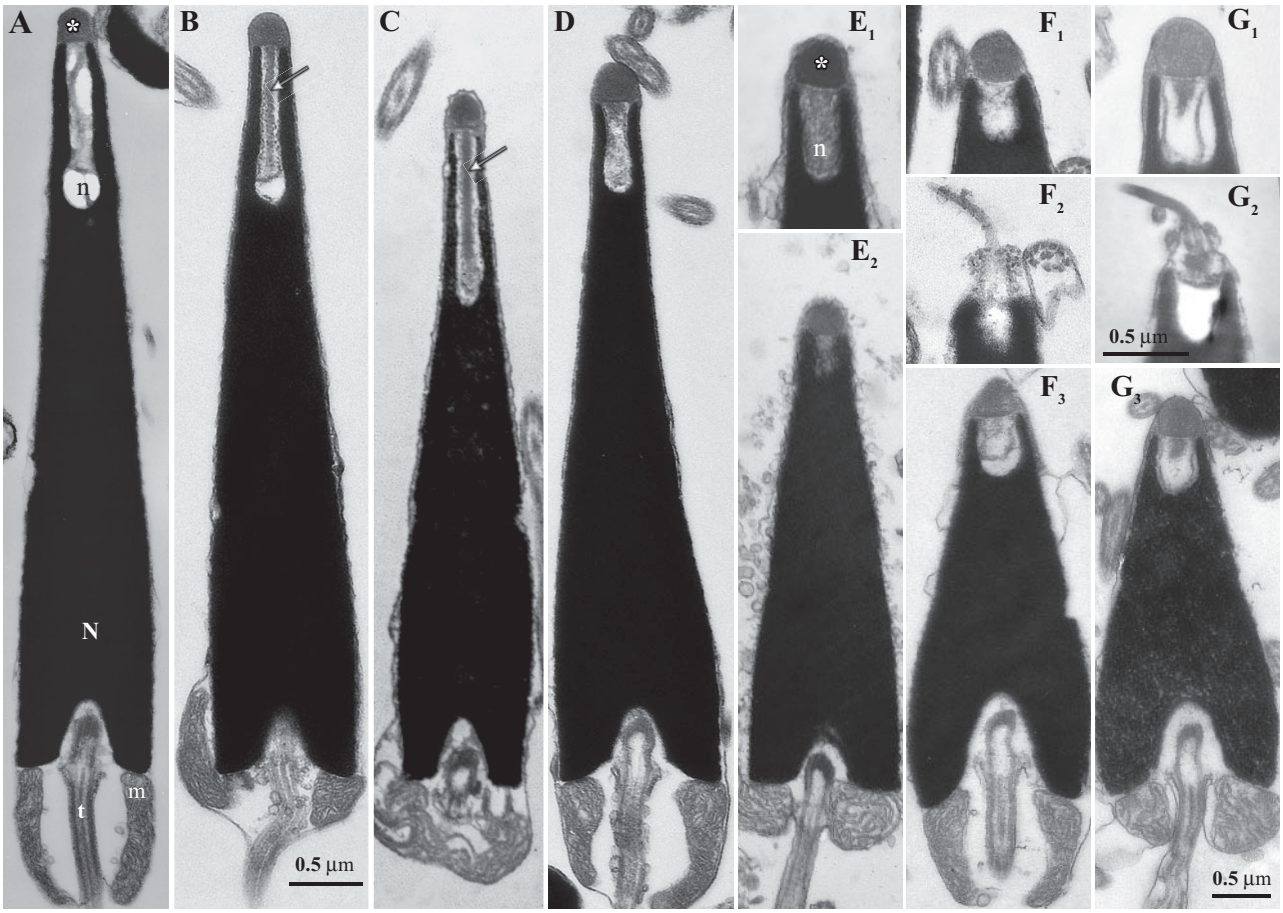

Figure 1. Transmission electron micrographs of *Strongylocentrotus droebachiensis* sperm heads (A–E), showing polymorphism among populations in nuclear size and shape, shape of the nuclear fossa, and for the localization of actin filaments in intact sperm. (A) general morphology; (B) Friday Harbor, WA, USA; (C) New Hampshire, USA; (D) Svalbard, Norway; (E) Bergen, Norway. Sperm also differ from the closely related *S. pallidus* (F_1–F_3: F_1 intact tip, F_2 acrosome-reacted) and *S. purpuratus* (G_1–G_3: G_1 intact, G_2 acrosome-reacted). Asterisk = acrosomal vesicle, N, nucleus; n, nuclear fossa; m, mitochondrion; t, tail; arrow indicates the preformed acrosomal filament. (Scale bar in G_2 applies to all insets; scale bar in B applies to all other photos except G_3).

Fig. 1). The typical morphology of regular echinoid sperm (Fig. 1A) is explained only briefly here because it is well described elsewhere (*e.g.,* Chia and Bickell, 1983). Mature, intact sperm are composed of a conical head, a midpiece with a single, circular mitochondrion, and a flagellate tail. The head is composed largely of the cone-shaped nucleus, topped with a spherical acrosomal vesicle situated at the tip. Directly underneath lies an apical depression in the sperm nucleus, the subacrosomal nuclear fossa. Although all sperm examined in the present study followed this basic plan, the shape and size of the nucleus differed among populations. Sperm from the Friday Harbor population (Figs. 1A, B; 2A, B) were all strikingly needlelike, with an elongated nucleus; the nuclear fossa comprised an indentation about 20%–25% its length. Notably, the northwestern Atlantic population most closely resembled the Pacific (Friday Harbor) population, except that the nuclei from males examined here were slightly shorter (Fig. 1C). In contrast to the Pacific and northwest Atlantic populations, sperm from the Bergen population (Fig. 1E) had a short conical nucleus and a nuclear fossa that was at most 15% of nuclear length in unreacted sperm. The Svalbard population (Figs. 1D; 2C, D) was similar in morphology but the nucleus was somewhat longer, and individuals from this population exhibited a high degree of variation in length. Sperm heads from all *S. drobachiensis* populations differed from those of *S. pallidus* and *S. purpuratus*, both of which are invariably wider and much shorter (Table 1, Fig. 1F,G). The variance among and particularly within males was generally very low. However, statistical estimates of within- and among-population variance in sperm-head dimensions are not presented here due to small sample sizes in a few populations. Lipid droplets, described in several other species of echinoids, were not observed here.

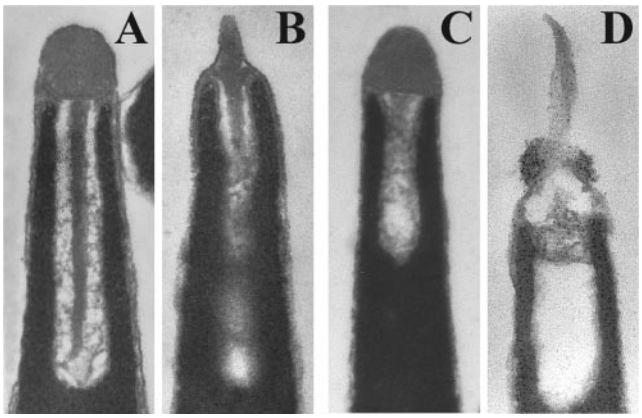

Figure 2. Transmission electron micrographs of the tips of *Strongylocentrotus droebachiensis* sperm heads from Friday Harbor, WA, USA (A, B) and Svalbard, Norway (C, D). A and C are intact; B and D are acrosome-reacted.

Fluorescent staining of intact sperm revealed that the extent of filamentous actin in *S. droebachiensis* sperm varies among populations (Fig. 3). Most strikingly, sperm from the Pacific population differed markedly from the northeast Atlantic populations. The long, needlelike sperm from the Friday Harbor population possessed a deep, narrow nuclear fossa with a long rod of actin filaments that stained brightly in both intact and acrosome-reacted sperm (see Fig. 3, A1 and A2). Sperm from the New Hampshire population were similar to those from Friday Harbor in ultrastructure (Fig. 1C), and the filaments seen in the fossa were highly suggestive of F-actin, but this was not confirmed through staining. Unreacted sperm from all other populations and species had a rounder, shallower nuclear fossa, usually filled with amorphous, granular G-actin (Figs. 1D–G; 2C), and here phalloidin stained only a small seed of F-actin, the actomere (Fig. 3, B1, C1, D1). Phalloidin-stained sperm from the Bergen population (not shown) were similar to those of the Svalbard population.

Intraspecific gene genealogies of both nuclear and mitochondrial DNA confirmed the close affiliation of populations from New England with those from the Pacific Ocean (Fig. 4). Eastern Atlantic *S. droebachiensis* formed a separate group that also included urchins from Iceland and Arctic Canada (Fig. 4). Hence the genetic subdivision mirrored sperm morphology: Svalbard and Bergen were similar to each other but deeply divergent from the Pacific/West Atlantic clade.

In the nuclear bindin gene, there was much more genetic diversity in the European/Arctic *S. droebachiensis* (mean within group distance 0.007 ± SE 0.001) than in the Pacific/West Atlantic urchins, whose bindin was much less polymorphic (0.002 ± 0.001; Fig. 4). The mean divergence between the geographic groups in bindin was 1.5% (0.015 ± 0.003). This figure would be much higher if insertions and deletions were included as substitutions (for example in the 3′ repeat region; Biermann, 1998), but homology is ambiguous in unalignable regions. The mitochondrial DNA varied very little within each clade (0.002 ± 0.002 European/Arctic and 0.003 ± 0.002 Pacific/West Atlantic), although it showed the geographic division most distinctly (average distance between groups = 0.035 ± 0.011; Fig. 4).

Discussion

Unique morphology

Sperm from the Friday Harbor and New Hampshire populations of *Strongylocentrotus droebachiensis* differed from that of all other echinoids examined to date in that they contained a large amount of F-actin prior to the acrosome reaction. The one possible exception in the literature is *Echinocardium cordatum*, which is described as having a stalked nuclear fossa that projects above the nucleus, topped with a round acrosomal vesicle (Afzelius, 1955, 2006). The long, tubular projection appears to contain filamentous material, but the distribution of actin in this species has not been studied. The localization of actin filaments in unreacted sperm from the Pacific *S. droebachiensis* population resembles that described in several molluscs, most notably *Haliotis discus*, described as having a preformed rod of actin filaments arranged in parallel array (Shiroya and Sakai, 1993). In Pacific *S. droebachiensis* sperm, the rod is further extended during the acrosome reaction, due to polymerization of G-actin in the periacrosomal cytoplasm. A completely preformed acrosomal filament is present in diverse groups and appears to have evolved independently several times (Afzelius, 2006).

Divergence in sperm morphology between closely related taxa has been associated with the co-evolution of female traits such as reproductive tract morphology (Presgraves *et al.*, 1999), egg size, and mode of development (Franzén, 1983). There is great intraspecific variation in egg size and egg-jelly thickness in *S. droebachiensis* and *S. pallidus*, with egg volume increasing nearly 5-fold along the Scandinavian coast (J. Marks, unpubl. data). It is unlikely that the marked variation in sperm morphology reported here is an adaptation to these egg characters, as eggs differ less between than within oceans and genetic clades (see Table 1), and sperm morphology seems consistent within populations. Sperm morphology in the family Strongylocentrotidae is not correlated with egg size; for example, *S. purpuratus* (egg diameter ≈80 μm) and *S. pallidus* (≈160 μm) have blunt sperm heads, whereas those of *S. droebachiensis* (egg diameter ≈160 μm) and *S. franciscanus* (≈130 μm) are more slender (Strathmann, 1987; Biermann *et al.*, 2003; Marks and Biermann, unpubl. obs.). Nor are gamete attributes like egg size correlated with phylogenetic position within strongylocentrotid sea urchins (Biermann *et al.*, 2003). We have additionally observed interocean

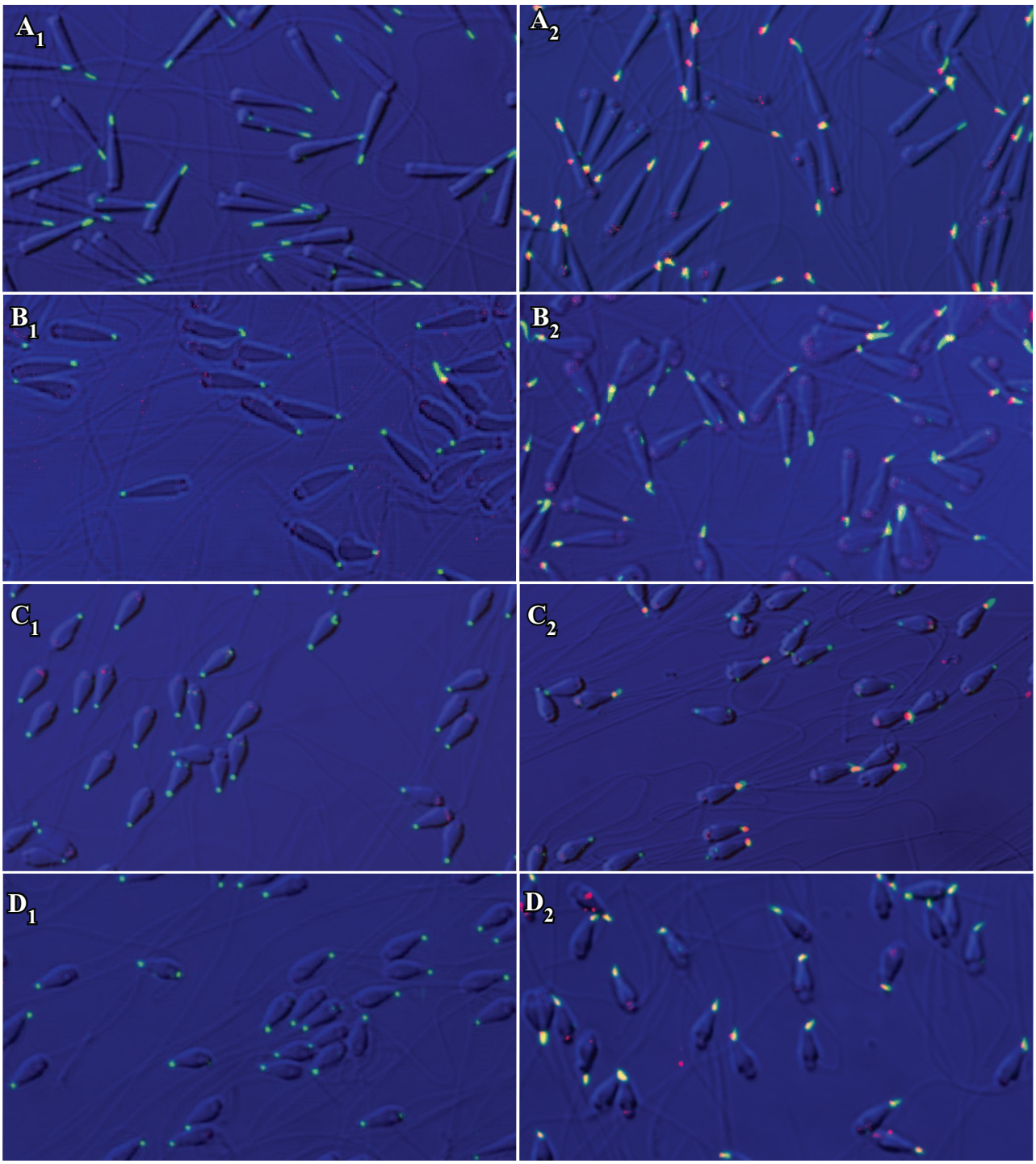

Figure 3. Confocal images of intact (A1–D1) and acrosome-reacted (A2–D2) sperm of *Strongylocentrotus* spp. treated with the F-actin–specific stain phalloidin (green) and anti-bindin antibody (red; indicates the acrosome reaction has occurred). Pacific sperm from Friday Harbor (A1) stained brightly with phalloidin, even when the acrosome was intact, and F-actin staining increased only slightly when acrosome reacted (A2). In contrast, intact *S. droebachiensis* sperm from Svalbard (B1), and *S. pallidus* (C1) and *S. purpuratus* (D1) from Friday Harbor contained only a small seed of F-actin, the actomere, which increased greatly in size in acrosome-reacted sperm (B2, C2, D2). The anti-bindin antibody assay served as a control to distinguish intact from acrosome-reacted sperm; the protein bindin shows up as bright red fluorescence at the tip of the sperm head (base of the acrosomal process) only after the acrosome reaction has occurred.

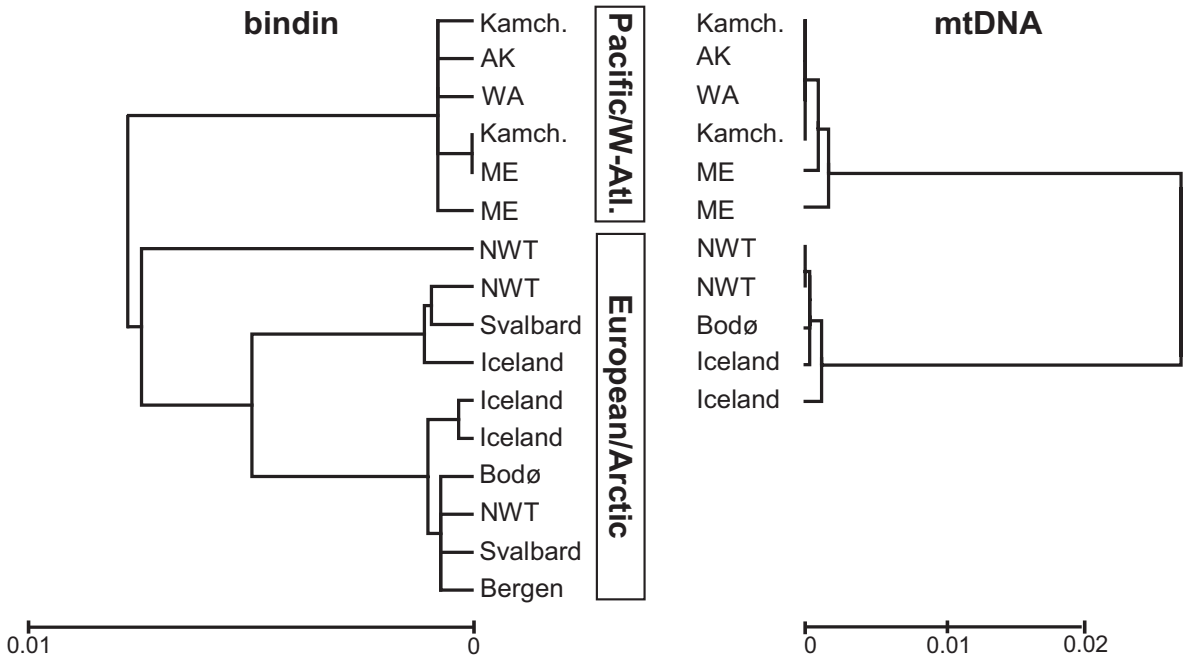

Figure 4. Genealogies of the nuclear gene for sperm bindin and a section of the mitochondrial ATPase 6 gene in *Strongylocentrotus droebachiensis*. Shown are strict consensus trees (based on maximum likelihood, maximum parsimony, and minimum evolution bootstrap consensus trees), outgroup-rooted with *S. pallidus*, and linearized in MEGA. For complete trees, detailed methods, and bootstrap values, see Appendix. Scale bars indicate the number of substitutions per site. Abbreviations: AK, Alaska; Kamch., Kamchatka, Russia; ME, Maine; NWT, Northwest Territories (Resolute); WA, Washington State (Friday Harbor).

differences in gamete compatibility that are independent of differences in egg and jelly size (Biermann and Marks, 2000; Marks and Biermann, unpubl. obs.).

Functional consequences

How might actin polymerization have functional consequences for fertilization? The acrosome reaction consists of two steps—polymerization and lengthening of the acrosomal process with subsequent exocytosis of the acrosomal vesicle, which exposes bindin; bindin then coats the acrosomal process and aids in sperm-egg attachment and fusion. Hirohashi and Vacquier (2003) showed that these events can be decoupled in sea urchin sperm by blocking one of the two calcium channels that are stimulated by sulfated fucans in the egg jelly. However, although polymerization of actin is not necessary to induce acrosomal exocytosis in sea urchins, anomalies in acrosomal process formation could still interfere with normal fertilization, and the role of polymerization in determining fertilization success remains unknown. In humans, actin polymerization seems to play an important role in the zona pellucida-induced acrosome reaction; blocking polymerization inhibits the acrosomal reaction, and thus subsequent zona penetration and fertilization (Liu *et al.*, 1999, 2002; Sun and Schatten, 2006).

Sperm structure and genetic affiliation

The geographic diversity in sperm morphology reported here is consistent with patterns of genetic divergence among *S. droebachiensis* populations. Northeastern Atlantic populations of *S. droebachiensis* are genetically divergent from populations in the northwestern Atlantic and northern Pacific. Norwegian populations show significant divergence from their Pacific counterparts in both mitochondrial DNA (Addison and Hart, 2005) and nuclear DNA (sperm bindin: Fig. 4; microsatellites: Addison and Hart, 2004, 2005). The unusual sperm morphology we describe here for *S. droebachiensis* occurs in the north Pacific Ocean, where strongylocentrotid sea urchins originated (Biermann *et al.*, 2003)—historically a much more stable and species-rich environment than the Atlantic (Vermeij, 1991). We found similar sperm in the western Atlantic; this close relationship in sperm ultrastructure of *S. droebachiensis* from New England and the Pacific is consistent with the DNA data. In contrast, the putatively younger (Addison and Hart, 2005) European populations of *S. droebachiensis* exhibit the more common, plesiomorphic sperm morphology—blunter and without preformed acrosomal filaments.

Our results indicate that sperm structure and genetic affiliation are correlated on a large geographic scale. The

high degree of genetic divergence among *S. droebachiensis* populations (more than 1.5% in bindin and 3.5% in mtDNA) is greater than that separating several species pairs within the Indo-West Pacific sea urchin genus *Echinometra*, where the difference between species can be as low as 0.9% (Landry *et al.*, 2003). Unlike the rapid differentiation in genetic signature and gamete morphology described for the *Echinometra* group, the intraspecific sperm divergence we describe here may have evolved over several million years.

Northwestern Atlantic *S. droebachiensis* populations contain a mixture of alleles from both northern Pacific and European sources (Palumbi and Wilson, 1990; Addison and Hart, 2004, 2005; Harper *et al.*, 2007). It will be interesting to examine sperm morphology in conjunction with genetics in fine-scale sampling of *S. droebachiensis* from the Arctic and northwestern Atlantic, to see whether this admixed population exhibits one or both distinct sperm types, or intermediates.

The links between gamete morphology, reproductive isolation, and genetic divergence remain elusive (*e.g.,* Birkhead *et al.*, 2005). Marine broadcast spawners offer many opportunities to study the functional significance of gamete traits, for both intraspecific competition and species-specific fertilization. In combination with genetic data and data from ongoing studies on fertilization success within and among species and populations, patterns of variation in sperm morphology may shed light on the mechanisms by which species recognition evolves.

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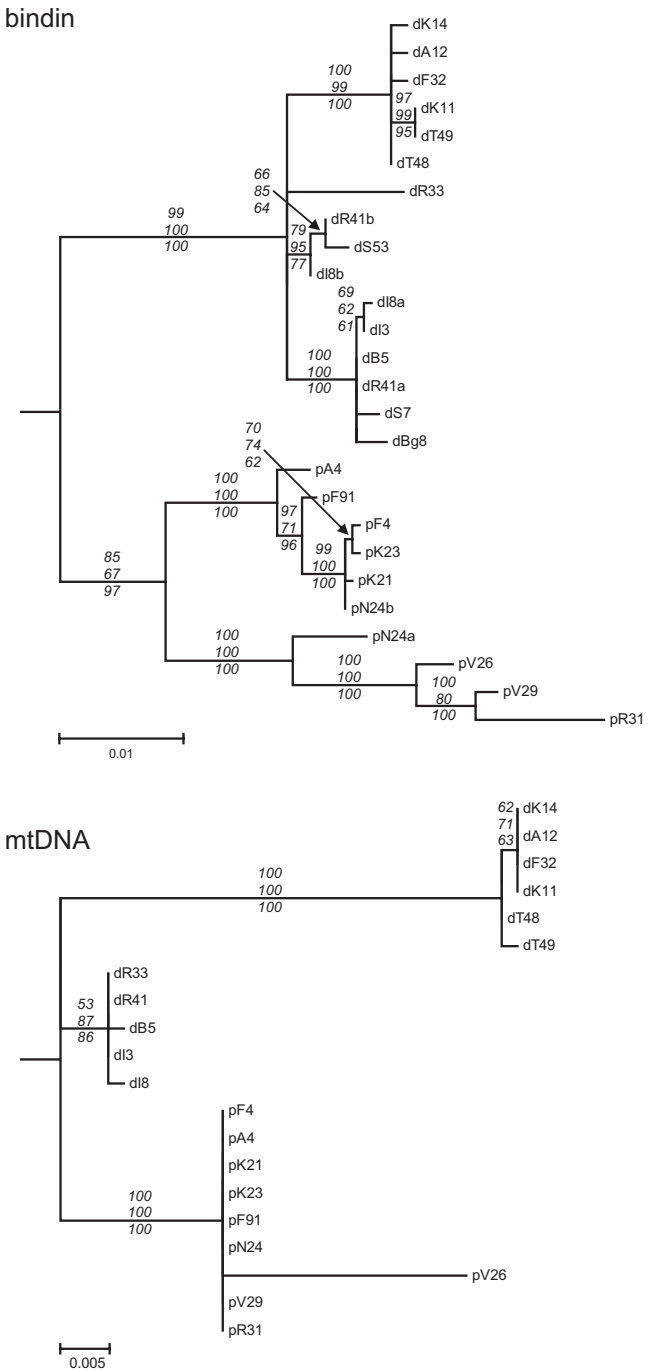
Appendix

Genealogies of sperm bindin and mitochondrial DNA sequences in geographic samples of the sea urchins *Strongylocentrotus droebachiensis* and *S. pallidus*

Strongylocentrotus droebachiensis (O. F. Müller) and *S. pallidus* (G. O. Sars) were sampled from both sides of the North Pacific and of the North Atlantic, and from Alaska, the Barrow Strait in Arctic Canada, and Iceland (see legend to Appendix Fig. 1). DNA was isolated from ethanol-preserved gonad or muscle tissue, and sequenced after PCR



Appendix Figure 1. Gene genealogies for the two circumarctic sea urchin species *Strongylocentrotus droebachiensis* (denoted "d" in the taxon labels) and *S. pallidus* (denoted "p"). The upper tree is based on DNA sequences of the nuclear gene for mature sperm bindin, the lower on a segment of the mitochondrial ATPase 6 gene. Sampling locations were Kamchatka, Siberia (denoted "K"); Alaska (A); Friday Harbor, Washington State (F); Resolute near the magnetic North Pole, Canada (R); the Grand Banks, Newfoundland (N); Thread-of-Life, Damariscotta River estuary, Maine (T); Breidafjord, Iceland (I); Rossøya, Svalbard (S); and Tromsø (V), Bodø (B), and Bergen (Bg) from mainland Norway. The letters "a" and "b" after the individual number denote two alleles from a heterozygous individual. Each tree is a strict consensus of three bootstrap consensus trees, calculated by maximum likelihood (ML), minimum evolution distance (ME), and maximum parsimony (MP), respectively. The bootstrap proportions are written on each branch: top—ML, middle—ME, bottom—MP. The scale bar for each tree indicates the number of substitutions per site as estimated by ML.

amplification (Biermann, 1997, 1998). The mitochondrial DNA fragment was sequenced directly, whereas bindin PCR products were cloned before sequencing because of the frequency of heterozygous length mutations. At least two clones were sequenced in their entirety for each allele. Most samples were sequenced manually (with Sequenase, Amersham), except samples S and Bg, which were sequenced on an ABI 377. We recovered 16 bindin alleles from 14 individuals (*i.e.,* two heterozygotes) of *S. droebachiensis*, and 10 bindin alleles from 9 individuals of *S. pallidus* (one heterozygote).

The section of the mitochondrial ATPase subunit 6 that we sequenced (first 312 nucleotides, positions 8640–8952 in Jacobs *et al.*, 1988) had no insertions or deletions. The ATPase 6 gene is one of the most variable regions available since echinoid mitochondrial DNA does not have a control region. The sequences for the mature sperm bindin gene (1839 nucleotides after alignment, about 950 of which constitute the intron) were aligned with one sequence of the outgroup species *Hemicentrotus pulcherrimus* (Biermann, 1998). The alignment was done with Clustal (in MEGA, Tamura *et al.*, 2007) with small manual corrections. We included all sites in the following analyses, except for a 535-nt insertion in the bindin intron found in three of the Pacific *S. droebachiensis* specimens (Biermann, 1997). Its inclusion had no discernible effect on the tree topologies.

The mtDNA sequences have been deposited in GenBank under accession numbers AF133311–AF133330, and the mature bindin sequences under AF077311–AF077314, AF077318, AF133794–AF133812, and EU663623–EU663625.

Phylogenetic analyses were conducted in PAUP* 4.0b10 (Swofford, 2003), and included parameter estimations by maximum likelihood (ML), plus bootstrap analyses under the criteria of ML, distance (minimum evolution, ME, with Tamura-Nei distances), and maximum parsimony (MP).

Because of the uncertain branching order among the five most closely related strongylocentrotid species (Biermann, 1998; Biermann *et al.*, 2003), *Hemicentrotus pulcherrimus* was used as an outgroup to root the bindin genealogies. The ML parameter estimates for the nuclear bindin gene were transition/transversion ratio 1.034, proportion of invariable sites 0.33475, and the shape parameter for the γ-distribution $\alpha = 0.8302$. We used the default settings for all other tree-building options. The number of bootstrap replicates for bindin were ML, 150; ME, 1000; and MP, 1000.

For the mitochondrial ATPase sequences, we estimated a

transition/transversion ratio of 15, the proportion of invariable sites as 0.5, and equal rates at variable sites. The number of bootstrap replicates were ML, 1000; ME, 750; and MP, 1000. The mitochondrial trees were rooted by making *S. pallidus* individuals a monophyletic sister group to *S. droebachiensis*.

For each of the two gene regions, we calculated a separate bootstrap consensus tree using each of the inference methods (ML, ME, MP); each bootstrap consensus was by the 50% majority rule. The three bootstrap consensus trees were then combined into an overall strict consensus tree for each locus. The branch lengths for these final trees were estimated by ML.

The genealogies were well resolved (unlike the branching order between strongylocentrotid species; Biermann, 1998), and were only partly congruent between species and gene regions. The bindin locus showed strong geographic structuring: in both species, North Pacific alleles formed a robust cluster with West Atlantic alleles. Interestingly, the European/Arctic *S. droebachiensis* individuals appeared paraphyletic with respect to this Pacific clade, even though the species originated in the North Pacific (Biermann *et al.*, 2003; Addison and Hart, 2005). The mitochondrial sequences confirmed the geographic population subdivision, but only in *S. droebachiensis*; they were virtually monomorphic in *S. pallidus*. These findings highlight the importance of making biogeographic inferences from multiple species and loci.