FACETS
a multidisciplinary open access science journal

RESEARCH ARTICLE

Cryptic species diversity and reproductive isolation among sympatric lineages of *Strongylocentrotus* sea urchins in the northwest Atlantic

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Abstract

Distinguishing between intra- and inter-specific variation in genetic studies is critical to understanding evolution because the mechanisms driving change among populations are expected to be different than those that shape reproductive isolation among lineages. Genetic studies of north Atlantic green sea urchins *Strongylocentrotus droebachiensis* (Müller, 1776) have detected significant population substructure and asymmetric gene flow from Europe to Atlantic Canada and interspecific hybridization between *S. droebachiensis* and *Strongylocentrotus pallidus* (Sars, 1871). However, combined with patterns of divergence at mtDNA sequences, morphological divergence at gamete traits suggests that the European and North American lineages of *S. droebachiensis* may be cryptic species. Here, we use a combination of cytochrome *c* oxidase subunit I (*COI*) sequences and single nucleotide polymorphisms (SNPs) to test for cryptic species within *Strongylocentrotus* sea urchins and hybrids between *S. droebachiensis* and *S. pallidus* populations. We detect striking patterns of habitat and reproductive isolation between two *S. droebachiensis* lineages, with offshore deep-water collections consisting of *S. pallidus* in addition to a cryptic lineage sharing genetic similarity with previously published sequences from eastern Atlantic *S. droebachiensis*. We detected only limited hybridization among all three lineages of sea urchins, suggesting that shared genetic differences previously reported may be a result of historical introgression or incomplete lineage sorting.

Key words: ddRAD, mtDNA, genetics, marine invertebrate, *Strongylocentrotus droebachiensis*, north Atlantic

OPEN ACCESS

Citation: Addison JA and Kim J-H. 2018. Cryptic species diversity and reproductive isolation among sympatric lineages of *Strongylocentrotus* sea urchins in the northwest Atlantic. FACETS 3: 61–78. doi:10.1139/facets-2017-0081

Handling Editor: Vance L. Trudeau

Received: June 26, 2017

Accepted: September 26, 2017

Published: January 29, 2018

Published by: Canadian Science Publishing

Introduction

Broadcast-spawning marine invertebrates offer unique prospects for studying reproductive isolation, because the opportunities for allopatric separation are fewer in the ocean, and both divergent selection along ecological boundaries and the evolution of gamete incompatibilities are expected to be key mechanisms driving speciation in the sea (Bowen et al. 2013). Genetic studies aimed at assessing the ecological and evolutionary processes that initiate divergence among populations hinge on the capacity to recognize (and target) species of interest, because the mechanisms driving intraspecific diversification can be different from those driving interspecific divergence (e.g., Palumbi 1994). Recent studies on marine invertebrates (e.g., Vogler et al. 2008; Ladner and Palumbi 2012; Pérez-Portela et al. 2013; Pante et al. 2015; Warner et al. 2015) have identified cryptic species in what were once

thought of as divergent populations. In such cases, unrecognized diversity within or among populations containing cryptic species influences the partitioning of genetic variation among allopatric samples and can bias the estimation of population divergence. Thus, patterns of genetic structure are open to being misinterpreted as a result of restricted gene flow and increased genetic drift while overlooking additional extrinsic and intrinsic factors driving genetic differentiation among species.

Differentiating between intra- and inter-specific diversity in sympatric lineages also provides the opportunity to identify introgressive hybridization when reproductive isolation is incomplete. Recent empirical studies have resurrected ecological factors as a primary driver of marine speciation (Choat 2006; Bowen et al. 2013), and the ecological differences between adjacent habitats represent a middle ground between sympatric and allopatric isolation (Doebeli and Dieckmann 2003). Identifying hybrid zones that form across such habitat disjunctions provides a "natural laboratory" in which we can observe the evolutionary process of speciation. Offspring of mixed ancestries contain different combinations of parental genotypes that are tested by natural selection (Barton and Hewitt 1985), and the introgression of loci among hybrids will vary with their effect on fitness. Thus, hybrid zones offer important settings for studies of selection, gene flow, and genetic drift, because the introgression of foreign alleles is a direct measure of the degree of reproductive isolation.

Sea urchins have long been a model organism for the studies of developmental biology, and species in the genus *Strongylocentrotus* are becoming an increasingly popular in the study of population genetics and evolution (Addison and Hart 2004; Addison and Hart 2005; Addison and Pogson 2009; Pujolar and Pogson 2011; Kober and Bernardi 2013; Norderhaug et al. 2016). Genetic studies of *Strongylocentrotus droebachiensis* (Müller, 1776) detected local panmixis but significant population substructure between the east and west coasts of the north Atlantic, where the eastern populations had low genetic diversity at both microsatellite loci and mtDNA sequences (Addison and Hart 2004, 2005; Harper et al. 2007). Individuals of eastern descent were detected at low frequency in more diverse western populations, and the patterns of genetic subdivision were interpreted as the result of limited intraspecific gene flow and genetic drift. However, subsequent research on patterns of morphological variation in sperm traits have identified significant differences among populations of *S. droebachiensis*, with sea urchins from the northwest Atlantic and Pacific having longer and narrower sperm nuclei than those from the northeast Atlantic (Marks et al. 2008). Similarly, Manier and Palumbi (2008) reported that overall sperm morphology (head length, head width, axoneme length, and mid-piece area) was most divergent between the Pacific and the northeast Atlantic, and comparisons of quantitative divergence (Q_{ST}) for sperm characters relative to divergence at neutral microsatellite markers (F_{ST}) suggest that directional selection may be accelerating the evolution of sperm traits, especially between the Pacific and northwest Atlantic. Significant divergence in both genetic and morphological characters among allopatric populations across the north Atlantic suggests that lineages of *S. droebachiensis* may be distinct (cryptic) species, and the presence of mtDNA lineages from the eastern populations in the west may simply be a result of incomplete lineage sorting and not gene flow per se. Distinguishing between intraspecific gene flow and interspecific lineage sorting in north Atlantic populations of *S. droebachiensis* will help identify the relevant scales to which population subdivision is an evolutionarily driver in this system.

In addition to potential for gene flow between the east and west lineages of *S. droebachiensis*, patterns of genetic diversity within Atlantic populations of *S. droebachiensis* may also be influenced by introgressive hybridization with the congener *Strongylocentrotus pallidus* (Sars, 1871). *Strongylocentrotus droebachiensis* and *S. pallidus* are circumpolar and have overlapping depth preferences, with *S. dreobachiensis* typically found in shallow habitats (0–30 m) and *S. pallidus* in deeper

habitats (>30 m). Mixed aggregations of both species have been observed throughout their range at depths >15 m (Vasseur 1952; Swan 1953; Gagnon and Gilkinson 1994; Bluhm et al. 1998), and as both species broadcast sperm and eggs, opportunities exist for their gametes to interact in the plankton. However, levels of hybridization and introgression detected in natural populations (Addison and Hart 2005; Harper et al. 2007; Addison and Pogson 2009; Pujolar and Pogson 2011) are much lower than predicted based on their propensity to hybridize under laboratory conditions (Strathmann 1981). Analyses of nuclear DNA sequences detected asymmetric introgression (from *S. pallidus* into *S. droebachiensis*) in approximately 5% of individuals sampled in the northeast Pacific (Addison and Pogson 2009). Genetic studies of *S. droebachiensis* in the northwest Atlantic are consistent with the low asymmetric gene flow of mitochondrial genes from *S. pallidus* (Addison and Hart 2005), but a lack of resolution at nuclear loci (four microsatellites; Addison and Hart 2002, 2004) makes it difficult to distinguish between incomplete lineage sorting and recent hybridization.

Our goal with this study is to examine northwest Atlantic populations of *Strongylocentrotus* sea urchins for evidence of cryptic species and hybridization. To do this, we evaluated mitochondrial and nuclear DNA polymorphisms in both coastal populations of *S. droebachiensis* (10–30 m) and offshore populations of *S. pallidus* (65–120 m). If contemporary gene exchange occurs between *S. pallidus* and *S. droebachiensis* (interspecific hybridization) or among genetically distinct populations of *S. droebachiensis* (intraspecific gene flow), then patterns of mtDNA haplotype sharing among lineages should correspond to admixture at nuclear loci. Alternatively, if mtDNA haplotype sharing results from incomplete lineage sorting or historical introgression, then we predict limited admixture across the nuclear genome. Finally, the absence of both mtDNA haplotype sharing and admixture across the nuclear genome would suggest complete reproductive isolation among the lineages. Overall, this study contributes to a better understanding of reproductive isolation among *Strongylocentrotus* sea urchin lineages in the north Atlantic and provides a framework for distinguishing between mechanisms driving intra- and inter-specific divergence.

Methods

Specimen collection

Green sea urchins (*S. droebachiensis*; $n = 60$) were collected from coastal habitat near Duncan Cove, Nova Scotia (44.2954°N, 63.3120°W), ranging in depths from 10 to 32.5 m using SCUBA ("shallow" populations). We sampled along a depth gradient at one location in an attempt to identify hybrid individuals across the range of shallow habitats dominated by *S. droebachiensis* and deeper coastal habitats where (at least in the Pacific) individuals of mixed ancestry have been detected (Addison and Pogson 2009). Pale sea urchins (*S. pallidus; n* = 70) were collected from offshore populations using a fixed gear dredge ("deep" populations) at 65 m (42.2348°N, 64.1282°W), 72 m (43.3588°N, 64.4279°W), 92 m (43.4862°N, 67.0702°W), and 120 m (43.4367°N, 62.5083°W) (**Fig. 1**). Shallow-water specimens were confirmed as being *S. droebachiensis* based on colour characteristics (i.e., test, tube feet, and aboral spines) that strongly correlate with detailed identification keys (e.g., Jensen 1974; Gagnon and Gilkinson 1994). Deep-water samples were generally pale in color, but as they had been frozen at sea, most were in poor condition, and confirming species identity through secondary characteristics such as the color of the tube feet or aboral spines was difficult. Therefore prior to DNA extraction, these samples were categorized as *S. pallidus* following the morphological studies of Gilkinson et al. (1988) and Gagnon and Gilkinson (1994) who showed that *Strongylocentrotus* sea urchins sampled at depths >60 m in Atlantic Canada were *S. pallidus*. Gonad tissue was preserved in 95% ethanol, and total genomic DNA was extracted using DNAeasy Blood and Tissue columns (QIAGEN) following the manufacturer's recommended protocols.

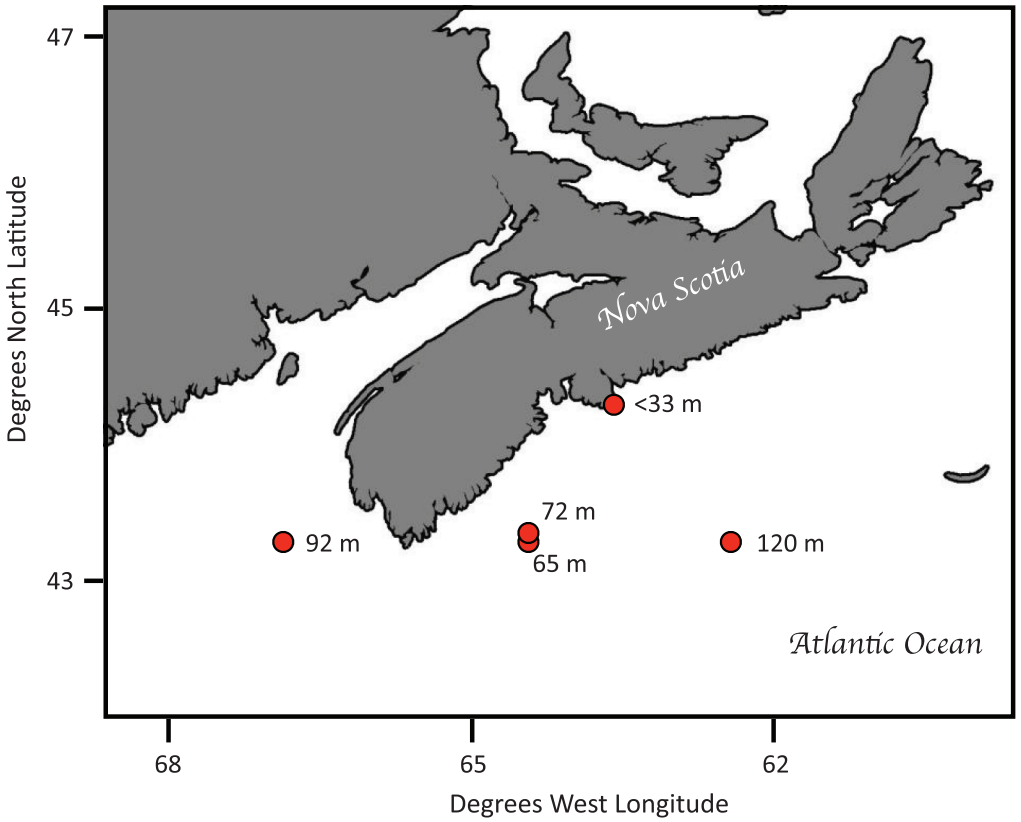

Fig. 1. Sample sites of *Strongylocentrotus* sea urchins. Coastal samples were collected using SCUBA, and offshore samples were collected using a fixed gear dredge. Map data ©2016 Mapbox (mapbox.com/about/maps/).

COI genotyping

A fragment of the cytochrome *c* oxidase subunit I (*COI*) mitochondrial gene was amplified using the polymerase chain reaction (PCR) primers COIJ and COIC (Edmands et al. 1996). Amplification was performed in a 30 µL volume consisting of 4 ng DNA, 1× ThermoPol reaction buffer (New England Biolabs, NEB), 0.2 mmol dNTPs (NEB), 2.0 mmol $MgSO_4$, 0.5 µmol forward and reverse primers, and 1.0 unit of *Taq* polymerase (NEB). Thermal cycling conditions were 95 °C for 3 min, followed by 39 cycles of 95 °C (30 s), 45 °C (30 s), 72 °C (60 s), and a final extension at 72 °C for 3 min. Amplicons were separated using agarose gel electrophoresis and visualized under UV light prior to sequencing using forward, reverse, or both PCR primers at the Genome Quebec Innovation Centre (McGill University, Montreal, Quebec, Canada).

Sequences were aligned, edited, and trimmed using CLC Genomics WorkBench (CLC GW; QIAGEN) (GenBank accession No. MG098337-MG098440). For samples collected at each depth, we computed measurements of nucleotide diversity (π), number of segregating sites (S), number of haplotypes (H), and haplotype diversity (h) using Arlequin v3.5 (Excoffier and Lischer 2010). To examine our data for introgressive hybridization, we conducted a phylogenetic analysis of mtDNA and designated each haplotype as either *S. droebachiensis* or *S. pallidus*. First, we constructed a gene tree and estimated the posterior probability of the nodes with MrBayes 3 (Ronquist and Huelsenbeck 2003). We used the HKY model of substitution selected by the Bayesian implementation

criterion implemented in the program jModelTest 2.1.3 (Darriba et al. 2012) and ran independent analyses of 10 chains (temp = 0.1) for 3 million generations, sampling trees every 1000 steps. Convergence was achieved by running the analysis long enough to ensure that the standard deviation of the split frequencies of both independent runs was <0.01. A second phylogenetic tree was inferred by maximum likelihood using PhyML 3.1 (Guindon et al. 2010), with an HKY85 substitution model, gamma distributed rate heterogeneity at sites, and an SPR tree search. Nodal support was estimated using nonparametric bootstrap analysis with 1000 replicates. We rooted both trees using the conger *Strongylocentrotus intermedius* (Agassiz, 1864) (see Kober and Bernardi 2013). Finally, to simultaneously visualize both the phylogenetic relationships and the frequency of each haplotype, we constructed 95% parsimony networks using the computer program tcs 1.18 (Clement et al. 2000).

SNP genotyping

We collected ddRAD-seq data following the protocol described by Poland et al. (2012). We selected a combination of restriction enzymes *Mlu*CI (common cutter; 5′-AATT-3′; NEB) and *Sau*96I (rare cutter; 5′-GGNCC-3′; NEB) to reduce the complexity of the genome and increase the sequencing depth at similar sites among individuals (Poland and Rife 2012). In addition, double digests using this combination of enzymes generated fragments in the size range of 200–300 bp, thus eliminating the need for a size selection step in the construction of the library. We used custom adapters that included 48 multiplex-identifier (MID) barcodes (4–9 bp), with a *Sau*96I overhang on one end (A1) and a common Y adapter with a *Mlu*CI overhang at the other end (A2). We included four different MID tags on Adapter 2 (3–8 bp) to increase sequence complexity during the first few bases to possibly improve resolution of captured florescent image in base-calling.

We double-digested 100 ng of genomic DNA in CutSmart® Buffer (NEB) by incubating 10 units of *Mlu*CI and five units of *Sau*96I in a single 20 µL reaction for 2 h at 37 °C, followed by 20 min at 65 °C. Adapters were ligated to the digested DNA in the same tubes that included 0.1 µmol/L A1, 15 µmol/L A2, 0.5× CutSmart® Buffer, 1 mmol/L ATP, and 400 units of T4 ligase. The 40 µL reaction volume was incubated at 22 °C for 2 h, 65 °C for 30 min, and 4 °C overnight. We pooled 12 individuals (10 µL each) and purified the mix using QIAquick PCR Purification kit in one column following the manufacturer's protocol and eluting using 50 µL EB buffer (10 mmol/L Tris-Cl at pH 8.5; QIAGEN). Pooled ligation products were amplified in 50 µL volumes containing 10 µL DNA template, 1× NEBNEXT High-Fidelity PCR Master Mix, and 0.8 µmol/L each of the forward and reverse sequencing primer. Thermal cycling protocol consisted of 98 °C for 30 s, 15 cycles of 98 °C (10 s), 65 °C (30 s), 72 °C (30 s), and 72 °C for 5 min. To construct the final libraries, we pooled four PCR reactions (48 individuals total) and purified with a QIAquick PCR Purification kits and eluting with 50 µL EB buffer. We constructed a total of three libraries (48-plex) and each was pair-end sequenced (100 bp) on the Illumina HiSeq2000 platform at the Genome Quebec Innovation Centre (McGill University, Montreal, Quebec, Canada).

Bioinformatics

Raw Illumina reads were imported into CLC GW 8.0.3 (QIAGEN) and demultiplexed based on Y-adapter and unique barcodes. Reads lacking a complete barcode were discarded, and we filtered for quality by retaining reads with a minimum PHRED score of 30, a limit of two ambiguous nucleotides, and a minimum length of 30 bp. Pairing reads that include a minimum of seven overlapping nucleotides at the beginning or end of sequences were merged to improve quality in read mapping by increasing the sequencing length. We then mapped all the reads to the *Strongylocentrotus purpuratus* (Stimpson, 1857) reference genome (Sea Urchin Genome Sequencing Consortium et al. 2006) using the CLC Assembly Cell 4.3 algorithm with default parameters, a length fraction of 0.5, and similarity fraction of 0.8. A consensus sequence was extracted from this mapping and used as the reference for

read mapping for SNP variants with the same parameters except a length fraction of 0.9. We detected variants for each individual and for the reference as a whole, using CLC's Basic Variant Detection tool. We removed all polymorphisms except single nucleotide variants (SNVs), and we retained only SNVs with at least eight reads of coverage for each individual. Finally, SNPs were filtered for a minor allele frequency of ≥ 0.05.

SNP analysis

Summary statistics, including indices of nucleotide diversity (π), observed (H_o) and expected (H_e) heterozygosity, average gene diversity over loci (π_n), and F_{ST} values, were calculated in ARLEQUIN v3.5. Locus-specific F_{ST} values were calculated in GENEPOP (Rousset 2008). We tested for the presence of distinct genetic lineages and hybrid individuals using the clustering method in STRUCTURE v2.4.3 (Pritchard et al. 2000; Falush et al. 2003) to assign individual multilocus genotypes to k groups. We performed the analysis in STRUCTURE using $1 < k < 10$ assuming an admixture model, uncorrelated allele frequencies, and no population priors. We ran Bayesian MCMC searches for 1 million steps with an initial burn-in of 100 000. For each analysis, we performed five replicates and used the method of Evanno et al. (2005) to find the best-fit value of k.

We further tested for the presence and nature of hybrids using the Gensback command in STRUCTURE. We assumed that individuals in each cluster had pure ancestry, but that some individuals may have ancestry from one or more of the other clusters. This analysis uses an informative prior based on k (from the admixture model) to calculate the posterior probability that an individual has an immigrant ancestor in the last G generations, where $G = 0$ corresponds to the individual being an immigrant itself. In this analysis, we limited k to 3 (see below), set the prior probability of migrants to 0.01, and allowed the detection of migrants up to four generations before the present by setting $G = 4$. The calculations were performed on the final 100 000 runs with an initial burn-in of 10 000.

Results

mtDNA variation

We obtained 399 bp *COI* sequences for 104 of the 110 sea urchins collected from shallow ($n = 49$) and deep-water ($n = 55$) habitats for which we obtained high-quality genomic DNA (**Table 1**). There were 36 variable sites and a total of 22 unique haplotypes. Phylogenetic analysis identified three well-supported clades (**Fig. 2A**) consisting of the expected lineages of *S. droebachiensis* and *S. pallidus* from the shallow and deep habitats, respectively, as well as an unexpected cryptic lineage of *S. droebachiensis* detected only in the deep habitat. Mean genetic divergence (Kimura 2-parameter) among all the sequences was 3.1%, whereas the pairwise divergence between the shallow and deep-water populations of *S. droebachiensis* was 2.3%.

Population-level analyses of the *COI* data revealed two instances of potential hybridization in which shallow-water *S. droebachiensis* individuals produced mtDNA that grouped within the well-supported clade of deep-water *S. pallidus* haplotypes (**Fig. 2B**). We failed to detect shallow-water (northwest Atlantic) *S. droebachiensis* haplotypes in individuals sampled from the deep-water habitat, but 17 of the 55 (31%) deep-water samples produced mtDNA haplotypes similar to the northeast Atlantic *S. droebachiensis* lineage (see Addison and Hart 2005; **Fig. S1**).

ddRAD-seq library results

Sequencing of the ddRAD-seq libraries generated a total of 889.0 million raw read dataset from 110 individuals (**Table S1**). A total of 372.7 million reads were retained following quality filtering, removal of adapter sequences, and merging pair-end reads. The total length of the processed (trimmed and

Table 1. A priori species identification, sample depth, number of samples (n), and molecular diversity indices for *Strongylocentrotus* sea urchins.

		nDNA				mtDNA				
Putative species	Depth	n	H_o	H_e	π_n	n	S	π	H	h
S. droebachiensis	10	14	0.268 (0.206)	0.260 (0.155)	0.157 (0.076)	11	22	0.012	6	0.800
	13	7	0.335 (0.225)	0.316 (0.146)	0.161 (0.082)	6	23	0.020	4	0.800
	17	7	0.347 (0.226)	0.321 (0.145)	0.159 (0.080)	7	4	0.003	3	0.524
	21	7	0.346 (0.226)	0.318 (0.146)	0.160 (0.081)	7	3	0.004	2	0.476
	28	11	0.301 (0.223)	0.280 (0.154)	0.157 (0.078)	11	2	0.002	2	0.436
	32	7	0.366 (0.232)	0.311 (0.147)	0.155 (0.078)	7	0	0.000	1	0.000
S. pallidus	65	9	0.283 (0.225)	0.312 (0.147)	0.238 (0.119)	8	19	0.012	3	0.464
	72	19	0.241 (0.180)	0.314 (0.137)	0.263 (0.127)	19	21	0.019	7	0.667
	92	14	0.277 (0.213)	0.306 (0.142)	0.248 (0.121)	13	19	0.013	5	0.583
	120	15	0.218 (0.175)	0.316 (0.153)	0.259 (0.126)	15	20	0.023	6	0.790
Total	—	110	0.199 (0.134)	0.277 (0.132)	0.277 (0.131)	104	36	0.029	22	0.801

Note: Values in parentheses are the standard deviation. Diversity indices: H_o, observed heterozygosity; H_e, expected heterozygosity; π_n, average gene diversity over loci for the SNPs; S, the number of segregating sites; π, nucleotide diversity; H, number of unique haplotypes; and h, haplotype diversity.

merged) reads was 34.8 billion bases, corresponding to a length coverage of 37.1× against the purple sea urchin genome of 936.4 million bases (Sea Urchin Genome Sequencing Consortium et al. 2006). We mapped over 90% (342.0 million reads) of the Atlantic sea urchin ddRAD-seq dataset to the *S. purpuratus* genome, with an average coverage of 104.0× (total length of mapped reads per mapped region) (Table S2). Consensus genome sequences between Atlantic (*S. droebachiensis* and *S. pallidus*) and purple (*S. purpuratus*) sea urchins were extracted and used as a reference for variant detection. More reads were aligned to intergenic regions (55.8%) compared with genic regions (44.2%) of consensus genome of Atlantic–purple sea urchin (Table S3). We detected a total of 104 817 SNPs that were scored in all 110 individuals, not including indel polymorphisms, or multi nucleotide variants. After filtering for a minor allele frequency of 0.05, 13 186 bi-allelic SNPs remained. In contrast to the results from read mapping, more were detected in genic regions (10 137; 76.9%) compared with those located intergenic regions (3049; 23.1%), respectively. We focused our analyses on polymorphisms in putatively neutral intergenic regions to limit the impact of loci potentially under strong ecological or sexual selection among the lineages.

nDNA diversity

Measures of genetic variability at each sample for 3049 intergenic SNPs are summarized in Table 1. Observed heterozygosity (0.218–0.366), expected heterozygosity (0.260–0.321), and the average gene diversity over loci (π_n; 0.155–0.263) were similar when computed for each sample site or for samples grouped into the distinct genotype clusters identified using both the mtDNA and STRUCTURE analyses (see below). Global F_{ST} among all samples was 0.267, and pairwise F_{ST} between the three genetic clusters was 0.467 (*S. pallidus* and deep *S. droebachiensis*), 0.474 (*S. pallidus* and shallow *S. droebachiensis*), and 0.497 between shallow and deep samples of *S. droebachiensis* (for all comparisons $p < 0.001$). The frequency distribution of locus-specific F_{ST} values calculated among samples of all three lineages peaked at $F_{ST} = 0.10$–0.15, but there was a relatively even frequency distribution of loci with F_{ST} values between 0.5 and 1.0 (Fig. 3). A total of 796 (26.1%) of the SNPs had an $F_{ST} > 0.50$, and

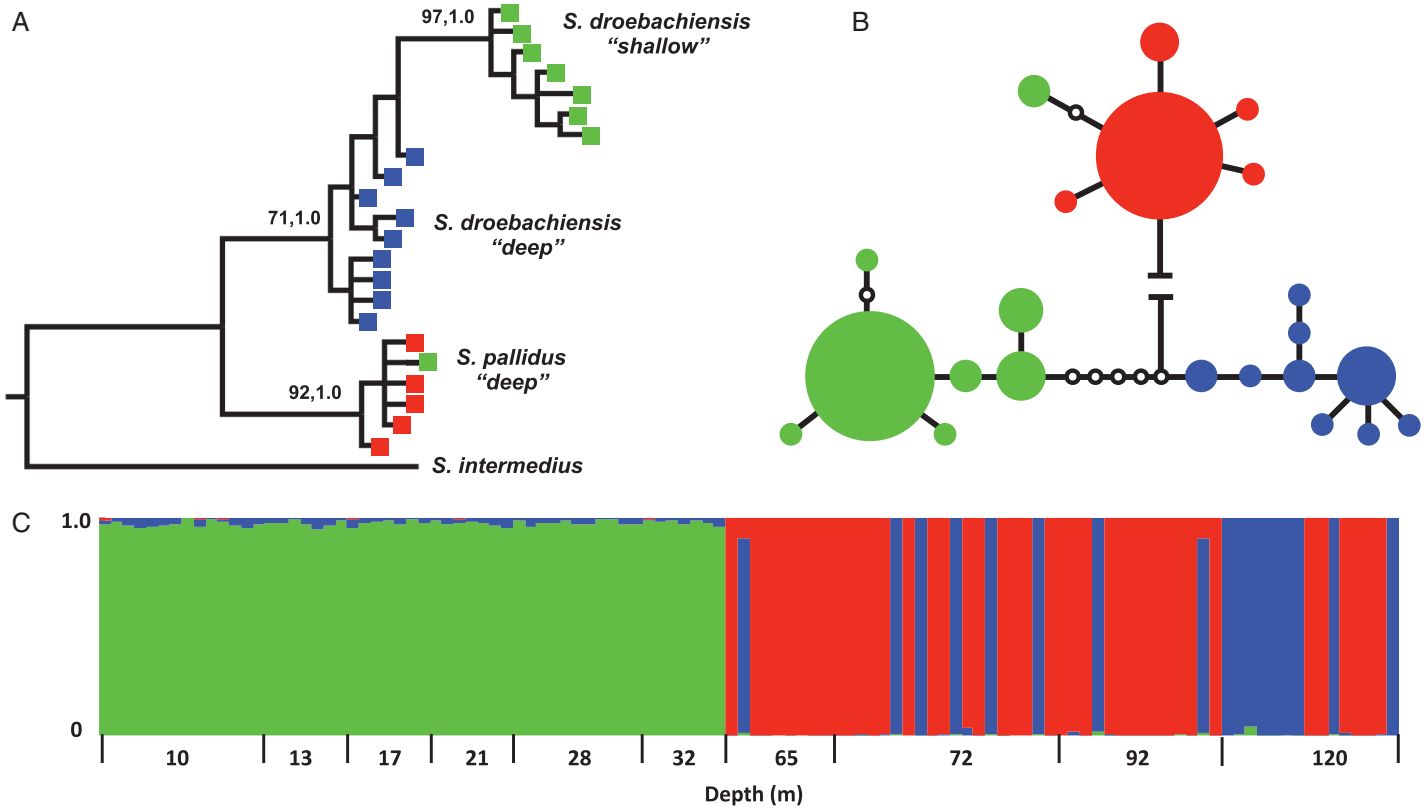

Fig. 2. Summary of *Strongylocentrotus* mtDNA haplotypes and SNP allele frequencies. A) Maximum likelihood tree of 22 unique *COI* haplotypes (399 bp) with nodal support indicated by nonparametric bootstrap (1000 replicates) and Bayesian posterior probability, respectively. B) TCS haplotype network for *COI* sequences ($n = 104$). Circle area is proportional to the number of haplotypes sequenced, lines connect haplotypes that differ by one mutation, small open circles represent mutations not observed in the data, and the line endpoints mark a connection that exceeds the number of steps calculated using the 95% parsimony criteria. C) Bar-plot of the STRUCTURE results for 3049 intergenic SNPs. The horizontal axis indicates individuals ($n = 110$) grouped by depth, and the vertical axis indicates the posterior probability of assignment to the $K = 3$ detected clusters.

304 (10%) had an F_{ST} value >0.80. There was no evidence of population structure among sample sites within lineages (F_{ST} not significantly different from zero), and frequency distributions of locus-specific F_{ST} values peaked at zero (Fig. 3) for all three lineages. Maximum locus-specific F_{ST} values ranged from 0.271 to 0.287 in shallow *S. droebachiensis* and *S. pallidus*, respectively. Frequent F_{ST} values >0.40 detected in the deep lineage of *S. droebachiensis* were largely driven by small sample sizes (≤ 2) in two of the four sample sites. Removing these two individuals revealed a similar intraspecific distribution of locus diversity with a maximum value of 0.373 (Fig. 3).

Using all 3049 SNPs, we tested for distinct genetic clusters (lineages) and the presence of hybrid individuals using the program STRUCTURE. The inspection of the log likelihoods identified three clusters of genotypes ($K = 3$; Fig. 2C) that corresponded to the shallow- and deep-water populations of *S. droebachiensis* and the deep-water species *S. pallidus*. Although Evanno's method of selecting the best-fit model of K identified $K = 2$, the low negative log-likelihood values from runs with $K = 1$ likely artificially inflated ΔK resulting in an underestimation of the number of distinct genotypes. A recent review by Janes et al. (2017) suggests bias towards $K = 2$ when using Evanno's ΔK correction. As likelihood scores reached an asymptote at $K = 3$ (Fig. S2), reanalysis of the STRUCTURE output after removing $K = 1$ identified the highest value of ΔK at $K = 3$. Based on a combination of the

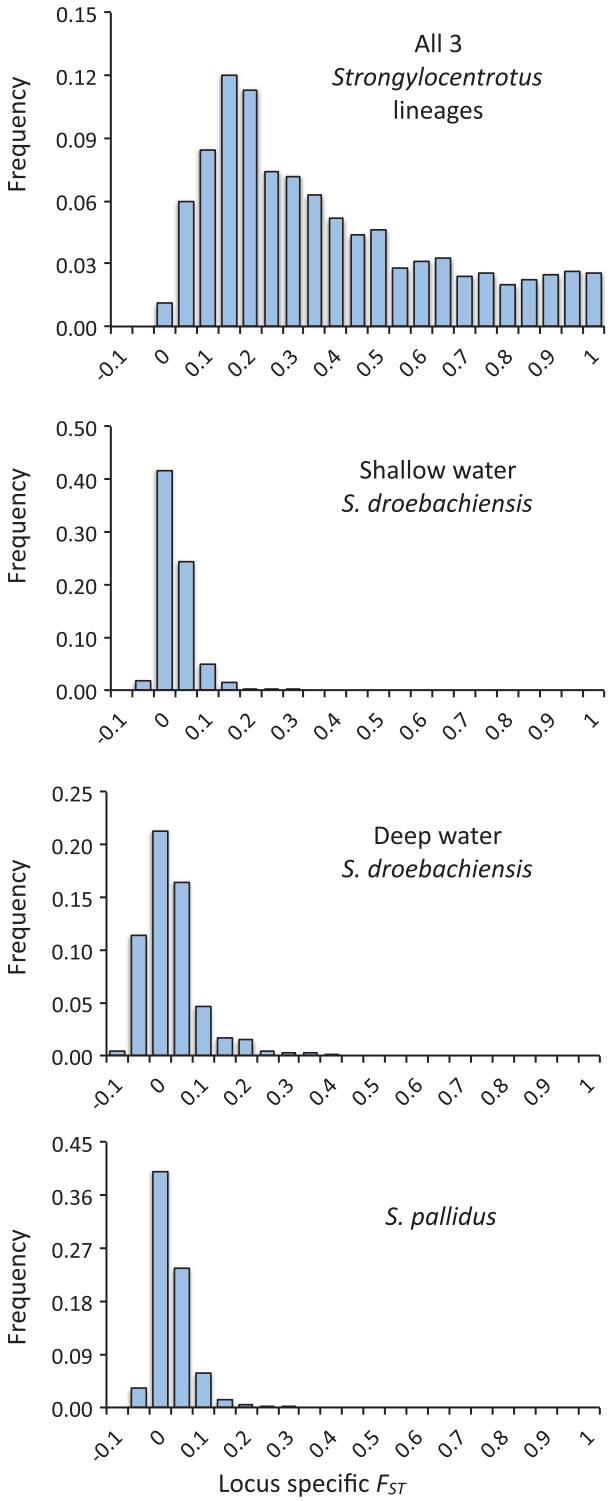

Fig. 3. Frequency distribution of locus-specific pairwise F_{ST} values calculated among samples collected at different depths.

log-likelihood scores from the STRUCTURE analysis, broad consistency of assignment of primary assignment to three clusters across simulations of $K = 1–10$ (Fig. S3), and the reciprocal monophyly observed in the *COI* data from deep and shallow lineages of *S. droebachiensis*, we chose $K = 3$ as the best-fit model.

The clustering pattern detected in the nuclear genome was broadly consistent with the results of the mtDNA analysis (Fig. 2), with the exception of the two mtDNA *S. pallidus* haplotypes recovered in the shallow-water *S. droebachiensis* population. We failed to detect hybrid individuals in the shallow-water *S. droebachiensis*, including the two individuals (at 10 and 13 m) that had an *S. pallidus* mtDNA haplotype but whose admixture proportions (*S. pallidus* into *S. droebachiensis*; 90% probability intervals) were only 0.035 (0.022–0.050) and 0.054 (0.039–0.091), respectively. We detected the presence of two putative deep-water *S. droebachiensis* × *S. pallidus* hybrids, one at 65 m with an admixture proportion (deep-water *S. droebachiensis* into *S. pallidus*) of 0.093 (0.094–0.108), and another at 92 m with an admixture proportion of 0.091 (0.075–0.108), respectively. Using the Gensback setting in STRUCTURE, the two admixed individuals were classified as third-generation backcrosses ($p = 1.000$).

Discussion

Our main goal was to assess northwest Atlantic populations of *S. droebachiensis* and *S. pallidus* for the presence of cryptic species and evidence of gene flow among them. Our data reveal a striking pattern of spatial segregation, genetic divergence, and reproductive isolation between two lineages of *S. droebachiensis*. Pairwise F_{ST} between shallow- and deep-water populations of *S. droebachiensis* was high (0.497, $p < 0.001$) and we failed to detect individuals of mixed ancestry using nuclear genetic markers. Shallow- and deep-water populations did not share mtDNA haplotypes, and the average genetic distance between the two mito-groups was 2.3% (K2P). Phylogenetic analysis of mtDNA identified the deep-water lineage as being genetically similar to sea urchins sampled in the northeast Atlantic (11 of the 17 haplotypes are identical to those found in Iceland and Norway, Fig. S1; Addison and Hart 2005). These results are consistent with earlier genetic studies in which significant subdivision between eastern and western Atlantic populations of *S. droebachiensis* was found for both mitochondrial (Addison and Hart 2005; Harper et al. 2007) and nuclear loci (sperm *Bindin*: Marks et al. 2008; microsatellites: Addison and Hart 2004, 2005). However, in combination with the significant morphological divergence identified in sperm traits (Manier and Palumbi 2008; Marks et al. 2008), the patterns of genome wide divergence and lack of hybridization we detected in this study suggest the presence of a cryptic species in north Atlantic *S. droebachiensis*. These results indicate that the patterns of significant genetic subdivision and asymmetric gene flow from eastern to western populations of *S. droebachiensis* (Addison and Hart 2004, 2005) may represent some degree of interspecific variation and cannot be strictly interpreted as limited intraspecific gene flow and genetic drift. Because both the eastern and western lineages of *S. droebachiensis* have been reported to co-occur in shallow-water populations at higher latitudes in the northwest Atlantic (e.g., Addison and Hart 2005), future genetic studies of these populations will provide insight evolutionary mechanisms driving both ecological and reproductive isolation.

Although studies in the Pacific Ocean have detected asymmetric hybridization and introgression from the pale sea urchin (*S. pallidus*) into the green sea urchin (*S. droebachiensis*) (Addison and Pogson 2009; Pujolar and Pogson 2011), our results suggest that northwest Atlantic lineages of these species are reproductively isolated. Divergence at mitochondrial (5.5% K2P distance) and nuclear loci ($F_{ST} = 0.474$) was high, and the admixture analysis of nuclear loci failed to identify hybrid individuals. We detected *S. pallidus* mtDNA haplotypes in two shallow-water *S. droebachiensis* individuals (4% of the samples), and this is consistent with the findings of Addison and Hart (2005), which reports a similar pattern in 3% of samples ($n = 132$) from Nova Scotia and Newfoundland.

However, limited admixture in the nuclear genome (<5%) in our samples suggests that the shared mtDNA lineages are result of historical gene flow or incomplete lineage sorting, and not recent hybridization.

We detected only weak evidence of hybridization between the deep-water lineages of *S. droebachiensis* and *S. pallidus*. Although the levels of mtDNA and nDNA divergence were high (4.2% K2P and F_{ST} = 0.467, respectively), our structure analysis identified two deep-water *S. droebachiensis* individuals of mixed ancestry whose nuclear genome consisted of ~10% *S. pallidus*. This level of introgression corresponds to a third-generation backcross, indicating that gene flow is contemporary and reproductive isolation between these two species may not yet be complete. Little is known about the spawning ecology of deep-water *Strongylocentrotus* sea urchins, but the relatively low frequency of hybrids detected in our deep *S. droebachiensis* population (~12%) and absence of F_1 or F_2 crosses indicate that gene flow between the species is rare. As population densities of sea urchins are generally low in the deep-water habitat of the northwest Atlantic (Gilkinson et al. 1988), the low frequency of hybrids could be explained by the low probability of heterospecific gametes interacting in the plankton. Alternatively, hybrids may form readily, but a moderate level of post-zygotic selection may act on early life stages (larvae, recruits, and juveniles) to maintain the barriers to gene exchange. More extensive surveys of deep-water populations are required to fully assess the extent to which the *S. pallidus* and *S. droebachiensis* hybridize throughout their range.

Given that hybrids readily form in laboratory crosses among Pacific (Strathmann 1981; Levitan 2002a, 2002b) and Atlantic (Biermann and Marks 2000) species of *Strongylocentrotus* sea urchins, the relatively low levels of hybridization in natural populations we detected in the northwest Atlantic may be due to a variety of pre-zygotic or post-zygotic mechanisms. Like most marine invertebrates, sea urchins broadcast spawn by simply releasing their sperm and eggs into the water column. Habitat or temporal isolation are effective pre-zygotic barriers among broadcast-spawning species (e.g., Pernet 1999; Coppard and Campbell 2005), but for sympatric species that spawn simultaneously, reproductive barriers may largely depend on the compatibilities of their gametes (Palumbi 1994; Coyne and Orr 2004). In the Pacific Ocean, *S. droebachiensis* co-occurs with *S. purpuratus* and *Mesocentrotus franciscanus* (nee. *S. franciscanus*; Kober and Bernardi 2013), all of which vary in their egg traits and susceptibility to fertilization under sperm limitation (Levitan 1993, 2002a, 2002b). *Strongylocentrotus droebachiensis* typically has a lower population density and produces eggs that are easily fertilized by both conspecific and heterospecific sperm (Levitan 2002a, 2002b). However, in the north Atlantic, *S. droebachiensis* is the only species of sea urchin in the shallow subtidal (<30 m) where population densities can reach 400 m^{-2} (Scheibling and Hennigar 1997). High sperm density in these populations can result in high variance in reproductive success caused by polyspermy (Levitan 2004, 2005), leading to sexual conflict as males are selected for fast fertilization and females are selected to prevent polyspermy by reducing fertilization rates (Levitan 2004; Levitan and Ferrell 2006; Levitan et al. 2007; Levitan and Stapper 2010). Under this scenario, the eggs of *S. droebachiensis* in northwest Atlantic populations may have co-evolved mechanisms that indirectly reduce the susceptibility to heterospecific sperm and thus prevent hybridization.

The rapid evolution of gamete recognition molecules has long been considered an important driver of speciation in Echinoderms (Vacquier 1998; Palumbi 2009; Lessios 2011; Vacquier and Swanson 2011). Both proteins and carbohydrates (sulfated polysaccharides) coating the sperm and eggs mediate gamete interactions in the plankton and are involved in sperm competition and mate choice (Biermann et al. 2004). Positive selection at the coding regions of some of these genes, including sperm *Bindin* (e.g., Biermann 1998), correlates with the strength of reproductive isolation between species (Zigler et al. 2005). If the divergence within *S. droebachiensis* corresponds with the

invasion of the east and west coasts of the Atlantic followed by population expansion throughout the ocean basin, then is it possible that continued selection at gamete signaling and recognition molecules continues to drive reproductive isolation (i.e., reinforcement selection; Lessios 2011). Patterns of divergence at sperm *Bindin* within Atlantic populations of *S. droebachiensis* support the importance of reinforcement selection in this system, but future studies are needed to link the function of genotypes with reproductive success and the presence of hybrids throughout the ocean basin.

Our failure to detect hybrids between *S. pallidus* and *S. droebachiensis* in adult sea urchins sampled from the shallow-water coastal habitat may also be a result of strong post-zygotic selection on individuals of mixed ancestry. As our sample site in Nova Scotia is close to the southern range limit of coastal populations of *S. droebachiensis*, decadal and seasonal variation in salinity (Breeze et al. 2002; Russell 2013; Bundy et al. 2014) may intensify the ecological gradient along which the shallow and deep lineages are adapted. Russell (2013) reported that over an eight year period the coastal salinity in New Hampshire frequently drop below 26‰ in March and April, with the lowest recorded salinity being 15‰. Laboratory studies have demonstrated that *S. droebachiensis* is one of the most tolerant echinoderms to hyposalinity (Russell 2013). Roller and Stickle (1985) showed that in the Pacific, larvae of *S. pallidus* failed to develop, and cultures experienced 100% mortality at salinities below 27.5‰. In contrast, although cultures of *S. droebachiensis* developed slowly at salinities ≤22.5‰ and below, survival was 100% at salinities as low as 20‰. In addition, hybrids generated by crossing female *S. droebachiensis* with male *S. pallidus* experienced 100% survivorship at salinities as low as 22.5‰. Himmelman et al. (1984) showed that *S. droebachiensis* collected in the Gulf of St. Lawrence tolerated salinities as low as 14‰. However in the same study, individuals collected from Nova Scotia experienced complete mortality when exposed to the same conditions, indicating that the geographical origin and possibly the genetic composition of populations contributed to the ability to tolerate hyposmotic stress. As both pure and hybrid *S. pallidus* are more susceptible to salinity <22.5‰, it is possible that seasonal variation in salinity could either limit the dispersal of hybrid larvae produced in overlapping populations in deeper habitats or impose strong post-settlement selection on newly metamorphosed recruits or juveniles. Future studies should aim to evaluate these ecological factors as primary drivers of both geographic range limits of and genetic divergence among north Atlantic lineages of *Strongylocentrotus* sea urchins.

Our results are consistent with a recent barcode survey of 145 Canadian Ecninoderm taxa by Layton et al. (2016), in which the authors detected 23 lineages that could not be identified to the species level. Of these individuals were two samples of *Strongylocentrotus* from the Arctic, and analysis of this confirms that these individuals cluster with others from the deep-water/northeast Atlantic lineage (**Fig. S1**). This finding suggests that the cryptic species is widespread throughout the Atlantic basin, segregating in sympatry with shallow-water *S. droebachinesis* at northern latitudes but being restricted to deeper habitats at southern latitudes. It is possible that the range and habitat limits are driven by similar ecological conditions discussed above, and future surveys of *Strongylocentrotus* populations throughout the Atlantic are required to better detail the species' distributions.

Conclusion

Strongylocentrus sea urchins are an ideal system for understanding how reproductive barriers evolve both from the initial accumulation of divergence between populations (e.g., reduced trans-Arctic gene flow in *S. droebachiensis*; Addison and Hart 2005), to the evolution of gamete recognition molecules driven by sexual conflict within species (e.g., *S. purpuratus*; Levitan and Ferrell 2006) and Darwinian selection driving changes between species (e.g., Pujolar and Pogson 2011). Our results identified a cryptic lineage of *S. droebachiensis* that shifts the interpretation of trans-Atlantic genetic diversity from limited gene flow and genetic drift to the interactions among reproductively

isolated species. We discovered the potential for strong ecological conditions to shape the distributions of shallow- and deep-water lineages of sea urchins in the northwest Atlantic and renewed the importance to further assess the biogeography, population genetics, and barriers to gene exchange among all three lineages in the Atlantic Basin.

Acknowledgements

We thank Don Clark at the Department of Fisheries and Oceans St. Andrew's Biological Station and Karen Filbee-Dexter and Bob Scheibling at Dalhousie University for sample collections. Funding for this research was provided by grants to JAA from Natural Sciences and Engineering Research council (Discovery Grant), Canadian Foundation for Innovation, New Brunswick Innovation Foundation (Leaders Opportunities Fund; Research Assistant Initiative), and the University of New Brunswick.

Author contributions

JAA, J-HK conceived and designed the study. JAA, J-HK performed the experiments/collected the data. JAA, J-HK analyzed and interpreted the data. JAA contributed resources. JAA, J-HK drafted or revised the manuscript.

Competing interests

The authors have declared that no competing interests exist.

Data accessibility statement

All relevant data are within the paper, the Supplementary Material, Dryad (SNPs; doi:10.5061/dryad.dn160) and GenBank (ncbi.nlm.nih.gov/genbank/; sequence data, accession No. MG098337-MG098440).

Supplementary material

The following Supplementary Material is available with the article through the journal website at doi:10.1139/facets-2017-0081.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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