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Clones or clans: the genetic structure of a deep-sea sponge, *Aphrocallistes vastus*, in unique sponge reefs of British Columbia, Canada

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Abstract

Understanding patterns of reproduction, dispersal and recruitment in deep-sea communities is increasingly important with the need to manage resource extraction and conserve species diversity. Glass sponges are usually found in deep water (>1000 m) worldwide but form kilometre-long reefs on the continental shelf of British Columbia and Alaska that are under threat from trawling and resource exploration. Due to their deep-water habitat, larvae have not yet been found and the level of genetic connectivity between reefs and nonreef communities is unknown. The genetic structure of *Aphrocallistes vastus*, the primary reef-building species in the Strait of Georgia (SoG) British Columbia, was studied using single nucleotide polymorphisms (SNPs). Pairwise comparisons of multilocus genotypes were used to assess whether sexual reproduction is common. Structure was examined 1) between individuals in reefs, 2) between reefs and 3) between sites in and outside the SoG. Sixty-seven SNPs were genotyped in 91 samples from areas in and around the SoG, including four sponge reefs and nearby nonreef sites. The results show that sponge reefs are formed through sexual reproduction. Within a reef and across the SoG basin, the genetic distance between individuals does not vary with geographic distance ($r = -0.005$ to 0.014), but populations within the SoG basin are genetically distinct from populations in Barkley Sound, on the west coast of Vancouver Island. Population structure was seen across all sample sites (global $F_{ST} = 0.248$), especially between SoG and non-SoG locations (average pairwise $F_{ST} = 0.251$). Our results suggest that genetic mixing occurs across sponge reefs via larvae that disperse widely.

Keywords: deep-sea, genomic duplication, glass sponge reefs, next generation sequencing, population structure, single nucleotide polymorphisms

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Introduction

The deep sea, once a remote habitat, is increasingly accessed for exploration and industry. With access has come knowledge about the seascape and connectivity of animal populations. Unlike on land however, isolation is less by geography than by seascape ecology (McClain *et al.* 2012). Food sources and substrate limit species distribution, with vents, seeps and whale falls providing 'stepping stones' for many animals (Glover *et al.* 2005; Breusing *et al.* 2015). Bacterial chemosynthesis at vent sites supports much of that life, and although associations of sponges with thioautotrophic bacteria may be more common than once thought (Nishijima *et al.* 2010), generally benthic suspension feeders such as sponges or corals tend to be associated with regions of downwelling (Pile & Young 2006), resuspension (Bett & Rice 1992) or enhanced flow such as is found over seamounts (Genin *et al.* 1986; Mehl *et al.* 1994).

Although barriers to dispersal do not exist in the deep sea, specific oceanographic characteristics

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generated by currents and topography can separate communities. Studies of deep marine benthic communities in Antarctica, for example, suggest that particular environmental conditions of the deep Weddell Sea foster distinct shelf and slope assemblages (Brandt *et al.* 2007). Deep-water communities can refresh shallower populations and vice versa but generally deep-water species have more in common with those in other oceans than with their shelf or slope counterparts. The same has been found for deep and shallow shelf environments in the Norwegian Sea (Costa & Bisol 1978). What unfolds appears to be a pattern of stable deep-water environments with mixing of the gene pool over the long-term via a stepping stone mechanism, and potentially less stable or perhaps more specialized shelf habitats that may be temporally ecologically isolated.

Deep-sea coral and sponge reefs are special cases. Coral reefs are restricted to particular locations on shelves at 200–300 m, yet colonies can live in isolation in deep (1000 m) and shallow (20 m) habitats. Whereas modern scleractinian corals evolved some 237 Mya, azooxanthellate species such as *Lophelia pertusa* which form deep-water reefs throughout the Atlantic and Pacific oceans only evolved 20 Mya. Like its shallow-water cousins, *Lophelia* spawns eggs and sperm and produces long-lived (up to 59 days) ciliated planula larvae that may be planktotrophic (Larsson *et al.* 2014). Despite the dispersal potential of such a larva through ocean currents, populations of *Lophelia* in the Atlantic are not panmictic but rather are structured at small spatial scales along the continental shelf, and distinct populations occur in fjord and shelf habitats (Le Goff-Vitry *et al.* 2004). Deep-water sponge reefs are a less well-known habitat that share many similarities with deep coral reefs. While sponges have existed since the Neoproterozoic, during the Jurassic (~200 Mya) sponges formed a several thousand km-long barrier reef along the margin of the Tethys Sea (Ghiold 1991). That reef became extinct 40 Mya and it was only in the late 1980s that modern analogs were found in the continental shelf waters of the north-eastern Pacific (Conway *et al.* 1991). Whereas the Jurassic reefs contained a diversity of species, the modern reefs are formed by only three species of glass sponge, which can, like *Lophelia*, live as isolated colonies in deep canyons and fjords. The modern reefs are dated at 6000–9000 years old, and so it is hypothesized that offspring of colonies in deeper water settled on exposed rock rubble left by the retreating glaciers on the Pacific coast of North America (Krautter *et al.* 2001; Conway *et al.* 2005).

Sponge reefs serve a major ecological role in generating habitat complexity, supporting increased diversity of infauna such as polychaetes, crustaceans and fish (Cook 2005; Chu & Leys 2010). As filter feeders, the sponges also contribute massively to benthic-pelagic coupling and can filter the equivalent of the entire water column (170 m) above a square metre of reef daily, removing bacteria with up to 95% efficiency, and recycling nutrients as carbon, ammonia and excreted material (Kahn *et al.* 2015). Trawling has already damaged large portions of the reef complexes in Hecate Strait, and while protection is planned (Jamieson & Chew 2002), knowledge of the effect of suspended sediments disturbed by trawling, as well as an understanding of the genetic connectivity between reefs and between reef and isolated colonies in fjords would be useful for management decisions.

Glass sponges (Class Hexactinellida) are generally a deep-water group (>1000 m), and the reefs are commonly found between 150 and 400 m depths, but individual glass sponges may be found as shallow as 16 m (Leys *et al.* 2004) where deep water is channelled upward in narrow passages. Glass sponges are thought to need temperatures lower than 14 °C (Uiblein *et al.* 1996; Leys & Meech 2006), high silica levels, low light and high amounts of bacteria, which is their primary food (Leys *et al.* 2004; Kahn *et al.* 2015). Because of their deep habitat, it has been difficult to gather information on the reproductive biology of glass sponges. Collections of reef-building species made by remote operated vehicle (ROV) over 10 years have revealed sperm but so far no oocytes and so reproductive periods remain elusive (Leys & Lauzon 1998; Kahn *et al.* 2016). Development has been described in only three species of glass sponge: *Vitrollula fertilis, Farrea sollasii* and *Oopsacas minuta* (Ijima 1904; Okada 1928; Boury-Esnault *et al.* 1999; Leys *et al.* 2006). The larvae from *O. minuta* are small, swim slowly, and although they typically settle within 1–2 days in dishes in the laboratory, settlement may be delayed up to 7 days (Leys *et al.* 2007, 2016). *Oopsacas minuta* provides the only clues to glass sponge larval behaviour so far, and details of the potential or realized dispersal of the larvae are still unknown.

Sponge larvae are nonfeeding passive propagules (Maldonado 2006), whose settlement is determined by light (e.g. Leys & Degnan 2002; Collin *et al.* 2010), gravity (Warburton, 1966), biofilms (Whalan & Webster 2014), water movement (Mariani *et al.* 2006) and changes in larval chemistry with time (Maldonado *et al.* 1997; Maldonado & Young 1999). Studies suggest most sponge larvae remain in the water less than 2 weeks (most settle within minutes to days) and few are encountered in offshore plankton samples (Maldonado 2006). Sponges can also reproduce asexually by budding, breaking or by a colony expanding in size and then shrinking again to leave satellite colonies (e.g. Blanquer *et al.* 2009; reviewed in Wulff 2012). Given the range of reproductive strategies, it is not surprising that

the degree of genetic structure in populations varies widely. Some species have predictably structured populations at small spatial scales due to asexual reproduction (Duran *et al.* 2004; Blanquer *et al.* 2009), and although sexual reproduction via larvae would be expected to result in genetic structure at larger spatial scales, this is not necessarily the case. In the same habitat, one species may be clonal over considerable distances (e.g. *Cliona delitrix* Chaves-Fonnegra *et al.* 2015), while other species have no evidence of asexual reproduction and no structure over large distances (e.g. Bell *et al.* 2014). Interestingly, some species such as the Great Barrier Reef sponge *Rhopaloeides odorabile* (Whalan *et al.* 2008) or the Mediterranean sponge *Chondrosia reniformis* (Lazoski *et al.* 2001) are genetically homogeneous over a scale of tens to thousands of kilometres suggesting that the larvae are capable of living much longer than previously thought. In these cases, it is possible the larvae use dissolved organic carbon for energy as shown by Jaeckle (1995) to reach such great distances.

One of the difficulties in comparing studies, however, is the use of different genetic markers (microsatellites or allozymes) and their ability to detect population structure. The use of single nucleotide polymorphisms (SNPs) is common for population genetics because of their abundance in the genome (1 per 300 bp), yet SNPs have yet to be used to analyse sponge population genetics. The abundance of SNPs in a genome is an advantage in species where single-copy microsatellite loci have been difficult to find, for example in barnacles (Barazandeh & Davis 2012), corals (Marquez *et al.* 2000) and lepidopterans (Zhang 2004), possibly from an association between duplicated portions of the genome and microsatellites (Behura & Severson 2013). Although it is expected that SNPs would also be duplicated in these genomes, their biallelic nature makes it easier to filter out duplicated loci, and their even spread throughout the genome may increase the probability that single-copy loci are obtained.

Sponge reefs offer an ideal opportunity to study connectivity at different spatial scales in a deep-water benthic species. Furthermore, the urgency to develop sound mechanisms for management of areas protected from trawling brings another level of importance to understanding connectivity in and between sponge reefs and nonreef populations at different spatial scales. The sponge larvae settle and grow on the skeletons of other individuals, and with time sediment buries the base to cement the reef structure together (Conway *et al.* 2005). Because the bases of individual sponges are not visible, distinct individuals cannot always be distinguished and so it has been speculated that the patchy distribution found across one reef alone (Chu & Leys 2010) could arise from either asexual reproduction such as budding, or from limited dispersal of larvae as with *Lophelia* (Larsson *et al.* 2014). In the Strait of Georgia, British Columbia, over 12 sponge reefs have been identified (Conway *et al.* 2007) and three have been studied in detail (Chu & Leys 2010; Chu *et al.* 2011; Leys *et al.* 2011; Kahn *et al.* 2015, 2016). Here genetic analysis of the principal reef-building sponge, *Aphrocallistes vastus*, was carried out to examine evidence of asexual reproduction by comparing multilocus genotypes and population structure at three spatial scales – within a reef, between reefs and between sites (both reef and nonreef) within and outside of the Strait of Georgia, a marginal sea near Vancouver British Columbia. The results greatly advance our understanding of the dispersal potential of an important deep-water foundation species, expand our knowledge of glass sponge reproduction and population connectivity and show the power of using next generation sequencing to determine patterns of reproduction in deep-sea animals.

Methods

Study region and description of the species

The Strait of Georgia (SoG) is a semi-enclosed basin 200 km long by 30 km wide that lies between the British Columbia mainland and Vancouver Island (Fig. 1a, b). The central Strait is 420 m deep with an average depth of 155 m. To the south, the SoG connects to the Pacific Ocean through the Strait of Juan de Fuca and to the north, it connects via Johnstone Strait and other very small channels to Queen Charlotte Sound.

Sponge reefs in the SoG are formed by two species of rigid (dictyonine), reef-forming glass sponge, *Aphrocallistes vastus* and *Heterochone calyx* (family Aphrocallistidae) (Fig. 1 c, d). Although reefs are separated by expanses of mud which are not colonized by dictyonine sponge species, the adjacent fjords and shelf regions contain 'gardens' of isolated colonies, and in some areas, they can be abundant without forming reefs (e.g. Leys *et al.* 2004). *Heterochone calyx* has a hydroid symbiont that often gives it a characteristic orange colour (Schuchert & Reiswig 2006); *A. vastus* is symbiont-free. Although they can appear very similar *in situ*, the two species can be differentiated by their spicule skeleton complement. We ensured only *A. vastus* was included in this study by avoiding orange coloured sponges and by confirming species identity by microscopy and genetic data.

Sampling

A total of 104 samples of *A. vastus* were collected between 2007 and 2011 at six sites (four reefs and two nonreef) and at low sample sizes (<5) at four other sites.

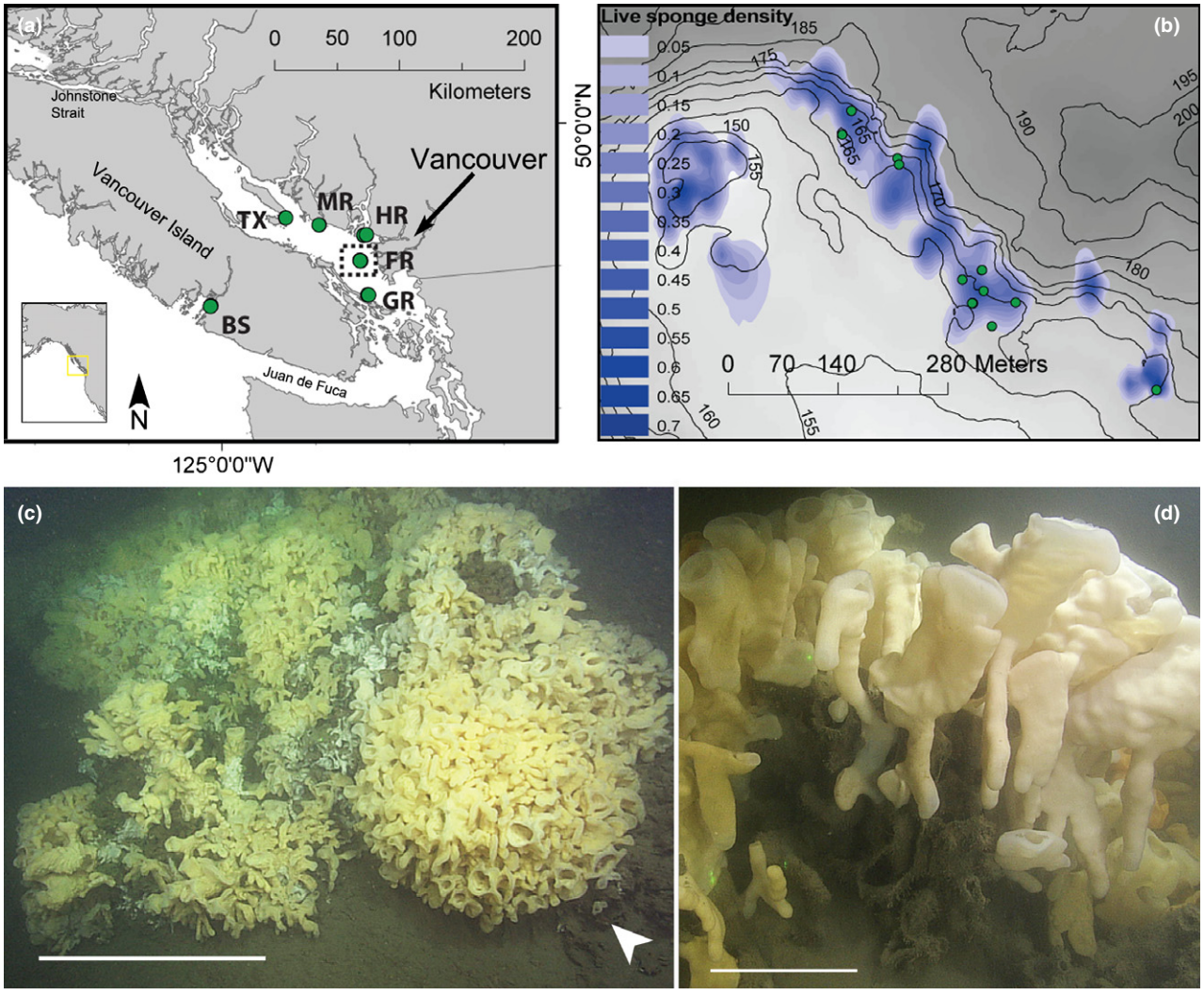

Fig. 1 (a) Map of sample sites: Texada Island (TX), McCall Reef (MR), Howe Reef (HR), Fraser Reef (FR), Galiano Reef (GR), Barkley Sound (BS). (b) Map showing detail of specific sampling locations (green dots, 1–5 N each) at Fraser Reef as an example of reef sampling, with density of live sponge cover in blue (from Chu & Leys 2010). (c) A clump of reef sponges including both *Aphrocallistes vastus* and *Heterochone calyx* is shown surrounded by sediment anchoring skeletons of past generations. A juvenile sponge growing on the skeleton of a dead sponge is indicated by the arrow. Scale bar, 1 m. (d) The base of sponges was typically dead due to accumulation of sediment during reef-building. Scale bar, 20 cm.

All samples were collected using the remote operated vehicle (ROV) ROPOS except for two additional samples (Saanich Inlet) which were collected at 35 m depths by SCUBA (Table 1).

Pieces of sponge approximately 5 cm^2 were collected using the ROV manipulator arms or a suction hose on the end of a 'wand'. At reefs, pieces were taken from each of five sponges in a clump approximately 1–5 m in diameter. This process was repeated for distinct clumps that were spaced 5 m to 2.2 km apart across each reef (Fig. 1b). Self–self controls were taken by sampling the same individual twice. Samples were placed into separate, closed collection boxes on the ROV. Precise spatial positioning recorded by the ROV provided GPS coordinates for all samples. High definition video and still images accompanied all sample collections. On the ship, using a new razor blade for each collection, a 2-cm^2 piece was cut from each sample and stored in 95% ethanol at −20 °C for transport to the University of Alberta. To determine whether the sponges we sampled were reproductive (and therefore could result in genotypes from multiple individuals), a second piece from each collection was preserved in Bouins' fixative for 1–2 days, rinsed twice in water, dehydrated through a graded ethanol series and embedded in paraffin wax. Sections 30 μm thick were stained with haematoxylin and eosin and viewed with a Zeiss Axioskop 2 plus microscope.

Table 1 Number of samples sequenced (N_{SEQ}), analysed for tests of clonality (N_{ID}), analysed for population-level tests (N_{POP}) and analysed for distance-based tests in the Strait of Georgia (N_{SOG}) sample depth range, number of observed alleles (Na) mean observed and expected heterozygosities (Ho, He) and probability of identity (PID) by sample. Mantel tests using the N_{SOG} data set were run with (numbers indicated in brackets) and without experimental duplicates

Site location	Site ID	Depth (m)	N_{SEQ}	N_{ID}	N_{POP}	N_{SOG}	Na	Ho	He	PID
Howe Reef	HR	65–115	23	22	19	19 [20]	129	0.342	0.323	1.03×10^{-19}
Galiano Reef	GR	70–98	17	15	13	13 [14]	127	0.420	0.320	1.82×10^{-19}
Fraser Reef	FR	164–172	20	17	17	17	127	0.369	0.319	1.82×10^{-19}
McCall Reef	MR	172–176	16	12	11	11	129	0.350	0.312	4.19×10^{-19}
Barkley Sound	BS	134–165	12	12	11		116	0.267	0.240	6.35×10^{-15}
Texada Island	TX	115–270	5	5	5	5	125	0.304	0.307	1.20×10^{-18}
		Subtotal	93	83	76					
Other sites			11	8						
Saanich Inlet			2	1						
Learmonth Bank			4	3						
Olympic Coast National Marine Sanctuary			4	3						
Coral Knoll			1	1		1				
		Total	104	91		66 [68]				

Of the 104 samples collected, 10 were used for SNP marker development (Brown *et al.* 2014) and 94 samples were genotyped here. Thirteen samples had poor data quality and were removed from further analysis (Table 1). The remaining 91 samples were used to examine evidence of clonality. Prior to population-level analyses, an additional 15 samples were removed: eight from sites with small sample sizes (two of which were from control pairs) and seven duplicate samples, one from each of an additional five control pairs and two experimental pairs with highly similar multilocus genotypes. Therefore, Hardy–Weinberg and linkage disequilibrium tests, probability of identity, F_{ST} and STRUCTURE analyses were performed on 76 samples (Table 1).

Amplification and sequencing

Single nucleotide polymorphism markers were discovered using a reduced genomic complexity next-generation sequencing approach described in Brown *et al.* (2014). Sequence-specific forward and reverse primers were designed in PRIMER3PLUS (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) with '*Glenn*' (forward) and *trPI* (reverse; Ion Torrent, Life Technologies Inc.) primer tails (see Table S1, Supporting information for tail sequences) to serve as primer binding locations in the second PCR. Eighty-one amplicons containing 93 putative SNPs were amplified in 94 *A. vastus* samples and a single *H. calyx* individual with a negative (no DNA template) control using four multiplexed reactions for each sample (Qiagen multiplex PCR kit). Multiplexed primer sets were created by sorting 81 primer pairs into four sets (3 × 20 and 1 × 21; Table S1, Supporting information) based on the length of the designed amplicon, clustering similar lengths while avoiding duplicate lengths to limit amplification bias by PCR and facilitate visualization of PCR products for optimization. Multiplexed PCRs in a final volume of 10 μL included 1× Type-It PCR master mix (Qiagen), 2 μM of each primer and 20 ng of template DNA. Primer pools 1 and 2 were amplified using an annealing temperature of 51 °C (15 min at 95 °C, 30x [30 s at 94 °C, 2 min at 51 °C, 60 s at 72 °C], and 30 min final extension at 60 °C) (Fig. S1, Supporting information). Primer pools 3 and 4 were run using touchdown PCR conditions which used the same settings except for the annealing stage: 16 cycles of decreasing annealing temperature (56–48 °C in 0.5 °C steps) followed by 14 cycles at 48 °C. The four multiplexed reactions were pooled for each individual and diluted 1:500. Ion Xpress™ Barcode Adaptors (Life Technologies Inc.) modified to contain 3′ '*Glenn*' (forward primer) tails were used along with *trP1* (reverse primer) in a second PCR to amplify 3 μL of the pooled, diluted PCR product in 10 μL reaction volumes (0.3 U Phusion High Fidelity DNA polymerase (New England Biolabs), 160 μM dNTPs, 3% DMSO, 1× Phusion HF buffer, 1.5 mM $MgCl_2$, 1.6 μM each of *trP1* and barcode primers; 5 min at 94 °C, 35x (30 s at 94 °C, 30 s at 55 °C, 60 s at 72 °C), and 10 min final extension at 72 °C). The amplification workflow is summarized in Fig. S1 (Supporting information). Small fragments (e.g. primer dimers) were excluded by sequential PCR purification (Qiagen Qiaquick PCR purification column) and gel purification (Qiagen Qiaquick gel extraction with 1% agarose gel) prior to sequencing.

Samples were sequenced on an Ion Torrent PGM using a 316 chip and a 200-bp read chemistry according to the manufacturer's protocol. Controls consisted of three samples used in the SNP discovery process (across-method controls; same DNA extractions carried through reduced complexity genomic sequencing [marker discovery phase] and the targeted amplicon sequencing [genotyping phase] to assess error in genotype calls between methodologies), one tissue sample extracted twice (in-run control; separate DNA extractions of the same tissue sample treated as distinct samples to determine error within targeted amplicon sequencing beginning with extraction), three self–self controls (oscula sampled twice in the field to discover potential error or contamination at sampling level), one different species (*H. calyx*) to look for identifiable differences between species and to verify that no samples were mis-identified and one negative control (no DNA template; to check for contamination in amplification and sequencing methodology). Genotyping error (targeted amplicon pipeline) was assessed using combined control data from three across-method pairs, one in-run pair and three self–self pairs.

Genotyping and analysis

Assembly and SNP genotyping were completed in SeqMan NGen, SeqMan Pro and Arraystar of Lasergene Suite 12 (DNASTAR, Inc). The minimum read depth required to minimize false homozygote calls (probability set at 0.01) was calculated for each locus separately based on observed heterozygosity and average read depth as described by Chenuil (2012). Maximum read depth was set to 1000. Genotypes not meeting the minimum read depth requirement were discarded. Individuals and loci with >10% missing data (genotypes either missing from sequence data, discarded within Lasergene due to low quality, or discarded due to insufficient read depth) were removed from analysis. Loci that were not polymorphic or bordered homopolymer sequences were also discarded (Table S1, Supporting information).

To examine evidence of clonality, multilocus genotypes of 91 individuals were compared in GENALEX 6.5 (Peakall & Smouse 2006, 2012) using the 'multilocus matches' function, which outputs a list of pairwise comparisons and the number of differing locus genotypes. Missing data were ignored. Genotypes at each locus were scored as either a 0 (perfect match) or 1 (different), totalled for each pairwise comparison, and graphed as a percent of total loci. A generalized extreme studentized deviate (ESD) test for outliers was performed on the experimental sample set (without controls) using the REAL STATISTICS RESOURCE PACK Software [Release 3.8: Copyright (2013–2015) Charles Zaiontz www.real-statistics.com].

Marker characteristics including number of alleles, allele frequencies, observed and expected heterozygosities, and probability of identity were calculated using GENALEX; these estimates were made for 76 samples from six sampling locations with five or more samples (removing one from each pair of controls and experimental duplicates). Deviations from Hardy–Weinberg equilibrium and pairwise linkage disequilibrium were tested using GENEPOP 4.2 (Raymond & Rousset 1995; Rousset 2008). Genetic distance between sample locations was assessed using *F* statistics, and within and between sample locations in the SoG using spatial autocorrelation analysis and Mantel tests in GENALEX. To perform these tests, a pairwise linear codominant genetic distance was calculated in GENALEX by weighting genotypic differences from 0 to 4 (0 being identical genotypes, 1 being a homozygote and heterozygote combination, and 4 being opposing homozygous genotypes) and pairwise linear geographic distance calculated in kilometre from decimal latitude and longitude, recorded by the ROV. A spatial autocorrelation analysis was performed on all samples with GPS coordinates in the SoG basin excluding one sample from each pair of highly similar multilocus genotypes ($N = 66$) using a single linear genetic matrix for all loci within a single population and even sample classes. Ninety-five percent confidence intervals were determined around the calculated *r*-values using 999 bootstrap replicates and around the null *r*-value of 0 using 999 permutations of the data. Despite recent concerns regarding the validity of Mantel tests on spatial correlations (Legendre *et al.* 2015), to provide an additional point of comparison Mantels were run on the same data set ($N = 66$) as well as including both samples in each pair of MLGs ($N = 68$) and provided as Supporting information. A Partial Mantel test ($N = 66$) based on Pearson's product-moment correlation was performed to determine the linear relationship between genetic distance and sample depth (m) while controlling for geographic distance (km). This was performed in R (R Core Team 2014) using the VEGAN package v2.3-3, mantel.partial function (Oksanen *et al.* 2016); significance was assigned using 999 free permutations. Samples were assigned to genetically homogenous populations (K) inferred using a Bayesian clustering algorithm without prior geographic information using STRUCTURE v2.3.4 (Pritchard *et al.* 2000). An admixture model was used with correlated allele frequencies and 1 000 000 MCMC iterations (burn-in of 50,000), repeated 20 times for each value of K from 1 to 8. The most likely value of K was determined using Evanno's *ad hoc* ΔK statistic (Evanno *et al.* 2005) calculated and plotted using STRUCTURE HARVESTER

web v0.6.94 (Earl & vonHoldt 2012). The 20 replicates of optimal K were aligned using the *FullSearch* algorithm in the software package CLUMPP v1.1.2 (Jakobsson & Rosenberg 2007), then visualized using DISTRUCT v1.1 (Rosenberg 2004). STRUCTURE analysis was run twice, again excluding one sample from each pair of highly similar multilocus genotypes ($N = 76$) and including both samples in each pair of MLGs ($N = 78$). Samples that assigned with >80% ancestry to the largest cluster (i.e. SoG) were isolated and run again through the same analysis to test for additional levels of substructure.

To determine the potential effect of low sample numbers, allelic richness at each site was investigated with an allele accumulation curve performed in R (VEGAN package, specaccum function). Each sample location was calculated independently using random draw and 1000 permutations to provide the allele count by number of samples randomly selected at each site. An additional STRUCTURE analysis (same parameters as above) was run on five randomly selected samples from each site (total $N = 30$).

Results

Species identity, reproductive status and evidence of clonality

All samples included in the study were identified as *Aphrocallistes vastus* using spicule complement. The single *Heterochone calyx* sample that was included as a control was genotyped at all 67 loci yet monomorphic at all loci and therefore distinct from all other samples. Microscopy confirmed that no individuals were reproductive at the time of sampling nor did any samples have unusually high heterozygosity (Fig. S2, Supporting information).

Genotypic variation, measured by the percent difference between multilocus genotypes in pairwise comparisons between *A. vastus* samples ($N = 91$, 4095 pairwise comparisons, Table 1), showed no identical multilocus genotypes outside of the control groups (Fig. 2). The DNA in-run control pair differed at 0% of loci, while the three across-method control pairs (using the marker discovery sequences) displayed 2.6%, 6.5% and 9.1% genotypic difference. The three sponge self–self control pairs (individuals that were sampled twice) differed at 1.3% (two control pairs) or 9.1% of loci (one control pair). Two pairs of samples that had few genotypic differences were found in two reefs. At Howe Reef, two sponges with 5.2% genotypic difference were less than 0.5 m apart and had dead bases that were connected by a mostly buried ridge of dead skeleton (Figs 2 and 3). At Galiano Reef, three closely packed oscula had similar multilocus genotypes: two differed from each other at 10.4% of loci, and the third differed from both of the others at 16.9% of loci (Figs 2 and 3). The two pairwise comparisons with 5.2% and 10.4% difference were the only statistically significant outliers within the experimental sample set ($P < 0.05$; ESD test for up to seven outliers). The 5.2% data point lies clearly within the

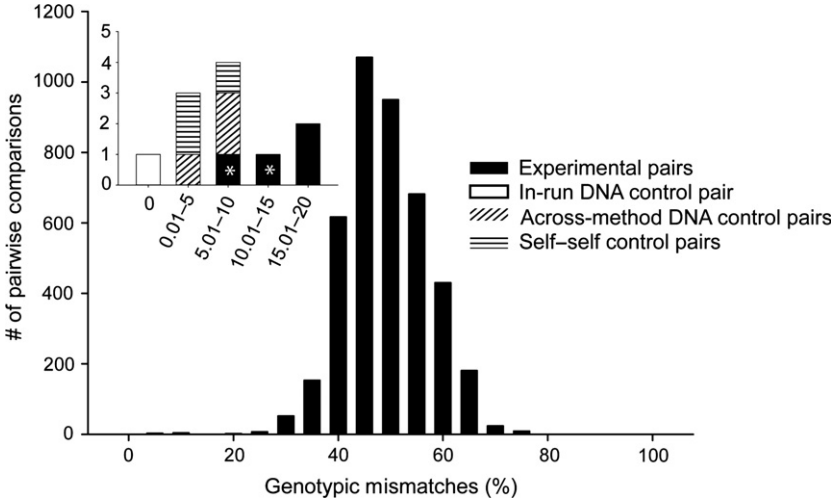

Fig. 2 Genetic identity and test for clonality in individuals of *Aphrocallistes vastus* as shown by the percent of genotypic differences between pairwise comparisons of individuals ($N = 91$). Genotypes between individuals are compared at each locus (of 76), where mismatches are totalled for the pair and presented as a percent. Starred experimental pairs indicate statistically significant outliers ($P < 0.05$; ESD test). Control pairs were created by extracting DNA twice from the same sample and sequencing in the same run (In-run), sequencing the same samples on different sequencing runs (Across-method) and sampling the same *A. vastus* individual twice (Self–self), and are presented as a comparison to the sample population.

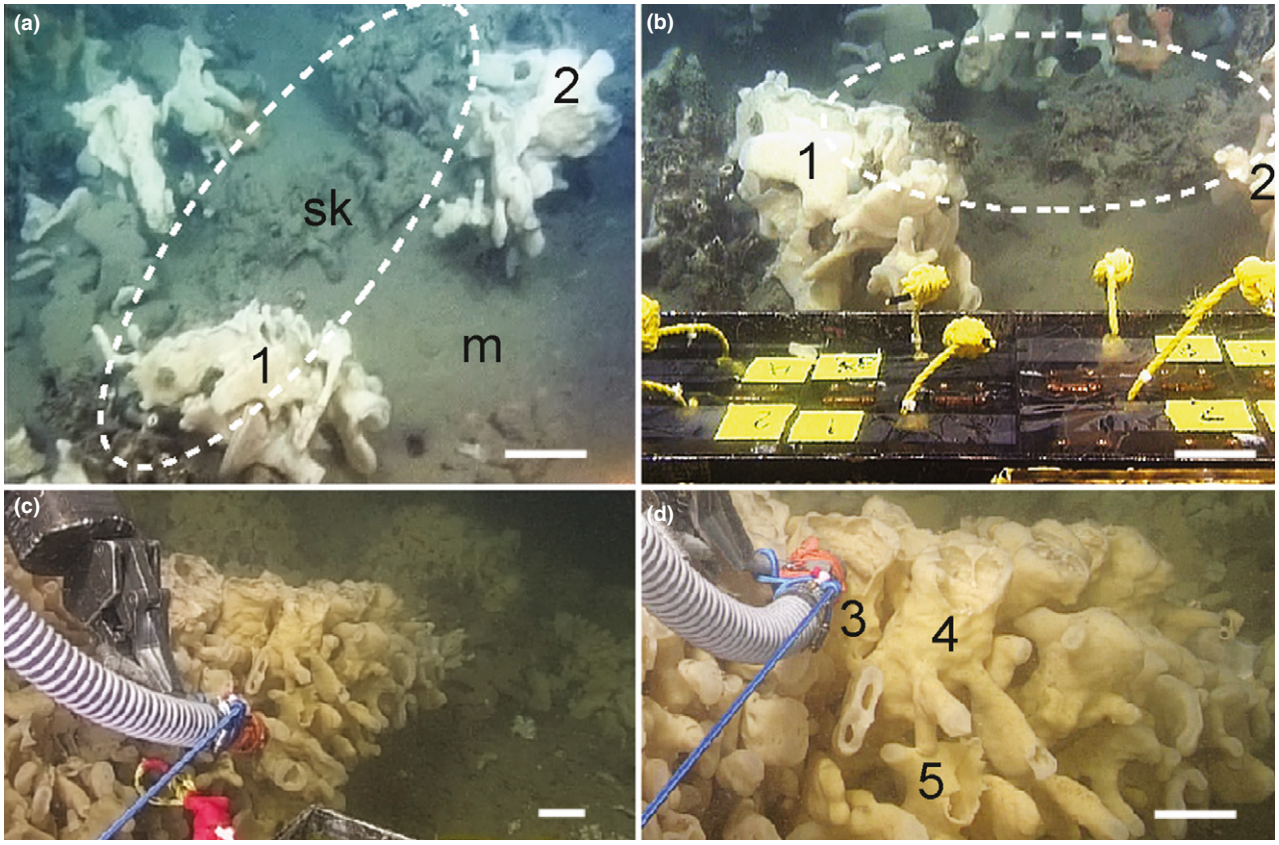

Fig. 3 Genetic similarity between closely positioned sponges. (a, b) 'Twins' – two samples (1 and 2) that differed at 5.2% of loci (within the range of paired controls) were separated by sediment (m) and partially buried skeleton (sk, dashed circle). The extent of the two live portions is shown in (a) with a closer view in (b) showing dead/live tissue connections and the sampling boxes in which each sample was stored separately after being collected by the manipulator arm of the ROV. (c, d) 'Triplets' – three samples (3, 4 and 5) that were closely grouped and had genotypic differences of 10.4–16.9% (outside the paired control range but with a high degree of genetic similarity). A large portion of the clump is shown in (c), with a close-up of the sampled trio in (d). Scale bars, 10 cm.

0–9.1% difference range seen in the control pairs and the 10.4% data point falls outside of the controls by a single allele, therefore neither were considered sufficiently different from the controls to be recognized as distinct individuals. Genotypic differences among samples averaged 46.5% (range 5.2–74.0%). Overall, adjacent samples had distinct multilocus genotypes with genetic distance equivalent to random sample pairs.

Genotypic variation

The number of observed alleles ranged from 116 to 129 per site (Table 1). Observed and expected heterozygosity ranged from 0.267 to 0.420 and from 0.240 to 0.323, respectively. Probability of identity ranged from 4.19×10^{-19} to 6.35×10^{-15} (Table 1). Although all loci used in analyses were in Hardy–Weinberg equilibrium and linkage equilibrium when samples were grouped by site after carrying out Bonferroni correction (Table 2), disequilibrium was observed at global and regional scales, suggesting that random mating occurs within sample sites but that there may be population structure at larger scales.

Genetic distance between and within sites

Global F_{ST} across all sites was 0.248 ($N = 76$, $P = 0.001$), indicating a high degree of population differentiation. Pairwise F_{ST} values between sample locations ranged from 0 to 0.056 inside the SoG and from 0.191 to 0.274 between Barkley Sound and SoG sites (Table 3). A model for two genetically homogenous clusters (K) had the highest likelihood determined by Bayesian clustering analysis ($N = 76$, STRUCTURE; Fig. 4a; Fig. S3a, Supporting information). Barkley Sound samples were distinguished from SoG samples by cluster assignment with samples from Texada displaying intermediate ancestry. No further population structure was found

Table 2 Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) values for three hierarchical sampling levels (Global: all samples; Regional: inside or outside the Strait of Georgia; Site: sample site. Exp: expected number of significant tests based on significance level; Obs: number of observed loci in disequilibrium at that significance level). Bonferroni corrected alpha value (original alpha = 0.05) shown in square brackets. No Result is due to monomorphism in HWE tests and one column or row in contingency table for LD tests

	Global HWE		Regional HWE		Site HWE	
Significance	Exp	Obs	Exp	Obs	Exp	Obs
0.05	3.35	16	6.7	10	20.1	11
0.01	0.67	9	1.34	3	4.02	2
Bonferroni	[0.00075]	4	[0.00037]	1	[0.00012]	0
No result		1		28		74
	Global LD		Regional LD		Site LD	
Significance	Exp	Obs	Exp	Obs	Exp	Obs
0.05	110.55	324	221.1	124	663.3	208
0.01	22.11	179	44.22	28	132.66	33
Bonferroni	[2.26E−5]	40	[1.13E−5]	1	[3.77E−6]	0
No result		2		1182		3266

Table 3 Pairwise F_{ST} values between six sample sites [Texada (TX), Fraser Reef (FR), Galiano Ridge (GR), Howe Reef (HR), McCall Reef (MR), Barkley Sound (BS)]. *P*-values are italicized above the diagonal (bolded values are significant after Bonferroni correction)

	TX	FR	GR	HR	MR	BS	*N*
TX	—	*0.035*	*0.080*	*0.188*	*0.253*	***0.002***	5
FR	0.056	—	*0.388*	*0.114*	*0.054*	***0.001***	17
GR	0.041	0.000	—	*0.193*	*0.068*	***0.001***	13
HR	0.016	0.013	0.010	—	*0.206*	***0.001***	19
MR	0.010	0.030	0.028	0.007	—	***0.001***	11
BS	0.191	0.274	0.269	0.252	0.269	—	11

after running the analysis on samples assigned with >80% ancestry to the largest cluster, which dominated the SoG ($N = 64$; Fig. S3b, Supporting information). The same analysis run with five randomly selected samples from each site showed an identical result (Fig. S4, Supporting information). Likewise, STRUCTURE runs either including both samples of each of the two similar genotype pairs or excluding one sample of each pair did not change clustering (not shown). The spatial autocorrelation correlogram ($N = 66$; Fig. 4b) showed low but significant *r*-values for the first (0–1 km, $r = 0.013$, $P = 0.002$) and third (21–37 km, $r = 0.014$, $P = 0.001$) of five distance classes. Very little correlation between geographic and genetic distance was also found by the Mantel test between populations within the SoG ($R^2 = 0.0156$, $P = 0.030$) or at the reef level within each reef (Fig. S5, Supporting information). A slightly greater effect of sample depth on genetic distance was detected while accounting for geographic distance ($N = 66$, Partial Mantel test, $r = 0.1467$, $P = 0.005$).

Discussion

Glass sponge reefs are massive bioconstructions covering tens of kilometres of seafloor that were only discovered on the Pacific coast of North America in the late 1980s. Although individual colonies in gardens can be reached by SCUBA, the shallowest reefs are 95 m deep and so collection of discrete samples by ROV is necessary. Discrete sampling at small spatial scales in the deep sea is therefore costly and limited by ship availability, but fortunately the proximity of the sponge reefs in the Strait of Georgia to major ports such as Vancouver, British Columbia, has allowed repeat collections during other work and has provided a unique opportunity to study dispersal at multiple scales in a deep-sea species.

Our results show high regional genetic structure in the reef-building glass sponge *Aphrocallistes vastus*, distinguishing populations in the SoG from those on the west coast of Vancouver Island; however, reef and nonreef sites within the SoG show little genetic differentiation, suggesting that larvae (and/or broadcast sperm) disperse extensively within this region. The genetic distinctness of adjacent sponges in a reef and the general absence of identical multilocus genotypes both imply that even small 1–5 m diameter clumps of sponges in a reef are the result of sexual reproduction.

Genetic distinction of individuals

Individuals in reefs were genetically distinct at all spatial scales (within clumps and between clumps), so clumps are formed of many different individuals rather than clones. The clustered pattern of sponges within a reef may therefore be a response to the high flow required to sustain considerable grazing rates (Kahn *et al.* 2015) or substrate availability. Two shared MLGs were observed between adjacent samples which appear in images taken by the ROV to be more likely two oscula of an individual rather than examples of discrete asexual reproduction (Fig. 3). In the first case, two samples appeared to be connected by dead skeleton, so the best explanation is that these two sponges represent reduced living portions of a large, older individual sponge (Fig. 3a, b). In *Crambe crambe,* an intertidal encrusting demosponge from the Mediterranean, clones from 24 lineages made up 76 of 177 sponges examined, with an average distance between clone mates of 20 cm (Calderon *et al.* 2007). This pattern was conjectured to have arisen by growth and recession of the sponge, and we suspect the same to be true of the two identical but spatially separated samples in our study. The three adjacent oscula with a genetic difference of 10.4–16.9% at Galiano Reef are another interesting result of our sampling design. Whereas samples taken within clumps were normally collected from what were considered to be distinct individuals that were clustered within 5 m of each other, this trio of samples was collected from three adjacent oscula that appeared to be the same individual, although the base could not be seen (Fig. 3c, d). Because the pairwise comparison of two oscula with 10.4% difference represents a statistical outlier and is virtually indistinguishable from an error rate of nearly 10%, those oscula are likely two branches of the same individual as would be expected. The third osculum differs from the previous two by 16.9%, outside of the error range, and was most likely a closely related individual. Although the general trend points to wide dispersal of larvae, this single example illustrates that larvae may also recruit locally. Further sampling within tightly clustered oscula presumed to be from the same individual could determine whether this is a rare occurrence or a common dispersal pattern.

The absence of clonality in the population is surprising because examples of asexual reproduction are common in benthic invertebrates, and range in degree from species like *Lophelia pertusa* that are predominantly sexually reproducing but use asexual reproduction when larval recruitment is low (Le Goff-Vitry *et al.* 2004), to species like *Pocillopora damicornis, Tubastrea diaphana* and *T. coccinea* which produce larvae asexually (Ayre & Resing 1986; Yeoh & Dai 2010). So far asexual production of sponge larvae is unknown, and yet some sponge populations (e.g. *Cliona delitrix*) show a significant number of identical genotypes suggesting they are potential clones (e.g. Chaves-Fonnegra *et al.* 2015), while others like *Xestospongia muta* show no identical genotypes (Bell *et al.* 2014). Austin (2003) proposed that *A. vastus* can reproduce asexually by portions of the branches 'dripping' off onto the substrate, but imagery from many years of ROV dives fail to confirm this happens even infrequently, and unique MLGs observed between adjacent individuals indicate that reefs must arise by sexual reproduction.

Individual heterozygosity was normally distributed across the sample set with no outliers (Fig. S2, Supporting information), which, combined with the fact that individuals sampled twice had the same MLG, suggests that separate individuals do not fuse to form chimeras as seen in some other sponge species (Blanquer & Uriz 2011). Genetic distinctness between individuals of *A. vastus* is further supported by an inability of tissue pieces from different individuals to fuse, whereas the syncytial tissue from self–self controls are able to fuse (Fig. S6, Supporting information). Fusion in glass sponges, which are entirely syncytial, means joining cytoplasm and nuclei under a single cell membrane.

Between-reef and within-reef homogeneity and genetic distance between regions

Within the SoG, no significant population structure was found based on F_{ST} calculations, although STRUCTURE assignment, spatial autocorrelation and Mantel correlations indicate subtle differentiation. Overall, individuals in clumps are not more genetically similar to each other than to sponges in other clumps of the same reef, or to sponges elsewhere in the SoG. This suggests that enough larval dispersal is occurring to maintain population connectivity over the widest and smallest spatial scales examined.

Sites on the west coast of Vancouver Island (Barkley Sound) are genetically distinct from the sites within the SoG. The distinction between the SoG and Barkley Sound populations could be caused by geography alone, because the distance from Barkley Sound to the closest sample site (Galiano Ridge) around the southern tip of Vancouver Island is 275 km. Samples within the SoG appear to belong to the same population, which may be self-recruiting or sourced from deeper waters in the Pacific, presumably arriving to the SoG via its southern entrance at the Strait of Juan de Fuca. Samples collected from Texada Island in Sabine Channel, which lies close to the centre of the SoG, showed mixed ancestry according to STRUCTURE analysis. This relationship, although weak, suggests that individuals in

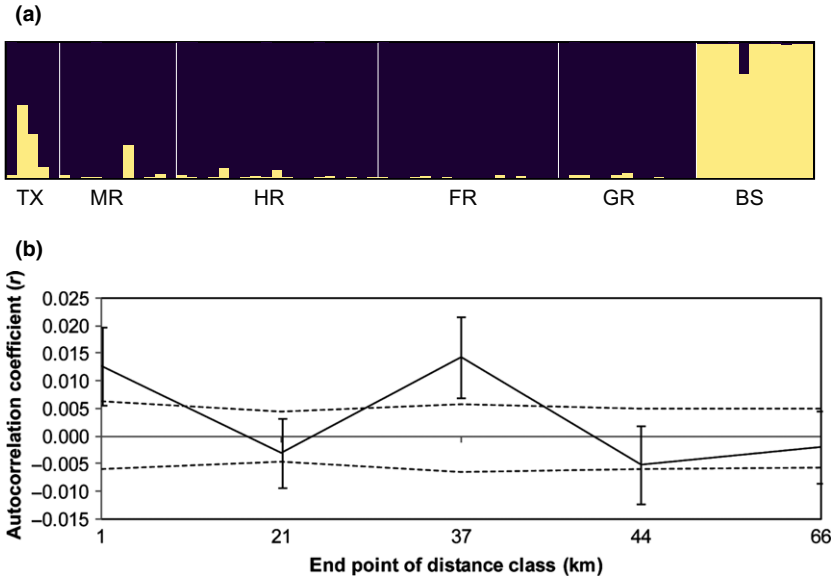

Fig. 4 Genetic structure of *Aphrocallistes vastus* in the Strait of Georgia. (a) STRUCTURE Bayesian analysis for two clusters (K) determined using the ΔK method (Evanno *et al.* 2005; Fig. S3, Supporting information). Each column represents a sample assigned proportionally to each genetic cluster. Sites include Texada Island (TX), McCall Reef (MR), Howe Reef (HR), Fraser Reef (FR), Galiano Reef (GR) and Barkley Sound (BS). (b) Multivariate spatial autocorrelation of samples collected within the Strait of Georgia generated within GENALEX using a single genetic matrix for all loci ($N = 66$). Distance classes were selected within the software as the optimal sample number distribution (even sample classes option). Error bars and dashed lines represent 95% CI around calculated and null *r*-values, respectively.

the northern SoG may be more genetically similar to west coast populations, and that the area around Texada Island represents a zone of admixture. Two tests show that this result is not affected by sample size: allele frequency curves show saturation at ≥5 samples and a STRUCTURE analysis with only five samples randomly chosen from each site gave the same result as that using the full set of samples (Fig. S4, Supporting information). The widely distributed deep-water coral *Lophelia pertusa* shows a similar pattern in structure between open North Sea and fjord populations, attributed to differences in geological and hydrological characteristics, or divergence in populations since the formation of the fjords (Le Goff-Vitry *et al.* 2004).

Evaluating the SNP sequencing approach

It was originally envisioned that microsatellite markers would be used to conduct this study, however during microsatellite development and testing portions of the genome were found to be duplicated (Jensen 2011). The ineffectiveness of COI as a population marker in sponges, consistent with low mitochondrial mutation rates in basally branching metazoans (Watkins & Beckenbach 1999; Shearer *et al.* 2002), was also confirmed in *A. vastus* and *H. calyx* through a paucity of intraspecific variation. SNPs were therefore chosen in combination with next-generation sequencing because they offered the advantage of having a large number of markers from which to choose and the ability to quickly identify duplicated markers. However, there were high rates of nonconcordance (≤9.1%) between multilocus genotypes in pairs of control samples. Differences in accuracy between methodologies are highlighted in three across-method control pairs, which consisted of DNA extractions that were sequenced twice using two methods (reduced representation library and amplicon sequencing), and differed at 2.6–9.1% of loci. In contrast, no difference was found between the in-run control pair consisting of DNA extracted twice from the same sample and sequenced with the same methodology (amplicon sequencing). Amplicon sequencing is more likely to provide accurate results than the reduced genome sequencing used for marker discovery through more selective amplification offering greater read depths. But there were also high rates of nonconcordance (1.3–9.1%) in the three self–self control pairs, sponge oscula sampled twice but sequenced in the same amplicon-sequencing run. These error rates, comparable to the across-method controls, suggest that high error rates may also arise from error inherent in PCR and sequencing, as well as genotyping calls. However, this may also be caused by genomic duplication which, despite stringent marker filters, could cause an increase in false genotyping calls due to skewed read percentages and sampling error. To compensate for the high error rates, the range allowed for genotypic difference of identical MLGs was relaxed, but this did not affect the inference of population structure (data not shown).

The *H. calyx* sample further supports the accuracy of the results and highlights an interesting relationship between species. In *H. calyx*, the 100% call rate paired with 100% homozygosity for *A. vastus* markers (all genotypes matched one of the two alleles identified in *A. vastus*) would be expected in comparing two closely related species with a divergence time of ~2–3 Mya (Miller *et al.* 2012). The phylogeny of the family Aphrocallistidae is uncertain; recent molecular analyses have

placed *H. calyx* and *A. vastus* as sister species with a recent divergence (Dohrmann *et al.* 2008, 2011) consistent with the above date.

Implications for larval dispersal in the Strait of Georgia

The results described here suggest larvae from glass sponge reefs are widely distributed in the SoG presumably by currents, supported by the stronger role of depth over geographic distance in describing genetic distance. In the SoG, there is a strong estuarine flow out to the Pacific Ocean fed primarily by the Fraser River, with a return flow at 50–200 m depth (Pawlowicz *et al.* 2007). Strong semidiurnal tidal currents reach 1.3 m/s through the Straits (Davenne & Masson 2001), although these apparently diffuse in the central SoG basin to an average of 0.5 m/s. Two eddies in the central SoG promote east–west transport across the Strait (Stacey *et al.* 1987), which may cause a degree of isolation at the northern Texada site. Currents accelerate over the ridges formed by the reefs by up to 0.5 m/s higher than surrounding flow (Bedard 2011), although flow at the level of the sponge oscula is much slower, from 0 to 0.15 m/s (Leys *et al.* 2011). High currents over the reefs may facilitate larval dispersal, while the extensive boundary layer may make settlement easier at the reefs. In a hypothetical situation of maintained currents that directly connected the reefs sampled, particle transport from one location to another could be accomplished in 11.7–38 h, the approximate larval duration time recorded in other sponges (Maldonado 2006; Leys *et al.* 2007, 2016). Given the dominance of tidal currents, which add to the complexity of flow along with local features such as eddies, it is expected that larval transport between reefs would take longer. A high-resolution circulation model for the SoG (Soontiens *et al.* 2016) will afford a more realistic idea of transport time and potentially elucidate source/sink relationships between sites but initial tests of dispersal from Fraser Reef using that model suggest that larval dispersal may be restricted to the SoG even within 30 days of release from the parent (Fig. S7, Supporting information). This preliminary result from the flow models supports our genetic analysis that flow might assist mixing of genotypes within the SoG but restrict mixing beyond. If larvae remain elusive, this approach may provide further hints of the larval behaviour and duration in this deep-sea species.

Implications for conservation

As slow-growing, sessile animals, glass sponges are vulnerable to damage by fishing activity (Freese *et al.* 1999; Heifetz *et al.* 2009), and slow to recover from large scale damage (Kahn *et al.* 2016). Marine protected areas have been proposed by the Department of Fisheries and Oceans Canada (DFO 2015) to limit damage to the sponge reefs by fishing and other anthropogenic disruptions, and management decisions, such as the selection of which geographic regions to protect, would benefit from knowledge of genetic structure of the reefs. The genetic similarity of *A. vastus* in adjacent reefs suggests the ability to disperse over spatial scales that connect reefs separated by tens of kilometres and the existence of a potentially self-sustaining population within the SoG. The fact that our results show two distinct populations of glass sponges – inside and outside the SoG – implies that it is important to protect sponge communities both outside and within the SoG basin to maintain genetic diversity. It should be noted, however, that our results do not resolve directionality of gene flow and so an exciting avenue for future work would be to determine sources and sinks of the reef populations.

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R.R.B., S.P.L. and C.D. designed the experiments. R.R.B. and S.P.L. collected the specimens. R.R.B. and C.D. carried out the data analysis. R.R.B. and S.P.L wrote the manuscript. All authors edited and revised the text.

Data accessibility

All data for this research are available through Dryad: https://doi.org/10.5061/dryad.sc0nf

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Characteristics of 93 SNPs.

Fig. S1 Four multiplex PCRs, each with twenty primer pairs, were performed with DNA template from 96 samples.

Fig. S2 Individual heterozygosity (n = 84).

Fig. S3 STRUCTURE likelihood plots for most likely number of genetically homogenous clusters (K): mean of estimated Ln likelihood with standard deviation bars for K = 1–8, and corresponding ΔK (Evanno *et al.* 2005) calculated with STRUCTURE HARVESTER.

Fig. S4 STRUCTURE Bayesian analysis for two clusters (K) determined using the ΔK method (Evanno *et al.* 2005), using only 5 randomly selected samples from each site (n = 30).

Fig. S5 Mantel tests comparing individuals of *A. vastus*.

Fig. S6 Self/non-self recognition experiments on *Aphrocallistes vastus* specimens from Fraser Reef and Howe Reef carried out in 2009.

Fig. S7 Future directions: larval tracking in an oceanographic model.