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Reproduction in Urbanised Coastal Waters: Shallow-Water Sea Anemones (*Entacmaea quadricolor* and *Stichodactyla haddoni*) Maintain High Genetic Diversity and Panmixia

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Abstract: Sea anemones are sedentary marine animals that tend to disperse via planktonic larvae and are predicted to have high population connectivity in undisturbed habitats. We test whether two sea anemone species living in two different tidal zones of a highly disturbed marine environment can maintain high genetic connectivity. More than 1000 loci with single-nucleotide polymorphisms (SNPs) were obtained with double-digest RADseq for 81 *Stichodactyla haddoni* and 99 *Entacmaea quadricolor* individuals to test for population genetic structure. We find evidence that both species predominantly propagate via sexual reproduction, and asexual reproduction is limited. We observe panmixia that indicates the absence of effective dispersal barriers for these species living in a highly anthropogenically disturbed environment. This is positive news for both species that are also found in the aquarium trade. More fundamentally, our results suggest that inhabiting different parts of a shallow reef may not affect a species' population connectivity nor favour asexual reproduction.

Keywords: fine-scale connectivity; ddRADseq; sea anemones; clonality

1. Introduction

The construction of artificial coastal structures and increased shipping traffic can potentially interfere with the population connectivity of marine species and are thought to have negative effects on sedentary species with limited dispersal abilities [1,2]. Fortunately, many sedentary marine species have planktonic larvae that aid in dispersal [3–6] given that they can be transported over large distances via oceanic currents before settlement [6]. Broadcast spawning in anthozoans is considered the predominant method of reproduction, used in both hermaphroditic and gonochoric species [5]. The range of pelagic larval dispersal could be dependent on factors like the influence of currents (passive distribution for longer distances). However, short-distance dispersal of pelagic larvae has been speculated to be dependent on behaviour i.e., larvae remaining close to the benthos [7]. A good example of sedentary marine organisms that use broadcast spawning for maintaining panmixia are sea anemones (Cnidaria: Anthozoa: Actiniaria) [5], but how effective is this reproductive mode when living in heavily

impacted marine environments? Reduced genetic diversity [8,9] and population connectivity [10] have been detected in populations settling on artificial structures. These are thought to be due to ecological and functional differences between natural and artificial structures and/or 'phenotype-environment' unsuitability. Pollution in heavily impacted environments is furthermore known to cause mutations [11] with sublethal effects [12], which suggests that marine species in heavily disturbed environments could face a bleak future especially if the population connectivity is low. It would interfere with re-population and lower the capacity to recover from disturbances. A decrease in the ability of populations to adapt to rapidly changing micro-conditions might occur [13–15], given that higher genetic diversity increases resilience of populations and the ecosystem to disturbances [16–18]. Population genetic data also allow for reconstructing demographic responses such as effective population size, genetic diversity and migration rates to contemporary stressors. The data could therefore also inform conservation prioritisation and management [19]. This is particularly important for economically important and endangered species [20–22].

Sedentary animals such as sea anemones have reproductive strategies that are selected to increase their chances of survival [5,23–25]. On the one hand, most species reproduce sexually, which increases the genetic diversity and allows for adaptation in dynamic and heterogeneous environments [26]. On the other hand, asexual modes allow for quickly reproducing successful genotypes that are well-adapted to prevailing stable and homogeneous environments [27,28]. In sea anemones, sexual reproduction primarily involves broadcast spawning while asexual reproduction may occur by pedal laceration, longitudinal fission or transverse fission [29,30]. In species with a predominantly sexual mode of reproduction, highly connected populations across different spatial scales and higher genetic diversity have been observed [31–33]. Conversely, species with asexual reproduction typically show reduced dispersal and stronger genetic isolation [34–37], because individuals are likely to attach to the first hard surface that they encounter [38].

Recent observational studies have demonstrated that most sea anemone species reproduce sexually [5,23,39–42]. For the sea anemone *Stichodactyla haddoni* (Figure 1a), there is no evidence of sexual reproduction—asexuality appears to be the main mode of reproduction [42]. On the other hand, sea anemones like *Entacmaea quadricolor* (Figure 1b) [5,23] and *S. gigantea* [33] occasionally perform asexual reproduction via longitudinal fission [23,30,43]. Unfortunately, our understanding of the reproductive biology of sea anemones is poor because obtaining high-quality observational data for long-lived and slow-growing anemones is time-consuming [39,44]. In the marine environment, difficulties to directly access, track and monitor marine species in situ further challenge the studies on these animals. Genetic tools on the other hand, can provide broad insights based on studying the outcomes of reproduction. These tools also allow for readily distinguishing between individuals resulting from sexual or asexual reproduction.

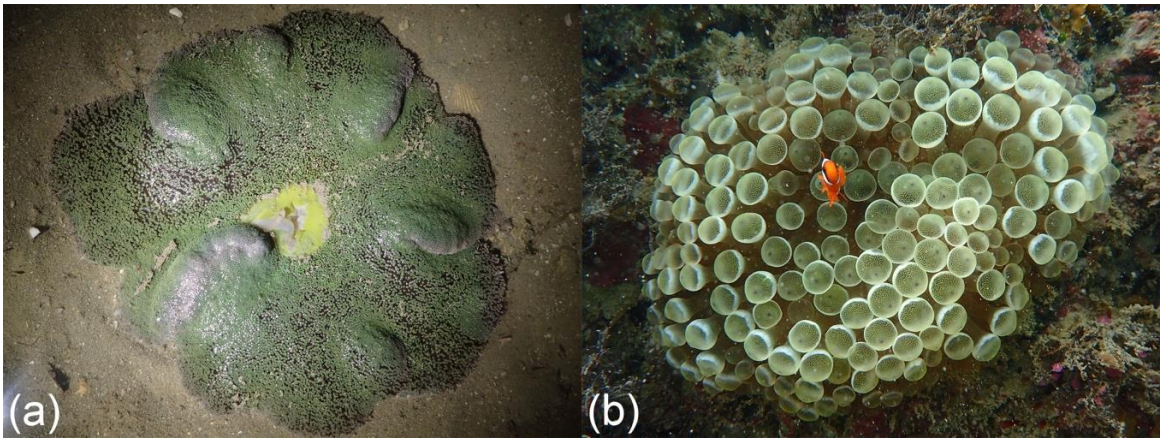

Figure 1. (**a**) *Stichodactyla haddoni* and (**b**) *Entacmaea quadricolor*.

Very little is known about the population genetics of sea anemones. Most published studies used enzyme electrophoresis (e.g., *Actinia bermudensis* [32]; *A. tenebrosa* [26,28,34]; *Bunodosoma caissarum* [32]; *Epiactis* spp. [45]; *Metridium senile* [27] or microsatellite markers (e.g., *A. tenebrosa* [37]; *S. gigantea* and *Heteractis magnifica* [33]), which provided limited information on population connectivity. Fortunately, the use of single nucleotide polymorphisms (SNPs) for sea anemones is in recent years becoming more popular [46,47]. Genetic markers with high levels of resolution are required especially to discern between reproductive methods. For anthozoans, this requires broad sampling of the genomes because they are known to have few DNA markers with good resolving power [48,49]. Genome-wide SNPs mined using restriction site-associated DNA sequencing methods are thus ideal for such taxa. They can be rapidly obtained even for non-model organisms as prior genomic information of the subjects is not required [50].

The present study assesses the population genetic structure of two sea anemone species living in urbanised environments using these SNP markers. *Stichodactyla haddoni* and *E. quadricolor* are shallow-water species thought to reproduce predominantly via broadcast spawning [5,41,51] and those inhabiting highly urbanised coasts such as Singapore's are likely under chronic stress because of the extensive coastal modifications [52,53]. It would be important to know whether they can maintain population connectivity and high genetic diversity under adverse conditions. The island nation has (1) increased in land area by 30% since the 1960s through land reclamation [54–57], leading to dramatic losses of natural habitats (e.g., 60% of coral reefs; [58]); (2) hardened its coasts with seawalls that constitute 63% of the coastline [53], which could lead to fragmentation and reduced connectivity [59]; (3) extremely high turbidity due to the reclamation and dredging [58,60]; and (4) extremely high shipping traffic (i.e., 2.85 billion gross tonnage of vessel arrivals in 2019 [61]) that could also disrupt larval dispersal and reduce connectivity.

High levels of connectivity are common in species with a planktonic stage and deeply linked to its pelagic larval duration ranging from less than one day to 90 days—e.g., ascidians *Ciona robusta*, *C. savignyi*, *Styela clava* [62]; the gastropod *Haliotis rubra* [63]; reef corals *Platygyra sinensis* [64] and *Acropora digitifera* [65]; the clownfish *Amphiprion polymnus* [66]; the sea star *Protoreaster nodosus* [67]; the bicolour damselfish *Stegastes partitus* [68]; and mussel *Mytilus edulis* [69]. However, unique population migration patterns and genetic divergence due to various biological and physical characteristics have been observed even at fine scales, e.g., in the coastal cod *Gadus morhua* [70], Asterinidae sea stars [71], American lobster (*Homarus americanus*) [72] and bivalve (*Brachidontes* sp.) [73]—where geographic distances can be as low as 40 km. Specific habitat choices (e.g., rocky surfaces, sandy platforms, seagrass meadows) play an essential role in determining the successful spread and establishment of populations in new environments [6,74] and can influence connectivity patterns. For instance, higher rates of genetic divergence have been observed in the intertidal compared to the more stable subtidal zones [75]: intertidal species not only have smaller windows of opportunity for dispersal, but are also under stronger, divergent selection pressures (e.g., high wave energy and greater sun exposure) [76]. In this study, we test the effects of different divergent selective pressures on two species that occupy different tidal zones [75]. *Stichodactyla haddoni* and *Entacmaea quadricolor* are commonly found in the coastal environments of Singapore [77]. While *S. haddoni* is found primarily in the intertidal zone in soft sand, *E. quadricolor* mostly grows on shallow subtidal coral reefs [77–79]. Larvae of *E. quadricolor* have been observed to survive up to 59 days after spawning in in situ experiments, with peak settlement of larvae at 10 days [42], granting them time to be thoroughly mixed within Singapore waters before settlement. Further research is required to determine precisely its pelagic larval duration that can affect its connectivity patterns [5]. In addition, Australian *E. quadricolor* has been observed to spawn annually at the start of the year [51]. However, the spawning periods and pelagic larval duration of *S. haddoni* remain unknown.

Stichodactyla haddoni and *E. quadricolor* are two of ten sea anemones known to associate with anemonefishes [42,80]. These are thus highly sought after in the aquarium trade, with high potential for overexploitation [81]. We sought to discover the population connectivity of two shallow-water

sea anemone species that live in different parts of the reef in urbanised waters and inferred the modes of reproduction of both species using genetic tools. Here, we provide the first information on genetic diversity and reproductive mode via genomic markers, which will potentially be important for managing the trade [42,82] and population assignment of confiscated animals.

2. Materials and Methods

2.1. Sample Collection

Stichodactyla haddoni was sampled from the intertidal zones at eight sites ($n = 86$) while *E. quadricolor* was sampled via SCUBA from subtidal zones at 12 sites ($n = 106$) in Singapore (Figures 1 and 2). Abbreviations for the sampling sites are defined in Figure 2, and full details of the collection sites and each sample can be found in the Supplementary Materials (Table S1). Each site was searched for three to five hours to ensure adequate sampling. Individual anemones were imaged in situ for morphological confirmation of species identity, as voucher specimens were not permitted under our collection permit (NP/RP15-088). Tissue subsamples were collected using forceps and scissors, sampling from the body column as much as possible instead of the tentacles to avoid high nematocyst and *Symbiodinium* densities. These were preserved in 100% molecular-grade ethanol and kept at −20 °C until further processing. At each site, a minimum of five individuals were collected and processed. Despite the increased sampling efforts, only three *E. quadricolor* individuals were observed at Raffles Lighthouse (RAH). However, RAH was retained as a key site that represents the southernmost offshore island of Singapore.

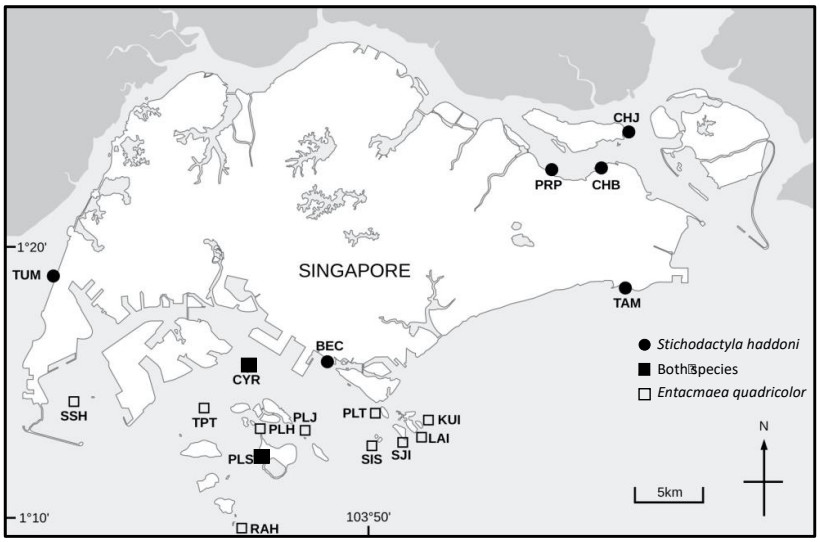

Figure 2. Sampling locations of *Stichodactyla haddoni* and *Entacmaea quadricolor*. BEC—Berlayer Creek, CHB—Changi Beach, CHJ—Chek Jawa, CYR—Cyrene Reefs, LAI—Lazarus Island, KUI—Kusu Island, PLH—Pulau Hantu, PLJ—Pulau Jong, PLS—Pulau Semakau, PLT—Pulau Tekukor, PRP—Pasir Ris Park, RAL—Raffles Lighthouse, SIS—Sisters Island, SJI—St Johns Island, SSH—Sultan Shoal, TAM—Tanah Merah, TPT—Terembu Pempang Tengah, TUM—Tuas Merawang Beacon.

All laboratory processing and data analyses were performed in a similar manner for both species, unless otherwise stated.

2.2. DNA Extraction

For each sample, genomic DNA was extracted from a 1 × 1 mm piece of the tissue sample using a modified hexadecyltrimethylammonium bromide (CTAB) DNA extraction protocol [83]. Each DNA pellet was eluted in 25–45 µL of molecular grade water.

2.3. DNA Barcoding for Species Confirmation

DNA barcoding was performed to confirm species identifications and ensure that population genetic analyses would not be contaminated by misidentified species. COIII DNA barcoding was first performed for all samples to ensure that the input samples for population genomic analyses were of the same species. This marker successfully identified *E. quadricolor* individuals but showed limited barcoding gap at species level for the genus *Stichodactyla*, and was hence unable to delimit *Stichodactyla* species. The internal transcribed spacer 1 (ITS1) marker was thus further barcoded for *S. haddoni*, and 28S rRNA was also sequenced to verify the morphological identification of *E. quadricolor*.

The internal transcribed spacer 1 (ITS1) marker was amplified for *S. haddoni* using the 18SUniv.fw primer [84] with a reverse primer designed for this study (STI_ITS1rev: 5′-GCG TTC AAA GAT TCG ATG ATT CAC T-3′). To allow for multiplex sequencing on the Illumina platform, a 9-bp tag was added to the 5′-end of these primers following Meier et al. [85]. The amplification cycling profile was 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 2 min. Each 25 µL reaction contained 1 × reaction buffer, 0.2 mM of each dNTP, 0.4 µM of each primer and 1 U BioReady rTaq polymerase and 1–2 µL extracted DNA. Successfully amplified products were pooled and purified using SureClean™ (Bioline, London, UK) following the manufacturer's instructions, and sequenced as part of an Illumina MiSeq 2 × 251-bp run. The reads were demultiplexed, and resulting barcodes filtered for quality using the barcoding pipeline and scripts in Meier et al. [85], as described in Wang et al. [86]. MAFFT ver. 7 was used to align ITS1 sequences. Objective clustering [85] was used to obtain a cluster dendrogram of barcodes, grouping sequences based on 3–5% pairwise-distances for all successfully obtained barcodes [87].

For *E. quadricolor*, the primers LSUrDNAF and LSUrDNAR [88] were used to amplify the 28S rRNA. Cycling conditions included a step-up reaction of four cycles of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 2 min, followed by 25 cycles of 94 °C for 30 s, 57 °C for 1 min and 72 °C for 2 min. Successfully amplified PCR products were purified using Sera-Mag™ Magnetic SpeedBeads (GE Healthcare, Chicago, IL, USA) in 18% PEG buffer (1M NaCl, 10mM Tris-HCl, 1 mM EDTA, pH 8) (hereon referred to as SeraMag-PEG). A SeraMag-PEG:DNA ratio of 0.96 was used to retain ≥300-bp fragments. The PCR products were prepared for Sanger sequencing using BigDye™ v. 3.1 (Applied Biosystems, Foster City, CA, USA), purified using PureSEQ beads following manufacturer's instructions, and sequenced on a 3730XL DNA analyser (Applied Biosystems). Sequences were assembled, edited and translated to check for stop codons, using Geneious v. 11 [89]. BLAST searches against the GenBank database were used to check for species identification.

2.4. ddRADseq Library Preparation and Sequencing

After omitting misidentified specimens and sites with small sample sizes, 81 *S. haddoni* and 99 *E. quadricolor* samples were retained for SNP analyses. Up to four DNA extraction replicates were included for each species to assess the repeatability of our methods, aid in the identification of biological clones and optimise bioinformatic pipeline parameters [90,91]. These were from BEC (STI063R), CHB (STI115R), CYR (STI129R), LAI (ANE083R), KUI (ANE039R), PLH (ANE055R), SJI (ANE077, a resample of ANE075). DNA quality and approximate quantity assessments were made using 1% agarose gels and the Qubit® dsDNA BR Assay kit with a Qubit Fluorometer (Invitrogen™, Carlsbad, CA, USA).

SNPs were randomly subsampled from across each genome using the ddRADseq method following the protocol in Tay et al. [67], with some modifications. Briefly, approximately 100 ng of DNA from each sample was simultaneously digested with restriction enzymes and ligated to Illumina-compatible adapters [92] at 37 °C for four hours, followed by an additional step of 65 °C for 20 min to inactivate EcoRI-HF and DNA ligase. This reduces the formation of chimeric DNA fragments that would become false loci. Each reaction contained 11.5 U EcoRI-HF, 2.3 U MspI, 10 × T4 DNA ligase buffer, 50 mM NaCl, 0.385 µM P1E adapter, 0.385 µM P2M adapter, 185 U T4 DNA ligase and 0.05 mg/mL bovine serum albumin. The products were pooled, as they could be identified by the different indexes and barcodes (Table S1), and purified using a 1.2 SeraMag-PEG:DNA ratio. A narrow selection for 400-bp

fragments was performed on the adapter-ligated products using the PippinPrep (2% dye-free cassette, EF Marker E v2), and purified again using SeraMag-PEG. Each pool of 46–48 samples was assigned a specific Illumina index (details in Table S1), and triplicate PCRs were performed per pool to amplify successfully ligated DNA fragments. Each 25 µL reaction contained 1 × Q5 reaction buffer, 800 µM dNTPs, 0.5 µM of each PCR primer and 0.2 U Q5® HF DNA polymerase (New England Biolabs, Ipswich, MA, USA). The PCR cycling protocol was 98 °C for 1 min, 12 cycles of 98 °C for 10 s, 68 °C for 30 s and 72 °C for 30 s, ending with 72 °C for 10 min. Products from each set of triplicates were pooled, purified using the SeraMag-PEG and quantified using the Qubit® dsDNA HS Assay kit before combining into a final pool. All 187 libraries were sequenced across three lanes of Illumina Hi-Seq 4000 platform (2 × 151 bp), with a 40% spike of whole-genome sequencing DNA libraries to increase the base diversity (Table S1).

2.5. Population Genomic Analyses

2.5.1. Single-Nucleotide Polymorphism Data

Read quality was assessed using FastQC v. 0.11.2 [93], and processed using STACKS v. 1.24 [94]. Read 1 of each sequencing run was demultiplexed using *process_radtags*, and trimmed to 134 bp according to read quality for each run. Reads for each individual were filtered for potentially chimeric sequences that may have been generated during the adapter-ligation step, by removing the reads that contained the restriction enzyme recognition sites (using a custom bash script from [67]). Loci were assembled and SNPs were called using the de novo pipeline in STACKS separately for each anemone species. To assess the sensitivity of the resulting SNP sets to SNP calling parameter settings, nine combinations of different settings were tested (Table S2). Parameter settings incorporated the recommendations by Paris et al. [95]. For each individual, putative loci were assembled from the reads that were demultiplexed and filtered in *ustacks*. A reference catalogue of consensus loci was created from across 18 *S. haddoni* and 22 *E. quadricolor* individuals, selected at random, as there were not closely related whole genomes available for mapping. Potential contaminants were identified and removed from this library by matching against genomes of likely contaminants from five datasets (bacteria, human, pomacentrid fishes, *Symbiodinium* sp., viruses) via BLAST (accession numbers can be found in Table S3). A low E-value of 10^{-1} was used to conservatively remove even the poor matches that may represent contaminants. To define the locus set per sample, the putative loci assembled in *ustacks* were then matched to the reference catalogue using *sstacks*, while SNPs were called using the module *populations*. The final SNP sets produced by STACKS were put through further filtering to ensure data robustness. Minor alleles that may have technical errors, were removed using PLINK/SEQ (0.09) (minor allele count, MAC = 3–206 for *Entacmaea quadricolor*, and 3–174 for *Stichodactyla haddoni*) (accessed from https://atgu.mgh.harvard.edu/plinkseq/download.shtml). Clones were identified through pairwise-similarity analyses [96] and removed to avoid overrepresentation by particular genotypes [46]. Missing data were filtered using vcftools (0.1.15, v4.1) [97], excluding individuals with more than 10% missing data. We also generated a dataset that excluded loci failing the Hardy–Weinberg equilibrium test ($p < 0.05$) with vcftools. Finally, potential outliers were identified using BayeScan v 2.1 [98] (False detection rate, FDR = 0.05) and removed. All file format conversions were performed using PGDSpider 2.1.0.2 [99].

2.5.2. Population Genomic Structure and Diversity Analyses

Genetic diversity indices such as expected and observed heterozygosities (H_S and H_O), inbreeding coefficient (G_{IS}) and pairwise F_{ST} values were estimated using GENODIVE 2.0b27 [100].

Signatures of genetic divergence among the sampled locations were first assessed using a principal components analysis (PCA), population model-free approach, to analyse relatedness via identity-by-descent measures as implemented in SNPRelate [101]. This was performed across all combinations of SNP assembly parameters, to assess the robustness of the biological results across

the different SNP calling parameter combinations. Subsequent analyses were performed on just one batch per species (sti_b1.10miss and ane_b3.10miss; Table S4) which maximised the number of SNPs retained, while ensuring that replicates were more than 98.79% and 94.21% identical for *S. haddoni* and *E. quadricolor* respectively (Tables S5 and S6). Other assessments of genetic structure performed include (1) STRUCTURE [102] and StrAuto [103]. Global ancestry can be estimated and underlying genetic structure among the individuals based on allelic frequencies can also be detected through analysing ten runs each per model of K genetic clusters from one to ten [104]. Individuals are assigned to genetic clusters probabilistically based on the assumptions of Hardy–Weinberg equilibrium between alleles and linkage equilibrium between loci [102,104]. To determine the most likely model of genetic clusters, the Evanno method [105] was implemented in the web version of STRUCTURE HARVESTER v0.6.94 [106]. CLUMPP 1.1.2 [90] was used to match the ten sets of genetic clustering results, and the final subpopulation assignment probabilities were visualised using DISTRUCT 1.1 [107]. (2) An analysis of molecular variance (AMOVA) based on an infinite allele model (F_s-analogue) with 10,000 permutations, with the sampling locations defined as groups, and (3) pairwise F_{ST} values was calculated using AMOVA F_S with 999 permutations in GENODIVE. While large samples sizes (more than six) were previously thought to be necessary to accurately infer F_{ST} values, small sample sizes can still be accurate if a large number of bi-allelic genetic markers is used (recommended number of SNP loci >1000; lowest SNP count here = 1196) [108].

3. Results

3.1. DNA Barcoding for Species Confirmation

Forty-five ITS1 barcodes were successfully obtained for *S. haddoni*, which included samples from all sampling sites (Table S7), after removing contaminating signals and samples with low coverage. One sample (STI020) matched one of two extraction negatives and one PCR negative passed the threshold. These were discarded before the clustering analysis was conducted. Objective clustering was performed and used for species identification as no ITS1 sequences were available on BLAST (Figure S1). Reads that were confirmed to be *S. haddoni* were used for analysis.

28S DNA barcodes for 100 *E. quadricolor* specimens (excluding all replicates other than ANE077) matching by at least 99% to *E. quadricolor* on GenBank were considered to be positively identified. The remaining six (ANE017, ANE025, ANE044, ANE049, ANE050, ANE052) had a 99% match to *Heteractis magnifica*, and in situ images further confirmed them to likely be misidentified (Figure 1, Figure S2). These were excluded from subsequent ddRADseq library preparations (Table S1).

All DNA barcodes obtained have been deposited in GenBank (Accession numbers 28S: MT05328-075433, ITS1: MT101752-101798).

3.2. Population Genomic Analyses

3.2.1. Quality Filtering of Data and SNP Calling

A total of 763,762,623 reads were obtained across all three sequencing runs, with most samples represented by at least 10^6 reads (Table S1). Coverage of putative loci for *S. haddoni* ranged from 14 to 134 reads (mostly >50) under the parameter settings m = 3, M = 2, N = 4 (batch 1, Table S8). For *E. quadricolor*, coverage ranged from 32 to 224 reads (mostly >50) under the parameter settings of m = 7, M = 2, N = 4 (batch 3, Table S8).

More than 90% of the SNPs called were retained for both species, after filtering for potential contaminants. These data have been deposited in the GenBank SRA (Accession numbers: SAMN15566063, SAMN15566064, SAMN15566065). Outlier analyses identified two loci potentially under directional selection for both species (i.e., outlier loci) (Figure S3).

The pairwise allelic similarity analysis to identify and remove potential clone-mates found high repeatability across all the sample replicate pairs (≥98.79% match for *S. haddoni* and ≥94.21% match

for *E. quadricolor*, Table S6). No clones were identified among the *Stichodactyla* samples. Three pairs of potential clones were identified for *E. quadricolor*, each from the same corresponding sites, Kusu Island (KUI), Terembu Pempang Tengah (TPT) and Sisters Island (SIS) (Table S6). Clonality in these pairs was further confirmed using in situ pictures of the individual anemones to ensure that they were not due to repeated sampling of the same individuals. ANE022 (TPT) and ANE024 (TPT) were confirmed to be different individuals and hence likely biological clone mates, while the two other pairs (ANE036 (KUI)-ANE095 (KUI) and ANE008 (SIS)-ANE079 (SIS)) were unconfirmed as there were no clear images. An additional pair, ANE065 (SIS) and ANE088 (Pulau Tekukor, PLT), was only 92.09% similar, lower than the percentage similarity of replicates (94.21%), but it remained possible that they might be biological clone mates from different sites as there was a large gap in percentage similarity between the ANE065-ANE088 pair and all other anemone pairwise comparisons (Figure S4 and Table S6). Replicate samples and potential clone-mates were removed for downstream analyses.

After filtering for minor alleles, missing data and clones/replicates, 1853 to 2289 SNPs were retained for *S. haddoni*, while 2758 to 4073 SNPs were retained for *E. quadricolor* (Table S4). The final set of samples analysed included 69 to 78 *S. haddoni*, and 89 to 93 *E. quadricolor* individuals which passed all filtering criteria. As all batches of SNP datasets with different parameter settings produced similar biological interpretations, we present results based on the smallest SNP datasets (sti_b1.10miss and ane_b3.10miss) (Table S4). Outlier frequencies per sampling site were also plotted (Figure S5).

3.2.2. Population Genomic Structure

Genetic panmixia was apparent for both species across all genetic clustering analyses as no clustering of individuals was observed in both PCA and STRUCTURE analyses for both species (K = 1; Figures 3 and 4, Figure S6). Dataset that excluded loci failing the Hardy–Weinberg equilibrium test also yielded the same result; hence, results presented were of datasets that included such loci.

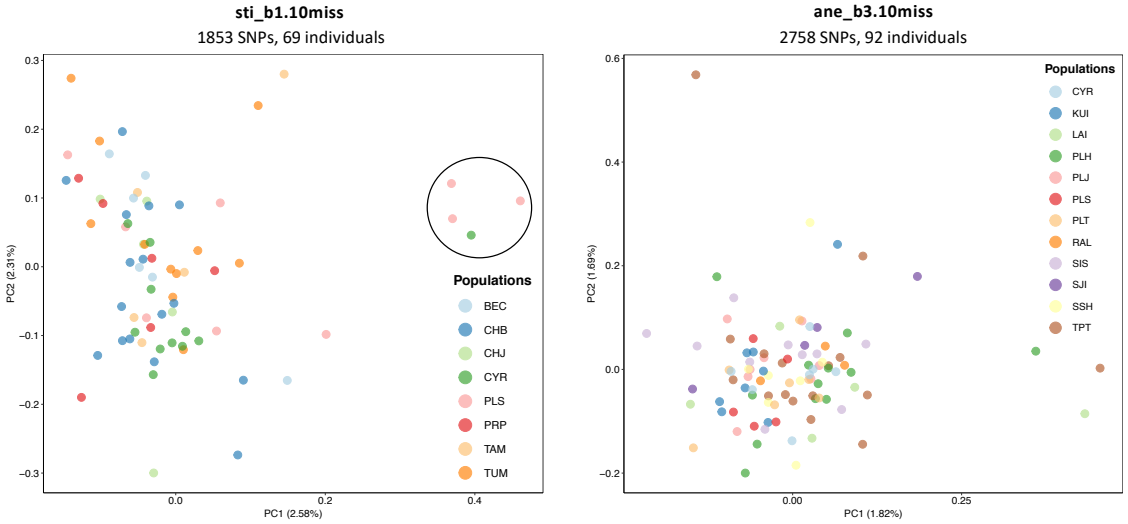

Figure 3. Principal coordinates analysis (PCA) of (**a**) *Stichodactyla haddoni* (sti_b1.10miss) and (**b**) *Entacmaea quadricolor* (ane_b3.10miss). Sampling sites are indicated with different colours. Four diverging individuals are circled.

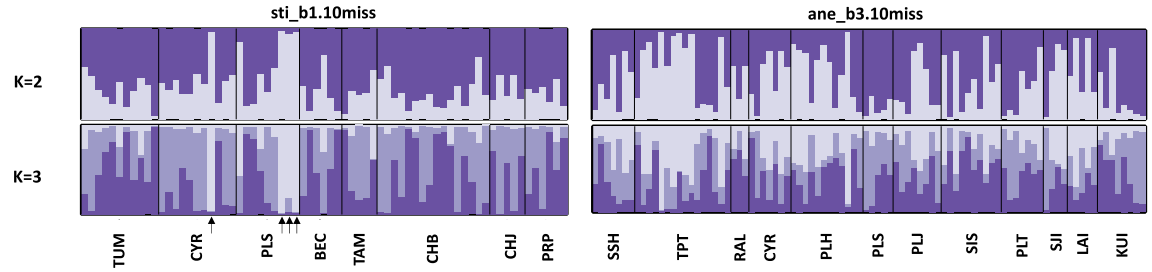

Figure 4. STRUCTURE plots of *S. haddoni* (sti_b1.10miss) and *E. quadricolor* (ane_b3.10miss) suggest a single genetic cluster across all the sampling localities in Singapore. Sites are arranged in a west-to east-ward order starting from Tuas Merawang (TUM) for *S. haddoni*, and Sultan Shoal (SSH) for *E. quadricolor*. The four diverging individuals are indicated with arrows.

Although the amount of variation captured in the first two PCs in both species was low (≤2.58%, Figure 3), the PCAs performed across all the datasets showed similar panmictic patterns. A tighter clustering of individuals was always observed for *E. quadricolor* as compared to *S. haddoni*. Interestingly, three individuals from Pulau Semakau (PLS) and one individual from Cyrene reefs (CYR) appeared to be diverging from the main genetic cluster of *S. haddoni* (Figures 3 and 4), and they were from the only offshore sites sampled for this species.

The pattern of genetic panmixia was also reflected in the partitioning of genetic variation, as most of the genetic variation (>0.818) was found within individuals, compared to the variation between populations which was the lowest (<0.003) for both species (Table S9). Similarly, low pairwise F_{ST} values were observed for both species, with only one pair of sites for *S. haddoni* being significant after Bonferroni correction (Table 1). This appears to be driven by four individuals, which appear to have slightly more distant genotypes as reflected in the PCA and STRUCTURE plots (Figures 3 and 4).

Table 1. Pairwise-comparison of sites and corresponding F_{ST} values (above the diagonal) and p-values (below the diagonal) of (**a**) *S. haddoni* (sti_b1.10miss) and (**b**) *E. quadricolor* (ane_b3.10miss). Significant p-values after Bonferroni correction are indicated with an asterisk (*).

(a)	CHJ	PRP	TUM	PLS	TAM	BEC	CHB	CYR
CHJ	–	0.006	−0.001	0.004	0.006	0.002	0	0.001
PRP	0.11	–	0.006	0.013	−0.003	−0.003	0.003	0.007
TUM	0.56	0.043	–	0.009	−0.007	−0.004	0.005	0.003
PLS	0.243	0.02	0.023	–	0.002	0.005	0.012	0.006
TAM	0.163	0.678	0.955	0.394	–	−0.016	−0.002	−0.003
BEC	0.362	0.805	0.872	0.172	1	–	−0.002	0.002
CHB	0.534	0.243	0.03	0.003*	0.731	0.698	–	0.002
CYR	0.377	0.046	0.153	0.058	0.77	0.291	0.137	–

(b)	PLH	SIS	PLJ	TPT	KUI	RAL	SJI	PLS	SSH	LAI	PLT	CYR
PLH	–	0	0.004	−0.002	0	−0.001	−0.004	−0.003	0.002	0.004	0.002	0
SIS	0.529	–	0.003	0.002	−0.002	−0.004	0.006	0.003	0.007	0.003	0.003	0.001
PLJ	0.059	0.216	–	0.003	−0.001	−0.002	0.001	0.002	0.005	0.006	0.006	0.001
TPT	0.84	0.204	0.16	–	−0.002	−0.005	−0.007	−0.003	−0.002	−0.006	0	−0.005
KUI	0.443	0.711	0.546	0.699	–	−0.008	−0.002	−0.019	0.002	0.001	−0.007	−0.005
RAL	0.59	0.675	0.697	0.79	0.887	–	−0.008	−0.012	0.002	−0.009	0.002	−0.002
SJI	0.796	0.14	0.472	0.92	0.567	0.92	–	−0.006	−0.001	−0.002	0.003	0
PLS	0.765	0.26	0.325	0.76	1	0.922	0.86	–	−0.005	−0.002	−0.005	−0.005
SSH	0.304	0.044	0.082	0.745	0.316	0.388	0.524	0.873	–	−0.002	0.004	0.001
LAI	0.149	0.268	0.073	0.898	0.409	0.889	0.711	0.681	0.65	–	0.002	−0.006
PLT	0.28	0.239	0.047	0.455	0.984	0.412	0.256	0.903	0.144	0.277	–	−0.003
CYR	0.534	0.401	0.323	0.935	0.923	0.627	0.492	0.838	0.469	0.95	0.763	–

3.2.3. Diversity and Reproduction

Our analyses suggested higher genetic diversity among *S. haddoni* individuals than *E. quadricolor* in Singapore. Pairwise allelic similarity analyses found one possible pair of biological clones, which indicated the possibility of clonal reproduction in *E. quadricolor*. *Stichodactyla haddoni* individuals were all genetically distinct, and no clones were discovered. Even with removal of these potential clone-mate pairs, diversity indices remained higher in *S. haddoni* compared to *E. quadricolor* (expected heterozygosity (H_S) *S. haddoni* H_S = 0.282 vs. *E. quadricolor* H_S = 0.239; Table 2). This was also reflected in the overall lower inbreeding-coefficients (G_{IS}) of *S. haddoni* (0.118) compared to *E. quadricolor* (0.201).

Table 2. Number of samples prepared for ddRADseq sequencing (*n*) per site and species and genetic diversity indices of *Stichodactyla haddoni* (sti_b1.10miss) and *Entacmaea quadricolor* (ane_b3.10miss), where Num = number of alleles observed, Eff_num = effective number of alleles in a population weighted for their frequencies, H_o = observed heterozygosity where 0 represents that all individuals are homozygous and 1 represents that all individuals are heterozygous, H_S = expected heterozygosity, H_t = total heterozygosity, H'_t = total heterozygosity corrected for bias from sampling limited populations, G_{is} = inbreeding coefficient; analogous to Wright's inbreeding-coefficient, F_{IS}.

	Population	*n*	Num	Eff_num	H_o	H_s	H_t	H'_t	G_{is}
S. haddoni	CHJ	8	1.692	1.4	0.241	0.272	0.272	—	0.113
	PRP	9	1.752	1.414	0.241	0.276	0.276	—	0.127
	TUM	7	1.851	1.429	0.24	0.276	0.276	—	0.131
	PLS	10	1.811	1.426	0.232	0.278	0.278	—	0.165
	TAM	7	1.687	1.393	0.226	0.271	0.271	—	0.167
	BEC	6	1.745	1.412	0.234	0.277	0.277	—	0.153
	CHB	17	1.887	1.422	0.229	0.268	0.268	—	0.145
	CYR	8	1.838	1.424	0.237	0.274	0.274	—	0.135
	Overall	**81**	**1.989**	**1.388**	**0.235**	**0.274**	**0.274**	**0.274**	**0.142**
E. quadricolor	PLH	12	1.806	1.364	0.198	0.238	0.238	—	0.17
	SIS	13	1.78	1.367	0.204	0.242	0.242	—	0.158
	PLJ	8	1.704	1.351	0.183	0.236	0.236	—	0.225
	TPT	17	1.842	1.363	0.173	0.237	0.237	—	0.268
	KUI	9	1.736	1.36	0.207	0.241	0.241	—	0.14
	RAL	3	1.504	1.331	0.199	0.245	0.245	—	0.186
	SJI	4	1.562	1.329	0.19	0.234	0.234	—	0.189
	PLS	8	1.598	1.333	0.175	0.233	0.233	—	0.25
	SSH	8	1.684	1.347	0.179	0.235	0.235	—	0.241
	LAI	5	1.645	1.36	0.21	0.247	0.247	—	0.15
	PLT	8	1.695	1.35	0.195	0.237	0.237	—	0.174
	CYR	7	1.697	1.352	0.196	0.238	0.238	—	0.176
	Overall	**99**	**2**	**1.2**	**0.192**	**0.239**	**0.238**	**0.238**	**0.194**

4. Discussion

Population connectivity is thought to be crucial for the long-term survival of a species, but it can be compromised by anthropogenic stressors. This study used genomic tools to understand the population demographics and assess the reproductive modes of sea anemones in a small but highly disturbed marine environment. While recent studies have suggested that urbanised sites with artificial surfaces might provide new niches and function as 'marine stepping-stones' that aid in population connectivity [69], such surfaces provide new environments for all species, native species and non-native invasive species alike [109]. Artificial surfaces such as seawalls that are ubiquitous along Singapore's coastline, could aid the colonisation and serve to connect populations for marine organisms like molluscs [110], crustaceans, polychaetes [111] and hard corals [57,112]. Seawalls serving as a 'stepping-stone' remains to be tested for sea anemone species that naturally prefer coral-dominated or sandy substrates, and not hard surfaces like seawalls [79]. At a small geographic scale such as in Singapore, connectivity depends heavily on the pelagic larval duration, which is undetermined for

both species here, but enough to maintain connected populations. Here, we find near-genetic panmixia and high genetic diversity are maintained in both species that live in environments with constant anthropogenic stress and inhabiting different tidal zones. Similar population genomic signatures have previously been described for Singapore's waters in other species belonging to very different taxa (e.g., the knobbly seastar *Protoreaster nodosus* and coral *Platygyra sinensis* [64,67]). The results of the present study thus add to the emerging pattern that having a planktonic dispersive stage is sufficient for maintaining panmixia in marine waters that are exposed to high levels of anthropogenic disturbances.

Effective dispersal of planktonic life stages is likely driven by the prevailing hydrodynamics. Unfortunately, the spawning periods for both anemone species in Singapore are unknown, but *E. quadricolor* and other scleractinian corals are known to spawn at the start of the year between January and April in eastern Australia [51,113]. If the anemones in Singapore were to spawn at a similar time, the high levels of connectivity observed would be driven by hydrodynamics and possibly chemical cues from the mass larval exchange among the southern islands of Singapore following coral spawning during the same period from March to April [114–116]. The general westward flow of currents in the southern islands of Singapore and semi-diurnal tides caused by strong hydrodynamic pressure gradients result in mixing between the low intertidal and subtidal zones of Singapore's reefs. This provides planktonic larvae with time and opportunity to mix thoroughly within Singapore's waters before settlement. This may explain the high connectivity of the sea anemone populations [88,116,117].

Kelly and Palumbi [75] previously found stronger population structuring in the intertidal compared to subtidal species, which they linked to the harsher intertidal environment. The present study, however, finds no such differences in the population genomic patterns of *S. haddoni* and *E. quadricolor* despite inhabiting largely the high intertidal and shallow subtidal areas, respectively. This suggests that the mixing of surface water provides sufficient opportunities for the planktonic larvae to travel between sites [88]. In addition, there may be some overlap in their distribution, as some *E. quadricolor* individuals may be visible at intertidal areas during (extremely) low tides, possibly due to the vertical compression of habitats along Singapore's coasts [118,119].

It is remarkable that even though there was no evidence for spatial structuring by site, there appeared to be some structuring at the individual level for *S. haddoni*. Three individuals from PLS and one from CYR seemed to be diverging from the main genetic cluster (Figures 2 and 3). These were collected from offshore sites away from the samples forming the main cluster of *S. haddoni*. This might be due to the arrival of recent immigrants from genetically distinct populations in Malaysian or Indonesian waters. These waters may harbour the intermediate genotypes that were not observed in our study. An alternative interpretation would be early signs for sympatric speciation. To test these hypotheses, further sampling across time focusing on the offshore sites in Singapore and beyond national boundaries are needed.

Overall, it appears that broadcast spawning is a good predictor for a species' population genetics in Singapore waters [64,67]. The genetic diversity was found to be high in both study species here (e.g., compare expected heterozygosity values in Table 2 to other sea anemone species in [46]), and also in other broadcast spawning species at this spatial scale [64,67]. This is surprising given decades of anthropogenic disturbances in Singapore's waters, including extensive land reclamation that has decimated entire populations and thus created genetic bottlenecks depressing diversity [52,53,120,121]. In addition, this study is a first attempt to predict sexual reproduction modes for sea anemone species based on genomic information. Higher genetic diversity and lower inbreeding coefficients in *S. haddoni* could have resulted from its tendency to reproduce only via sexual reproduction, while *E. quadricolor* likely reproduces both asexually and sexually. However, further studies focused on genetic diversity and its relation to reproduction methods would be important for comparing genetic diversity between other marine species, especially in larger scales and across biogeographical regions. The high genetic diversity in both species could possibly confer higher genetic resilience against potential anthropogenic disturbances. Being able to predict genetic diversity based on reproductive mode is thus also important for the management of sea anemone population and health.

Although asexual reproduction was not detected for *S. haddoni*, two pairs of clones were found in *E. quadricolor* which is consistent with the previous observations of clonal reproduction in *E. quadricolor* [5,23]. Scott [42] also suggested that *S. haddoni* only reproduces via broadcast spawning while *E. quadricolor* can reproduce via both broadcast spawning and asexual reproduction (longitudinal fission). Species that rely solely (or dominantly) on sexual reproduction can maintain and renew populations, but do so during short breeding seasons [23,39–41,51]. Since *S. haddoni* lives at a higher intertidal zone, the need for dispersal via broadcast spawning could conceivably be greater than that of the more submerged *E. quadricolor* where opportunities to disperse far are not limited by tide immersions.

DNA barcoding was used in the present study to confirm morphological identifications. We detected several misidentifications based on morphology (Figure S2). Such misidentifications by parataxonomists are not uncommon [122,123], and the present study provides further evidence for the importance of species confirmation via DNA barcoding in taxonomically challenging groups such as actiniarians. Such taxa may not have sufficient taxonomic expertise available, or could consist of many cryptic species complexes. For anthozoans, including sea anemones, there is low interspecific variability in the universal barcoding gene COI [48,124]. Therefore, 28S rDNA was here used to successfully distinguish *E. quadricolor* from closely related species found in this region (i.e., *H. magnifica*). *Stichodactyla haddoni* is often morphologically confused with *S. gigantea* and especially *S. tapetum* [77]. The genes COIII and 28S were used in preliminary tests but were unable to resolve species-level differences between closely related *Stichodactyla* species. Based on the ITS1 marker used here, *S. mertensii* and *S. haddoni* were clearly distinguishable (Figure S1). Even though there were two apparent clusters of *S. haddoni* (7.6% difference), these differences were not detected in the SNP data and PCA clusters and could be due to paralogy. While *S. tapetum* (STI102) clustered amongst other *S. haddoni* individuals, this individual could have been misidentified (i.e., *S. haddoni* juvenile that was mistaken as *S. tapetum*), or that there was low inter-specific variability between *S. haddoni* and *S. tapetum*. Since variable rates of evolution in other intronic regions (ITSII) have been shown for actiniarians generally [125], only additional sequencing of other *Stichodactyla* species would allow the determination of the absence/presence of a barcoding gap for this marker, and its usefulness for species identification in this group. As a precaution, STI102 was excluded from subsequent analyses. Nevertheless, the large pairwise difference between *S. mertensii* and *S. haddoni* (12%) suggests that ITS1 has potential for distinguishing *Stichodactyla* species.

In conclusion, this study represents the first fine-scale population genomics study and direct comparison of two sea anemone species in an environment that is heavily impacted by human activities. The species inhabit different habitats and tidal zones but we find support for the hypothesis that primarily broadcast spawning species like *S. haddoni* and *E. quadricolor* have highly connected populations at small spatial scales even under adverse conditions. High connectivity for broadcast spawners from different clades and living in different habitats is good news for managers, as these populations are more likely to be able to recover naturally from disturbances. Analysing more than 1000 genome-wide SNP markers, we were able to infer population connectivity and also the reproductive strategies of *S. haddoni* and *E. quadricolor* using genetic tools. Only *E. quadricolor* was found to also propagate asexually. Where it might not be feasible to directly observe reproduction in species that have undetermined breeding modes and sporadic temporal breeding seasons, genetic tools play a critical role in the assessment of reproductive modes here. There remains a need to explore genetic structuring between intertidal and subtidal populations of other marine taxa in relation to differential wave action and emersion gradients, which can affect larval retention and thus local adaptation [84].

Supplementary Materials: The following are available online at http://www.mdpi.com/1424-2818/12/12/467/s1. Figure S1: Cluster dendrogram of *Stichodactyla haddoni*, *S. mertensii* (STI134) and *S. tapetum* (STI102) based on the ITS1 barcodes. The numbers at each node indicates the percentage pairwise difference. Figure S2: (a) Images of *Heteractis magnifica* and (b) *Entacmaea quadricolor*. Figure S3: BayeScan analysis outputs for (a) *Stichodactyla haddoni* and (b) *Entacmaea quadricolor* that were selected for downstream analyses, where FDR = 0.05. Dots labelled with numbers that fall to the right of the line are loci or SNPs identified as potential outliers. Figure S4: Histogram of pairwise genetic similarity for (a) sti_b1.10miss (b) ane_b3.10miss. ANE065-ANE088 pair (*) and the gap for potential clonemates (bracket) are indicated in (b). Figure S5: Outlier loci frequencies per site (a) sti_b1.10miss (b) ane_b3.10miss. Figure S6: (a) sti_b1.10miss lnPK graph, (b) sti_b1.10miss deltaK graph, (c) ane_b3.10miss lnPK graph, (d) ane_b3.10miss deltaK graph.

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