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Comparison of sequence-capture and ddRAD approaches in resolving species and populations in hexacorallian anthozoans

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ABSTRACT

Genome-level sequencing is the next step in understanding species-level relationships within Anthozoa (soft corals, anemones, stony corals, and their kin) as morphological and PCR-directed (single-locus) sequencing methods often fall short of differentiating species. The sea anemone genus *Metridium* is a common northern temperate sea anemone whose species are difficult to differentiate using morphology alone. Here we use *Metridium* as a case study to confirm the low level of information available in six loci for species differentiation commonly sequenced for Actiniaria and explore and compare the efficacy of ddRAD and sequence-capture methods in species-level systematics and biogeographic studies. We produce phylogenetic trees from concatenated datasets and perform DAPC and STRUCTURE analyses using SNP data. The six conventional loci are not able to consistently differentiate species within *Metridium*. The sequence-capture dataset resulted in high support and resolution for both current species and relationships between geographic areas. The ddRAD datasets displayed ambiguity among species, and support between major geographic groupings was not as high as the sequence-capture datasets. The level of resolution and support resulting from the sequence-capture data, combined with the ability to add additional individuals and expand beyond the genus *Metridium* over time, emphasizes the utility of sequence-capture methods for both systematics and future biogeographic studies within anthozoans. We discuss the strengths and weaknesses of the genomic approaches in light of our findings and suggest potential implications for the biogeography of *Metridium* based on our sampling.

1. Introduction

The majority of marine invertebrate species are described following a morphological species concept, which defines new taxa based on unique aspects of anatomy or biology. Likewise, synonymies are generally justified through the demonstration of identity in these visible features. Although these data are certainly relevant for species delimitation in hexacorallian cnidarians, ecophenotypic variation can be very high (e.g., Hoeksema & Crowther 2011), and phylogenetic analyses have repeatedly highlighted the plasticity and high levels of convergence of key features in sea anemones (González-Muñoz et al., 2015; Rodríguez et al., 2012, 2014; Grajales & Rodríguez 2016; Daly et al. 2017; Gusmão et al. 2020) and other anthozoans (e.g., Bo et al., 2018; Budd et al., 2010; Cachet et al., 2015; Sánchez et al., 2003). This has cast doubt on the effectiveness of morphological characters as the arbiter of species boundaries within this lineage.

Beyond its historical importance and despite its practical problems, morphology has remained the primary system for species determination in anthozoans because genetic tools have not been very effective. Many anthozoans have a very slow rate of mitochondrial sequence evolution, and standard mtDNA barcodes like Cytochrome Oxidase 1 (CO-I) or 16S rDNA generally do not differentiate species (Shearer et al. 2002; Hebert et al. 2003; Huang et al. 2008; McFadden et al. 2011; but see Sanamyan et al. 2020). For sea anemones, the insufficiency of each traditional marker has been circumvented through the concerted evaluation of multiple traditional markers (e.g., Pereira et al. 2014; Grajales & Rodríguez 2016; Larson & Daly 2016; Titus et al. 2019a) and by attempts to identify new markers (e.g., Brugler et al. 2018) but these

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studies generally have shown low support and resolution for relationships at species level.

Genome-scale approaches reveal new possibilities for testing species boundaries in groups whose members cannot be differentiated with conventional PCR-directed loci. These efforts have included both size-based and sequence-based approaches. Size-based studies have effectively used Amplified Fragment Length Polymorphisms (AFLPs) and MIG-seq of inter-simple sequence repeats (ISSRs) to detect fine scale population genetic structure and species boundaries in anemones and other anthozoans (e.g. Reitzel et al. 2008; Thornhill et al., 2013; Suyama & Matsuki 2015; Richards et al. 2018; Takata et al., 2019). However, because AFLPs and ISSR markers are size-based rather than sequence based, they are subject to repeatability issues, hidden allelic diversity, and non-Mendelian inheritance that may bias analyses of population diversity and structure (reviewed in Mendelson & Shaw 2005; Holliday et al. 2018).

High-throughput sequencing methods such as Restriction Associated Digest methods (e.g., RAD, ezRAD, ddRAD) have since been used to evaluate connectivity or species boundaries in corals (e.g., Toonen et al. 2013; Bongaerts et al. 2017; Forsman et al. 2017; Devlin-Durante & Baums 2017; Leydet et al. 2018; Quattrini et al. 2019) and in a small number of sea anemones (Spano et al. 2018; Bellis et al. 2018; Titus et al. 2019b; Cornwell 2020; Porro et al. 2020), generally finding greater molecular diversity in the studied species than expected based on anatomy or taxonomy. For example, Titus et al (2019b) and Cornwell (2020) found significant differentiation within currently accepted species of *Bartholomea* and *Anthopleura*, respectively, but the diversity could be accommodated in existing species concepts. In contrast, Spano et al (2018) found that morphology-based species hypotheses failed to recognize significant, geographically-structured diversity within *Anthothoe chilensis* (Lesson, 1830 in Lesson and Duperrey (1830), and Porro et al. (2020) failed to find correspondence between genetic diversity in RAD sequences, color, or geography in the widespread species *Anemonia viridis* (Forsskål, 1775) despite significant genetic variation. Although their high variability and ease of use in novel systems are major benefits (Reitzel et al., 2013), allele dropout, batch-effects associated with the random amplification steps in RAD approaches, and variation in bioinformatic methods can be problematic (reviewed by Puritz et al. 2014; Shafer et al. 2017; Lee et al. 2018; Bresadola et al. 2020). Furthermore, RAD data are difficult to connect to studies at different levels of hierarchical organization and may be complicated to parse in species that harbor photosymbionts (Titus & Daly in review).

Among genome-scale methods, sequence-capture methods (see Faircloth et al., 2013; Lemmon and Lemmon, 2012) represent an alternative to RAD approaches. Sequence-capture approaches require existing genomic resources for species and entail some upstream development (reviewed in Harvey et al. 2016). Sequence-capture approaches generally work with DNA of lower quality and concentration compared to RAD methods, can be scaled and replicated more easily (see Faircloth et al. 2012), and the same loci can be targeted across different taxonomic scales (e.g., species to family to class). A set of sequence-capture baits developed for Anthozoa to resolve broader questions (Quattrini et al., 2018, 2020) has been adapted to address questions of narrower taxonomic scale (Erickson et al. 2020, Cowman et al. 2020, Untiedt et al. 2021). Initial studies of these newly-designed baits for species- and genus-level questions in hard/stony (Cowman et al. 2020) and soft corals (Erickson et al., 2020) show promise for resolving relationships at those scales in anthozoans.

Here we explore the potential of genome-scale sequencing approaches to decipher the genetic and geographic diversity in *Metridium* de Blainville, 1824, a genus of sea anemones found from the shallows to depths of 200 m in coastal waters across the Northern hemisphere. *Metridium senile* (Linnaeus, 1761) was first described from Scandinavia but is well-known throughout the British Isles and Northern Europe, the Atlantic and Pacific coasts of North America, Northern Japan, North Eastern China, and Eastern Russia (reviewed in Glon et al. 2020). Species of *Metridium* have been circumscribed and re-named based on anatomy, reproductive attributes, and geography, with as many as eight Northern Hemisphere species recognized in addition to *M. senile*; seven species are currently considered valid (Daly & Fautin 2020)

Because of their accessibility as intertidal animals and the known variation in color, body size, and biotic processes like incidence or asexual reproduction, *Metridium* was among the earliest groups of anemones to undergo genetic investigation. Using biochemical markers to examine allozyme variation, Bucklin & Hedgecock (1982) distinguished California (Bodega Harbor) *Metridium exile* Hand, 1956 from California (Bodega Harbor) *M. senile*. These markers further identified two distinct clusters of *M. senile*, one containing clonal and one containing solitary individuals, with the large and solitary form later being raised to species level as *Metridium farcimen* (Brandt, 1835) by Fautin et al. (1989). However, these markers were unable to distinguish the Atlantic (Maine) *M. senile* from the clonal variety of *M. senile* in the Pacific. Bucklin (1985) examined allozyme variation in Atlantic *Metridium* and failed to recover the distinction Stephenson (1935) had made between the British *M. s. dianthus* (Ellis, 1767) and *M. s. pallidum* (Holdsworth, 1855). These early perspectives have not been re-assessed with either single markers (e.g., DNA barcodes) or genome-level data.

The high intra-population variation in anatomy, reproduction, and genetics along with their geographic distribution make *Metridium* a compelling system in which to investigate the intersection of genetic, morphological, and geographic diversity. We evaluate the utility of conventional, PCR-directed markers for species identification in *Metridium* and then apply and explore genomic scale methods of ddRAD and sequence-capture to members of the genus. The results are used to understand how each of these genomic methods can contribute to understanding species diversity and connectivity across the distribution of these anemones. Our comparison between sequence-capture and ddRAD identifies strengths and challenges with each of these approaches to consider for future studies of Anthozoa. Our application of hexacoral-targeted sequence baits (Cowman et al. 2020) to explore phylogeographic structure and connectivity in a small sampling of *Metridium* individuals represents the first step in applying sequence-capture approaches to species-level relationships in Actiniaria.

2. Material and methods

2.1. Specimen collection

We collected *Metridium* throughout the known range in the Atlantic and Pacific (Appendix Table 1.A). Specimens were obtained mainly by hand by the authors or by collaborators from floating dock fouling communities, intertidal depths, or by SCUBA diving to depths of 30 m, and preserved with 99% EtOH. We attempted to minimize sampling multiple anemones from a single clonal group by collecting individuals separated from each other on discontinuous substrate or of different colors if in close proximity (see Hoffmann 1976; Shick et al. 1979). Individuals in the Atlantic Ocean include those considered to be *M. senile* (US and Canada; Norway) or *M. dianthus* (Ireland, Northern Ireland), though names are used somewhat interchangeably across the Atlantic. For clarity within this study, we apply only the name *M. senile* in the results and figures to individuals collected from the Atlantic. We included samples from the Gulf of Alaska and the Bering Sea collected during a 2017 NOAA trawl survey that were identified as *M. farcimen* based on their size and the depth where collected. The *M. farcimen* specimens collected from California and Washington were identified by their large size and solitary nature, as compared with the smaller and clonal-type *M. senile* collected from docks and intertidally along the Pacific coast. Because *Diadumene* Stephenson, 1920 is the sister clade to *Metridium* (see Rodríguez et al. 2014), *Diadumene lineata* (Verrill, 1869) and *Diadumene lighti* (Hand, 1956) were used as outgroup taxa. For all samples, we extracted genomic DNA using the Qiagen DNeasy Blood and Tissue Kits (Qiagen Inc.) or the E.Z.N.A. Tissue DNA Kit (Omega Bio-Tek,

Norcross, GA, USA) according to the manufacturer's protocol.

2.2. Sequencing PCR-directed loci

We amplified three nuclear (ITS, 18S, 28S) and three mitochondrial (COIII, 12S, 16S) loci (see Table 1 for primer information) which are commonly used in actiniarian systematics and show low to no intra-individual variation (e.g. Daly et al. 2010; Rodríguez et al. 2014). We used IllustraTM puReTaq™ Ready-To-Go™ PCR beads (GE Healthcare) to amplify these markers from 39 individuals (Appendix Table A.1.). The thermal cycle for all primers was: 95.0 °C for 2 min, 30 cycles of 95.0 °C for 15 s, 45.0 °C for 1 min, 72.0 °C for 1 min 10 s), with a final extension at 72.0 °C for 3 min. Samples were sequenced in both directions at the TacGen sequencing center (Richmond, CA). We edited, aligned, and gathered sequence statistics in Geneious R11 (www.geneious.com: Kearse 2012).

2.3. ddRAD library Preparation, Sequencing, and dataset assembly

We used double-digest RAD methods, in which SNPs are identified after sequencing the fragments generated by two rounds of restriction enzyme digestion. We followed the protocol of Titus et al. (2019b), which modified protocols originally developed by Sovic et al. (2016). Samples were quantified (ng/μL) using a Qubit fluorometer and standardized to 25 ng/μL in 10 ul of elution buffer. DNA was digested using the restriction enzymes *Eco*RI-HF and *Pst*I-HF and the restriction cut sites were annealed with Illumina compatible barcodes. Samples were manually size-selected to a range of 400–800 bp using gel electrophoresis and quantified via quantitative PCR prior to pooling into final libraries for sequencing on Illumina HiSeq 2000 for 100 bp, single-end reads at the Duke GCB Sequencing Shared Resource.

We used iPyrad v.0.7.27 and v.0.9.51 (Eaton 2014) to demultiplex and quality filter the raw Illumina reads. During the processing, we retained default parameters, keeping the maximum number of SNPs per locus at 0.2 (20%), the maximum number of shared heterozygous sites per locus to 0.5 (50%), and adjusting minimum depth parameters to 10. Additionally, we adjusted two of the stringency settings: the clustering threshold (clust_threshold) was loosened to 85% sequence similarity to allow for a less stringent pipeline (following Quattrini et al. 2019) and the minimum number of samples per locus for output (min_samples_locus) was set to 4 [10%; ddRAD_10] and 20 [50%; ddRAD_50] to allow comparison between two datasets; separate ingroup-only datasets were also built to obtain matrix statistics. We allowed our datasets to have a substantial amount of missing data (90% and 50%, respectively) as the aligned length of the resulting datasets offset effects of missing data; filtering too stringently across loci may drop important phylogenetic information (Huang & Knowles 2016; Eaton et al. 2017). All analyses were conducted using the computing resources of the Ohio Supercomputer Center (1987).

2.4. Bait capture library preparation, sequencing, and dataset assembly

Sequence-capture library preparation and sequencing was conducted at Arbor Biosciences (Ann Arbor, MI) following protocols by Quattrini et al. (2018) using a recently redesigned bait set for Hexacorallia (Cowman et al. 2020). The Cowman et al. (2020) bait set was subset to target actiniarians specifically, resulting in 17,268 baits targeting 2496 conserved elements (i.e., exons, ultraconserved (UCE) loci), which were synthesized by Arbor Biosciences (Ann Arbor, MI). Prior to sequencing, DNA quantity (ng/μL) and quality (260/280 & 260/230 ratios) were assessed for each sample using a Qubit 2.0 fluorometer and NanoDrop spectrophotometer, respectively. Up to 1000 ng DNA per sample was sent to Arbor BioSciences for library preparation and target enrichment. DNA was sheared to a target size of 400–800 bp and library preparation was performed using a Kapa Hyper Prep Kit, optimized for target capture, with universal Y-yoke oligonucleotide adapters and iTru dual-indexed primers (Glenn et al., 2016). Twelve libraries were pooled into equimolar ratios (100 ng) totaling 1.2 μg of DNA. Target enrichment was then performed following the MyBaits v. IV protocol using 500 ng/rxn concentration of baits. Target-enriched libraries were sequenced on Illumina NovaSeq (~1Gb per library, 150 PE reads).

Paired-end reads were cleaned using TrimGalore (https://github.com/FelixKrueger/TrimGalore) using a stringency of five and then assembled in SPAdes genome assembler v3.14.0 (Bankevich et al., 2012) using the –careful and cov-cutoff 2 parameters. We used the PHYLUCE pipeline (Faircloth 2016) to search for and match conserved element loci in the resulting assemblies to the bait set, following the online tutorial phyluce.readthedocs.io with some modifications (Quattrini et al. 2018). We used phyluce_assembly_match_contigs_probes with a min-coverage of 70% and min-identity of 70% to match baits to contigs. We then extracted loci using phyluce_assembly_get_match_counts and phlyluce_assembly_get_fastas_from_match_counts and then aligned loci with MAFFT (Katoh et al., 2002) using phyluce_align_seqcap_align. We then trimmed loci internally using Gblocks (phyluce_align_get_gblocks_trimmed_alignments_from_untrimmed) with default parameters. Summary data were obtained using phyluce_align_get_align_summary_data and phyluce_align_get_informative_sites. We obtained taxon occupancy matrices for both 50% and 75% completeness, as a comparison, and created separate ingroup-only datasets for calculating matrix statistics.

Because we also were interested in the versatility of using sequence-capture SNP data for population studies, we followed previously published SNP-calling pipelines (Zarza et al. 2016, 2018, Derkarabetian et al. 2019, Erickson et al. 2020). Scripts in this pipeline primarily use tools from PHYLUCE (Faircloth 2016) and GATK3 (McKenna et al. 2010). We used the individual in our dataset with the highest number of unique loci as a reference and mapped reads back to that reference individual. Final SNP matrices were produced and filtered according to parameters listed below.

Table 1
Sequence statistics and models used to calculate Maximum Likelihood analyses for the six regions.

Dataset	Primer Sequence	ML Model	Number taxa	Aligned Length	PI sites (ingroup)	% PI sites	Variable sites (ingroup)
ITS	Fwd: 5′-GGTTTCCGTAGGTGAACCTGCGGAA-3′ Rev: 5′-GTTCCCGCTTCATTCGCCATTAC-3′	HKY + G	36	731	11	1.50	14
18S	Fwd: 5′- AACCTGGTTGATCCTGCCAGT-3′ Rev: 5′- CAGACAAATCGCTCCACCAAC-3′	GTR	37	1805	1	0.06	7
28S	Fwd: 5′-GCCGACCCGCTGAATTCAAGCATAT-3′ Rev: 5′-TTCYGACTTAGAGGCGTTCAG- 3′	GTR + G	31	3163	18	0.57	49
COIII	Fwd: 5′- CATTTAGTTGATCCTAGGCCTTGACC-3′ Rev: 5′-CAAACCACATCTACAAAATGCCAATA- 3′	HKY + I	38	664	3	0.45	51
12S	Fwd: 5′- AGCCACACTTTCACTGAAACAAGG-3′ Rev: 5′- GTTCCCYYWCYCTYACYATGTTACGAC-3′	HKY + I	38	844	0	0.00	22
16S	Fwd: 5′- CACTGACCGTGATAATGTAGCGT-3′ Rev: 5′-CCCCATGGTAGCTTTTATTCG −3′	HKY	38	447	0	0.00	12
Concatenated		–	39	7654	33	0.43	155

2.5. Phylogenetic analyses

For our PCR-directed loci dataset, we evaluated the best model of evolution for the six loci using jModelTest (Posada 2008) under the AIC criterion. We then conducted separate maximum likelihood phylogenetic analyses in IQTree v1.6.1 (Nguyen et al. 2015), with 1000 UFBoot bootstraps for each locus and an analysis of the concatenated dataset using partitioned models as calculated above with jModelTest (Nguyen et al. 2015; Chernomor et al., 2016). For the ddRAD and sequence-capture datasets, we carried out likelihood analyses using RAxML-NG (Kozlov et al. 2019), as it has performed well in regards to speed and may produce equivalent or higher-scoring trees against methods including IQTree, using 200 Felsenstein bootstraps (FBP). Trees were rooted using *Diadumene* spp. The UCE and exon loci within the sequence capture dataset were not separated, as studies (Quattrini et al. 2020; Cowman et al. 2020) using this bait set have explored differing phylogenetic signal and found no difference in topology between UCE and exon loci. Phylogenetic trees were edited and mapped in R (R Core Team, 2020) using the ape (Paradis & Schliep 2019) and phytools (Revell 2012) packages, and outgroup taxa were removed from final figures to better visualize ingroup branch lengths.

2.6. Genetic cluster analyses

To compare the number of SNPs contained in each of the ddRAD and SC data matrices, we used vcftools (Danecek et al. 2011) to filter SNP data (iPyrad output .vcf file and SC SNP calling pipeline output). We applied separate filters and also a combination of filters to obtain SNP counts filtering to bi-allelic SNPs (minimum and maximum number of two alleles), thin 1000 as a proxy filter to simulate getting one snp per locus for SNP analyses, and a maximum of 25% and 50% missing data (–max-missing 0.75, 0.50).

We used unlinked SNPs (one SNP per 1000 bp) from each dataset to examine how ddRAD and sequence-capture (SC) SNP datasets recovered major genetic partitions across broad biogeographic space. To be consistent with current methodology using ddRAD data, we used the iPyrad .ustr and .usnps files for the ddRAD datasets and the unlinked SNP files for the SC data. We conducted a discriminant analysis of principal components (DAPC; Jombart et al. 2015) conducted in R (R Core Team, 2020) via the adegenet package (Jombart & Ahmed 2011). We assessed the number of principal components to retain using the K-means method (K = 10, retain all principal components) and defined the most likely number of genetic clusters using the lowest Bayesian Information Criterion (BIC) value. We determined the number of principal components to retain (optim.a.score) and assigned each individual to a cluster using DAPC. We plotted individuals to display their membership to each cluster and used a Principal Component Analysis (PCA) to display the clusters.

We ran STRUCTURE v2.3.4 (Pritchard et al. 2000) to infer population structure given *K* on the two ddRAD and one SC SNP datasets. We conducted five iterations over *K* = 1–8, with each MCMC chain for a single *K* running with a burn in of 100,000 and sampling over 200,000 generations. We analyzed and plotted the results of STRUCTURE using CLUMPAK (Clustering Markov Packager Across K), selecting the best *K* according to Evanno et al. (2005).

3. Results

3.1. PCR-directed dataset

Individually and in total, the six PCR-directed loci contain a low number of parsimony informative sites, with 0–18 sites per locus (<1.5%) and a combined total of 33 sites in the 7654 bp concatenated dataset (0.4%, Table 1). Each locus contributed strong support for grouping the *Metridium* samples together to the exclusion of *Diadumene*, but none individually provided strong resolution for subsets within *Metridium* (Appendix Fig. A.1.). Notable groupings from the trees based on individual loci are a clade of samples from Ireland (ITS, 28S), a clade of some California specimens (ITS), and a clade of *M. farcimen* samples (COIII). The tree from the concatenated dataset (Fig. 1) is largely unresolved and fails to group samples expected to have affinity to one another based on species identification. Only the clade of mainly *M. farcimen* at the base is supported (>95). For example, one specimen of *M. farcimen* did not resolve within the *M. farcimen* clade, while another specimen of *M. senile* resolved within the *M. farcimen* clade. The Northern Ireland individuals resolved within a poorly supported (bootstrap (BS) = 64) clade in the combined dataset as also suggested in the ITS and 28S trees, but the remainder of the Atlantic and Pacific *M. senile* were mixed, with no clear geographic or taxonomic structure.

3.2. ddRAD dataset

The ddRAD data were processed into two data sets: one in which the minimum percentage of samples for each locus was 10% (ddRAD_10) and one in which the minimum percentage of samples for each locus was 50% (ddRAD_50). Unsurprisingly, because it includes loci shared by a smaller number of samples, the ddRAD_10 data set had six times the

Fig. 1. Sanger multi-locus IQ-Tree Maximum Likelihood hypothesis based on 1000 bootstraps. Individuals designated in black are putative Metridium farcimen individuals; all other color designations are assumed M. senile or M. dianthus, and are colored based on geographic location: red (Japan), orange (Pacific), blue (western Atlantic), green (eastern Atlantic). Support values are provided only for backbone support, where present, with a support of 95% or higher considered support for confidence in the clade. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

number of loci and six times the aligned length of the ddRAD_50 data set (Table 2). However, the greater number of sequences and aligned length did not translate to an equally large number of informative sites (ddRAD_10 has 1.5x the informative sites than ddRAD_50) and the difference in terms of missing data is striking, with ddRAD_10 having more than twice the missing data of ddRAD_50 (Table 2). Two samples in the ddRAD_10 dataset (PAC3 and N27) dropped out of the ddRAD_50 dataset due to excess missing data.

RAxML phylogenetic analyses for the ddRAD_10 dataset resolved four well-supported clades (Fig. 2A): Atlantic *M. senile*, Pacific *M. senile*, *M. farcimen*, and the Bering Sea/Japan *Metridium*. Though there is strong support for the grouping of *M. farcimen* with the Bering Sea/Japan *Metridium*, the placement of this combined clade relative to the Pacific and Atlantic *M. senile* clades is unsupported (Fig. 2A). Within the *M. farcimen* clade, there are three subgroupings: south of Aleutians, Bering Sea, and Japan + two dock-collected specimens from the Pacific Northwest (Coos Bay, OR and Hammond Marina, WA). These relationships are most clearly seen in the ddRAD_10 tree (Fig. 2A). In the ddRAD_50 tree (Fig. 2B), despite greater matrix completeness, resolution and support are lacking: the only relationships that are well supported are the Atlantic as a single clade (95 BS) and lower latitude (below the Aleutian Islands) clade of *M. farcimen*. Although the BS support values are low, both the ddRAD_10 and ddRAD_50 trees resolve the same Bering Sea + Japan relationship. In the ddRAD_50 tree, the Pacific coast *M. senile* are a grade at the base of the tree, leaving relationships among Pacific individuals ambiguous.

3.3. Sequence-capture dataset

As with the ddRAD data, the sequence-capture data were processed into one dataset in which the matrix completeness was lower (SC_50) and another in which it was higher (SC_75). The number of loci in the SC_50 dataset was roughly twice that of the SC_75 dataset (Table 2), though the number of parsimony informative sites (25.05% vs. 24.86%) and missing data (38.36% vs. 30.05%) were not substantially different between the two. The mean locus length was 1019 bp (range: 479–1976) in both the SC_50 dataset and in the SC_75 dataset (range: 297–2045). The ddRAD and sequence-capture reads are contained within the NCBI BioProject accession PRJNA666413, with individual accession numbers contained within Appendix Table A.1.

Despite these differences in matrix completeness, the major clades within the phylogenetic trees produced by the two sequence-capture SNP datasets were identical, with minor variation in tip-level relationships presumably due to short branch lengths and the impact of missing data. Within these trees, a major clade of *M. farcimen* is present, although not all individuals (purple, Fig. 2D&E) assumed to be *M. farcimen* upon collection are included. Notable and consistent in these trees is that these specimens of *Metridium* from the Bering Sea resolved as well supported within the Atlantic *Metridium senile*, rather than associating with samples from Japan or *M. farcimen*, as might be expected based on geographic proximity and as recovered in the ddRAD analyses (Fig. 2A&B). Within *M. senile*, there are two well-resolved subclades which further partitions these samples into largely Pacific and Atlantic groups (Fig. 2D&E). The Atlantic clade of *M. senile* is roughly split into Western and Eastern Atlantic. The exception is a single individual from near Bergen, Norway (N27) which appears to be more closely related to the individuals from the Western Atlantic than those in Europe, and another individual from Egersund, Norway (N58) which is more closely related to individuals from the Bering Sea.

3.4. ddRAD and sequence-capture SNP data

The raw number of SNPs in the ddRAD datasets was about ten-fold more than in the sequence-capture datasets. However, because of the high level of missing data within the ddRAD datasets (80.17% and 56.84%), when applying the same SNP filters used on the sequence-capture dataset to the ddRAD data for unlinked SNPs and 75% complete matrices, the number of SNPs in the ddRAD datasets was reduced to less than half than those retained in the sequence-capture dataset (Table 3).

Using the *K*-means method, we identified 6 as the optimal *K* for both ddRAD datasets (Fig. 3, Appendix Fig. A.6), and 4 as the optimal *K* for the sequence-capture dataset (Fig. 4, Appendix Fig. A.6). However, as $K = 6$ also had a low BIC score for the sequence-capture dataset, we conducted additional DAPC analyses for $K = 6$. The optimal *K* using the Evanno et al. (2005) method was 5 for both the ddRAD_10 and ddRAD_50 datasets, and 7 for the sequence-capture dataset. Additionally, as a means of comparison between datasets and with the DAPC results, we used the STRUCTURE analyses (Figs. 3 & 4) using $K = 6$ for all datasets, as well as an additional $K = 4$ for the sequence-capture datasets.

For all datasets, *M. farcimen* was retained in one (STRUCTURE) or two (DAPC) major clusters. The relationship of samples from Japan, while consistent with their respective phylogenies, are conflicting between the ddRAD (closest to Bering Sea *Metridium*) and sequence-capture (closer to Pacific *M. senile*). The Pacific *M. senile* are clustered well together in the sequence-capture dataset, except where $K = 6$ and there seems to be some mixing between Japan and Pacific *M. senile* in only the DAPC plots. Likewise, the ddRAD data suggests little mixing, though ddRAD_10 does partition three individuals separately. There is little variation among the Atlantic individuals, with a few exceptions from Norway that are equally seen in the phylogenetic trees.

4. Discussion

Our results concur with previous studies of other taxa in finding that the traditional PCR-directed markers provide low levels of biogeographic resolution and do not consistently differentiate among major species of *Metridium* or populations sampled here. The resolution and support provided by sequence-capture data is encouraging for future studies, as the paucity of effective PCR-directed markers, combined with challenging morphological features (Fig. 5), has left many species level relationships unresolved in anthozoan phylogenetics. Below we detail the strengths and weaknesses of each of these methods as it relates to this study, how they may be applied to future phylogenetic studies in the Actiniaria, and the preliminary insights that these data provide into the history of *Metridium*.

4.1. PCR-directed loci

None of the six PCR-directed loci are suitable as barcodes or for distinguishing among the three putative species in *Metridium*. The six loci used in symphony with each other suggested a distinction between *M. farcimen* and the remaining *Metridium*, although this inference was

Table 2
Models and sequence statistics for the ingroup of both the ddRAD and sequence-capture (SC) datasets.

Dataset	ML Model	Number taxa (ingroup)	Number loci	Aligned Length	PI sites (total)	% PI sites	% Missing Sites in aligned matrix
ddRAD_10	GTR + FU + G4m	39	12,659	1,771,372	37,626	2.12	73.10
ddRAD_50	GTR + FU + G4m	38	1667	235,427	10,361	4.40	37.40
SC_75	GTR + FU + G4m	49	583	636,541	158,246	24.86	30.05
SC_50	GTR + FU + G4m	49	1192	1,215,640	304,626	25.05	38.36

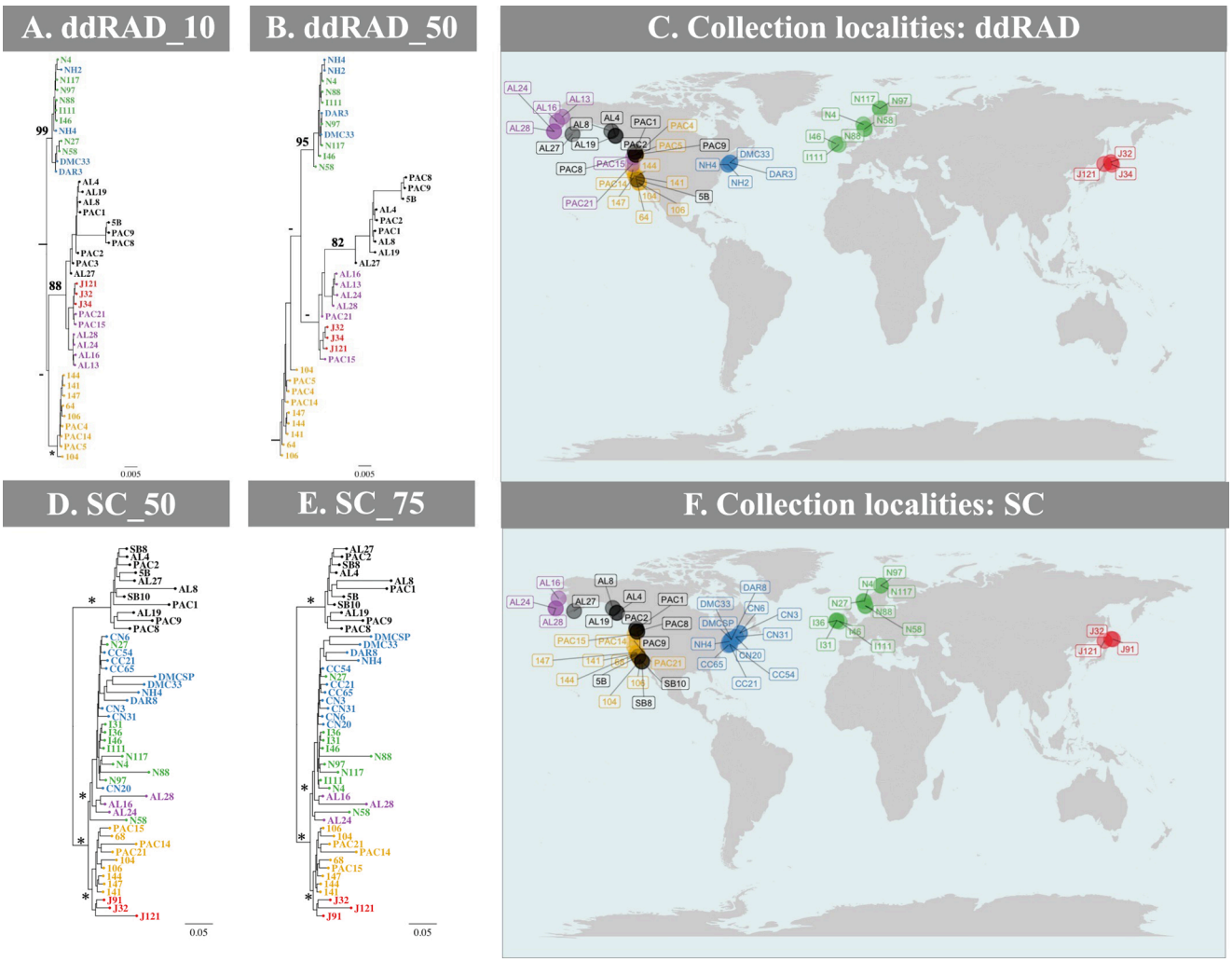

Fig. 2. RAxML-ng phylogeny with associated sampling locations for ddRAD and sequence-capture (SC) datasets: A) ddRAD_10, B) ddRAD_50, D) SC_50, and E) SC_75 datasets based on 200 bootstraps. Individuals designated in black and purple are putative M. farcimen individuals; all other color designations are assumed to be M. senile, and are colored based on geographic location: red (Japan), orange (Pacific), blue (western Atlantic), green (eastern Atlantic). Support displayed only for backbone relationships; full support values available in Appendix Fig. A.2-5. Bootstrap support of 100 designated as (*), <60 designated as (-). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Comparison of SNP information for ddRAD and SC datasets. SNP data from the two ddRAD final datasets and the SC dataset were filtered using identical settings with vcftools: bi-allelic SNPs only (min/max-alleles = 2), final SNP matrix filtered at 75% completeness, thinned to a single SNP every 1000 bp (=unlinked SNP filtering), and a combination of thinned and completeness matrices at 75% and 50%.

Dataset	Initial SNPs	Bi-allelic SNPs	75% Matrix	Unlinked SNPs	Unlinked and 75%	Unlinked and 50%
ddRAD_10	62,040	59,452	1497	9683	203	1615
ddRAD_50	13,212	12,330	1497	1619	203	1615
SC	22,668	22,047	16,434	1398	1164	1332

not consistent. Among the loci, the ITS and 28S trees displayed the most resolution, but the power of these to distinguish relationships was not equally distributed across the tree. The ITS region had the highest percentage of PI sites, but these were highly localized to the Ireland clade and were not able to distinguish *M. farcimen* from *M. senile*. Though 28S has the greatest sequence length, it contains only 0.5% PI sites. The 28S tree (see Appendix Fig. A.1) is the most resolved but most of its nodes are not retained in the concatenated tree, as was found more broadly for this marker (Daly et al. 2010). The six loci together appear to be useful for genus-level identification and at deeper taxonomic levels. Although equivalency of genetic divergence between sibling species in different lineages is as unlikely across sea anemones as it is in other lineages (e.g., Cádiz et al., 2018; Cognato, 2006), our results suggest that studies that distinguish subgeneric groups based on these markers (e.g., Pereira et al. 2014; Grajales and Rodríguez 2016; Titus et al. 2019a) are likely recovering relatively deep signal, and may fail to identify more recent genetic lineages within their study systems (e.g., cryptic species).

The majority of labs are well-equipped for standard DNA extraction and PCR; this combined with the affordability of Sanger sequencing and fast post-processing of data makes them an attractive first step in exploring intergeneric relationships in Actiniaria. However, there is high variation across studies of Actiniaria using the same markers; these resolve intergeneric and interspecific relationships inconsistently. Titus et al. (2019a) resolved superfamily relationships in clownfish hosting

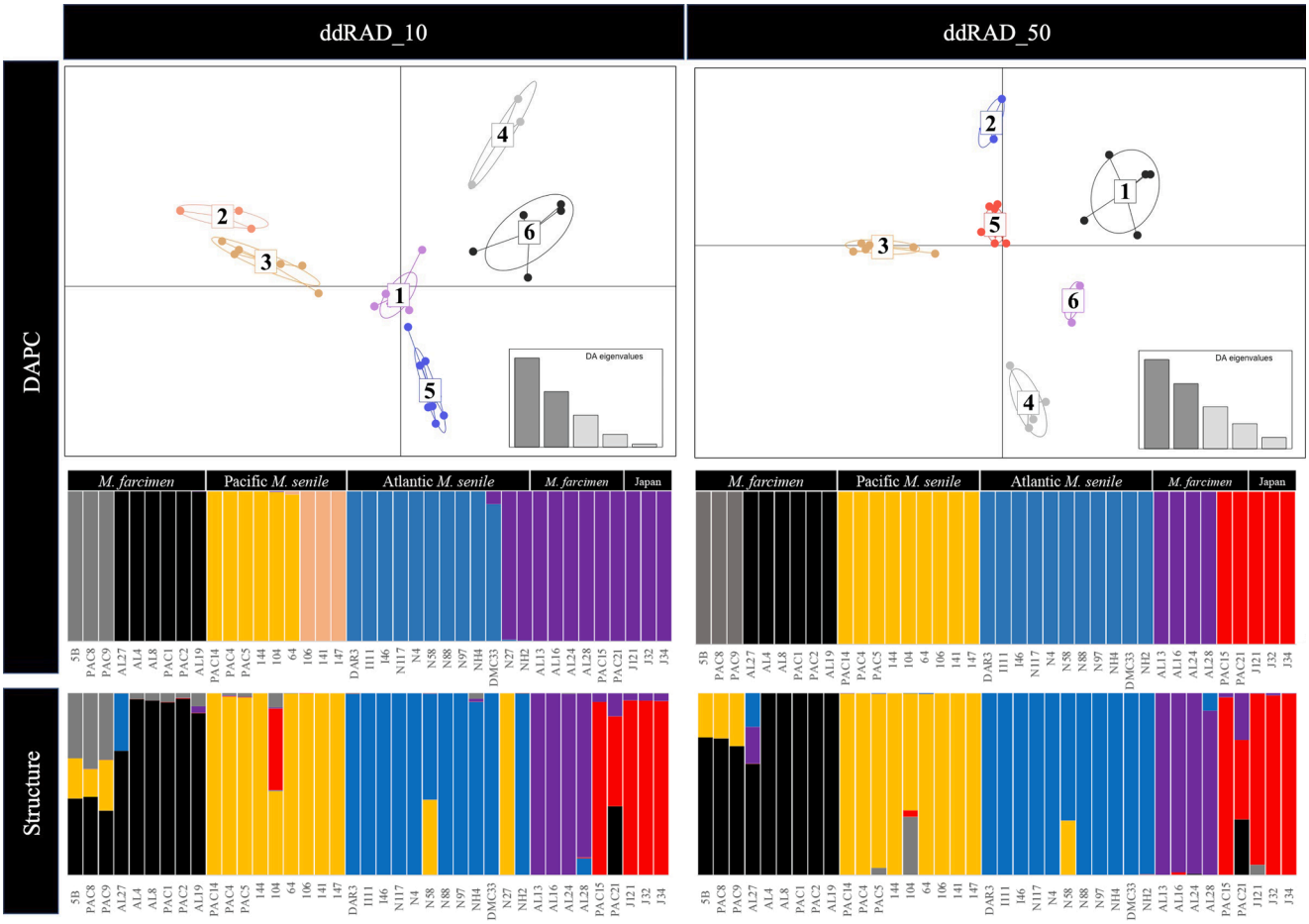

Fig. 3. DAPC scatter and cluster plots of the ddRAD_10 and ddRAD_50 SNP datasets. Both datasets retained six PCs, and are based on six clusters for comparison. Structure plot (K = 6) included below with colors most closely matching the major clusters resolved in the DAPC.

anemones but lacked support and resolution for relationships at the familial, intergeneric and interspecific level. Larson and Daly (2016) were able to provide support at the family level but had reduced support at the intergeneric level. Within the Aiptasiidae, Grajales & Rodríguez (2016) recovered major genera with relatively strong support and distinguished *Exaiptasia brasiliensis* (Grajales & Rodríguez, 2016) from *Exaiptasia pallida* (Agassiz in Verrill, 1864) but failed to resolve intra-generic relationships in other genera within the family; genomic methods later confirmed the separateness of *E. brasiliensis* from *E, pallida* (Bellis et al. 2018).

4.2. ddRAD sequencing

The ddRAD methods generated the greatest number of potentially useful sites, but the proportion of missing data was high. This commonality across ddRADseq datasets is often overcome by the strength of reduced representation approaches in that these methods are capable of producing large quantities of informative loci. However, in *Metridium*, the fraction of sites that are parsimony informative and usable after processing is much smaller than the raw number of potential SNPs (Table 2). Furthermore, the success rate of this method was lower than any of the other methods in terms of sample success. Though likely due to low quality DNA and allelic dropout, only 41 of 71 samples passed through the iPyrad pipeline with enough loci (>3000) to be retained in the final dataset. Building a ddRAD library requires good quality, high molecular weight DNA, which is not always obtainable from previously collected specimens. Differences in sequencing platforms or lab conditions may contribute to issues while integrating old data sets with newly sequenced individuals. As ddRAD data sets show both high individual and allelic dropout post-processing (e.g., Andrews et al., 2016; Gautier et al., 2013), it is difficult to combine studies or expand a ddRAD data set out to broader phylogenetic investigations at deeper evolutionary scales. Despite these challenges, ddRAD library preparation requires less development in terms of probes, primers, or other technical elements, and it can easily be used in lineages lacking genomic resources. The cost of preparing a library is generally lower (~$40–80 per sample) for ddRAD than sequence-capture (~$75–150 per sample), and it may be quicker to prepare libraries depending on the protocol and method used.

In terms of the resolution of relationships, while the ddRAD trees do support four major groupings (*M. farcimen* + three *M. senile* clades of Atlantic, North American Pacific, and Japan/Bering Sea), they lack support for the positioning of these clades to each other. Although the currently accepted taxonomy is not supported sufficiently, support is relatively high towards the tips (Fig. 2), suggesting that ddRAD is well-suited to population-level and fine-scale analyses in this group. Branch lengths are short and the high within-region support values may be inflated by missing data. The outgroup taxon (*Diadumene lighti*) in the ddRAD datasets retained only 55 loci, which may have contributed to the lack of support at the base of the ingroup. Although it is possible in some cases to extract additional data from ddRAD sequencing events, including nearly complete mitochondria (Stobie et al. 2019), the shared variation we see between samples of *Metridium* and of *Diadumene* suggest that this dataset would be difficult to expand taxonomically to include individuals outside of very closely related species in *Metridium*.

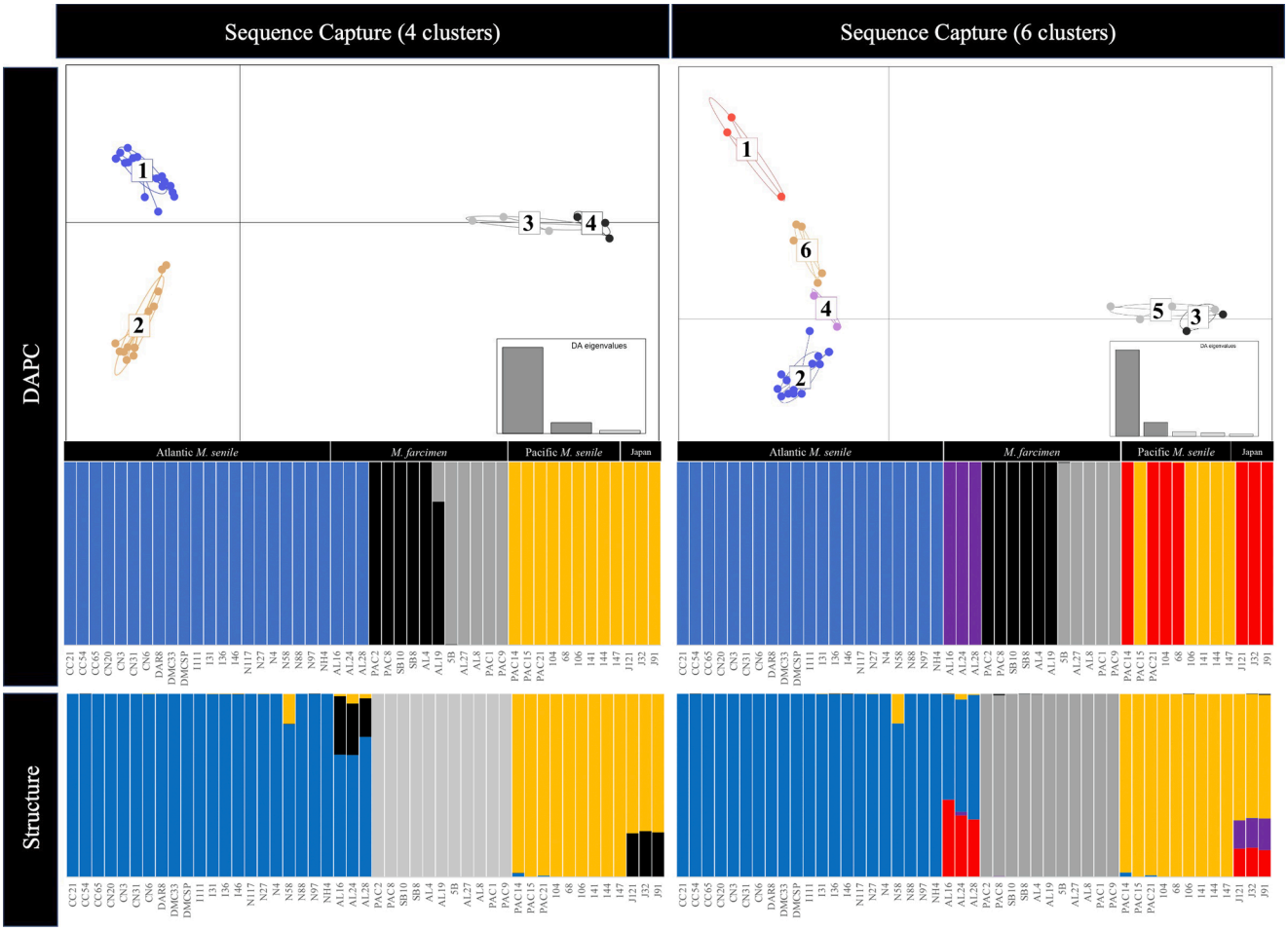

Fig. 4. DAPC scatter and cluster plots of the sequence-capture (SC) SNP dataset for four and six K-clusters. Structure plot (K = 4, K = 6) included below with colors most closely matching the major clusters resolved in the DAPC.

4.3. Sequence-capture

Although we recover fewer loci overall with sequence-capture than with ddRAD, the lower percentage of missing data and increase in PI sites allowed the sequence-capture data to outperform ddRAD in *Metridium*. These results are most apparent when the final filtered number of SNPs are compared between the datasets. Overall, the geographic regions and taxonomic groups were clearly resolved and supported consistently in the sequence-capture results. The well-supported and congruent backbone relationships in both sequence-capture datasets demonstrate that these are suitable for both phylogenetic questions and for the study of populations for actiniarians. Collins et al. (2018), who performed *in silico* comparisons, as well as Harvey et al. (2016), Manthey et al. (2016), and Erickson et al. (2020), found that for their focal taxa, ddRAD and sequence-capture both produced consistent and well supported topologies. In this study for *Metridium*, however, sequence-capture provided higher confidence in the topology than ddRAD.

As with ddRAD, there are important practical and logistical considerations in using sequence-capture. Bait sets are readily available for anthozoans and several other broad taxonomic groups (https://www.ultraconserved.org; Quattrini et al. 2018, Erickson et al. 2020, Cowman et al. 2020); however, designing bait sets requires having genomes and/or transcriptomes available for a suite of taxa within the lineage of interest. As sequence capture targets positions on the genome rather than shearing DNA with enzymes, the protocols are repeatable with greater overlap across efforts, experience less allelic dropout, and retain more individuals after processing and filtering steps. The PHYLUCE pipeline that we used is well-established for processing sequence-capture reads; however, recovering SNPs from sequence-capture data requires an additional step (e.g., Erickson et al. 2020) whereas an unlinked SNP file is a standard output by iPyrad. Though the initial effort and cost are high, the reproducibility of the sample preparation is valuable and the ability to target loci, unlike in ddRAD, enables the expansion out to include deeper relationships, creating the ability to re-use data for multiple studies which increases efficiency particularly in labs that commonly study closely related taxa. Furthermore, there is a potential for single locus discovery followed by directed PCR within the sequence-capture datasets, which can augment loci currently used in systematics of actiniarian sea anemones and further reduce the long-term cost in using targeted loci (see Ramirez-Portilla in review). Finally, this method can be used to obtain genomic data for relatively old (up to 50 yrs,) and fluid-preserved anthozoans in museum collections (Untiedt et al. 2021), highlighting the promise of this approach for integrating type material into systematic research.

4.4. SNP data analysis

The DAPC for both datasets largely confirmed the genetic structure among species and populations seen in the trees. DAPC groups form particularly tight clusters for the sequence capture data, recovering both species and populations. The STRUCTURE plots are also more clearly defined for the sequence-capture datasets. Because of the emphasis on quantity over reducing missing data for ddRAD methods, the SNP data in the ddRAD datasets may be influenced by noise (Leaché et al. 2015) or inconsistent levels of genetic signal related to uneven missing data (see

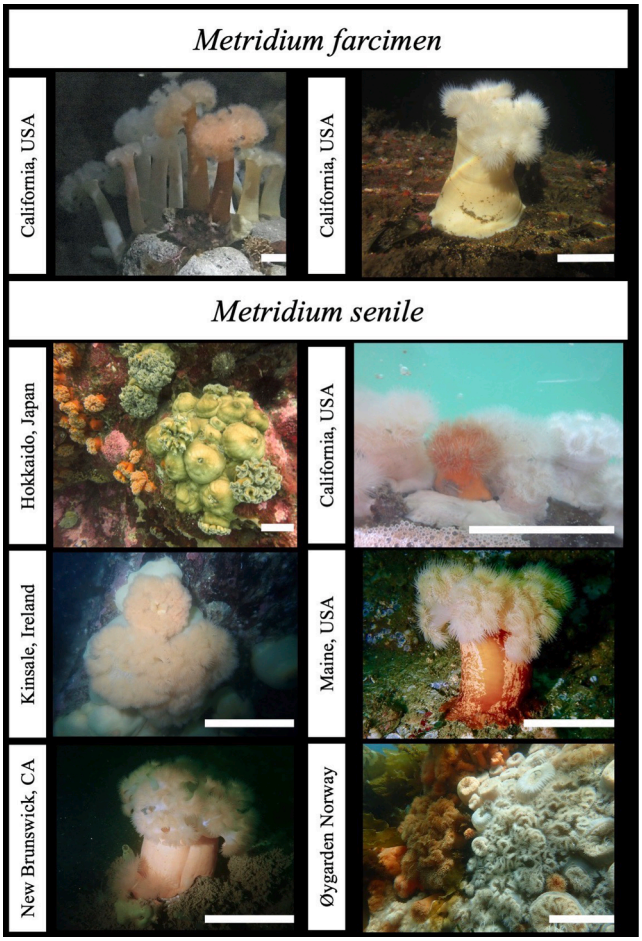

Fig. 5. Photographs of Metridium farcimen and Metridium senile displaying the variation in color, size, and clonal groups. Scale bars represent approximately 10 cm. Photographs by H. Glon.

(Collins et al., 2018), and therefore may show varying membership to each of the major clusters depending on the individual.

4.5. Taxonomic and biogeographic considerations in metridium

Our genomic-scale data serve as a first step towards resolving phylogenetic relationships and biogeography of *Metridium* across the globe. We recognize that some of the variation in topologies may be due to slight differences in geographic sampling between datasets and the limited sampling (though approximately equal in each dataset) relative to the wide distribution range of *Metridium*. More individuals from additional locations are needed to fully resolve phylogeographic patterns and relationships within this genus. Furthermore, we acknowledge that coalescent-based approaches are required to quantify and delimit species boundaries.

The contiguous habitat and ranges of the putative species of *Metridium* has historically complicated assessing species boundaries. The name *M. senile* has been used for populations on both the Northwestern and Northeastern Atlantic coasts and for populations in the North Pacific. We sampled broadly across oceans to test preliminary affinities and distinctions between Atlantic and Pacific populations and between large and solitary *Metridium* and smaller and clonal *Metridium*. Despite our small sample size, the results clearly differentiate the large and solitary form from the smaller and/or clonal forms, which supports the distinctiveness of *M. farcimen*. This distinction is widely supported across data sets and methods. We also find clear support for the separation of the smaller, clonal *Metridium (M. senile)* into Pacific and Atlantic clades.

Based on our limited sampling for this study, we do not recover any distinction between the two smaller, clonal *Metridium* on the Pacific coast of North America, contradicting the expectation of a distinction based on allozymes (see Bucklin & Hedgecock 1982). Although the major clade that corresponds to *M. farcimen* is largely upheld in both the sequence-capture and ddRAD datasets, we see a distinction between those collected south of the Aleutians through California and a small number of samples collected in the Bering Sea that had been presumed upon collection to be *M. farcimen* and now appear to be *M. senile*. The affinity of samples from shallow waters in Alaska (ADK7, ADK13, MEFI) is inconsistent across data sets. The placement of the individuals from Japan differs between the ddRAD data and sequence-capture analyses, which requires greater sampling to determine whether they are more closely related to the Pacific *M. senile*, *M. farcimen*, or Bering Sea *Metridium*.

The Northern Hemisphere habitats in which species of *Metridium* are found share deep-water connections through the Arctic, but habitat availability and connectivity were likely different in the past. Connections between Pacific and Atlantic populations are possible given present distributions and have historical precedent. The passage through the Bering Strait opened during the early to mid-Pliocene (~3.5 and 5.5 MYA), allowing exchange from the Pacific to the Atlantic (Durham & Macneil, 1967; Marincovich and Gladenkov, 2001; Gladenkov et al., 2002), and slowed or halted exchange between oceans until after the last glacial maximum, around 13–11 ky ago (Kaufman and Brigham-Grette 1993, Anderson et al. 2006, Laakkonen et al. 2020). Based on our STRUCTURE results for the sequence-capture data and the ddRAD DAPC results, the Japan and Bering Sea *Metridium* populations may represent two intermediate steps connecting the Eastern Pacific and the Atlantic *M. senile*. We interpret the directionality of dispersal to be from the Pacific to the Atlantic because in every tree, a Pacific-only clade (or series of clades) is sister to the clade comprised of Atlantic and Pacific populations of smaller and clonal *M. senile*. However, these biogeographic patterns warrant further investigation with increased sampling.

The mixing of individuals within the Atlantic clade (New England, Ireland, and Norway), particularly in the ddRAD datasets, does not support a distinction between *M. senile* and *M. dianthus* as had been proposed in the past and which is implied by the use of separate names for these populations. The intermingling of Western and Eastern Atlantic populations of *Metridium* is also contrary to the findings of Bucklin (1985) but does support her conclusion that *M. senile* and *M. dianthus* are ecophenotypes and not distinct species. The propensity of *Metridium* individuals for being transported (see Glon et al. 2020) has undoubtedly impacted population structure in the Atlantic. Anthropogenic transport may also explain two individuals (Hammond Marina, OR and Coos Bay, OR) that are resolved as sister to the Japan clade; these locations see high shipping traffic from Japan, and in Coos Bay a ship has been documented as carrying *M. senile* larvae in the ballast water (Carlton & Geller 1993; see Glon et al. 2020).

5. Conclusions

Both ddRAD and sequence-capture approaches are capable of distinguishing *M. farcimen* from *M. senile* and show promise for differentiating populations within *M. senile*. The preliminary insights discussed above support the recognition of two species, with a potentially complex history of *M. senile* in the Pacific Ocean. At least for the taxa studied here, the sequence-capture results better distinguish groups of populations and have higher support for intermediate nodes than the ddRAD results. The ability of sequence-capture approaches to recover a highly resolved and supported topology within *Metridium*, a group that has been historically difficult taxonomically and biogeographically, underscores the broader utility of the anthozoan target-capture bait set (Quattrini et al. 2018, Cowman et al. 2020).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2021.107233.

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