

A novel target-enriched multilocus assay for sponges (Porifera): Red Sea Haplosclerida (Demospongiae) as a test case

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Abstract

With declining biodiversity worldwide, a better understanding of species diversity and their relationships is imperative for conservation and management efforts. Marine sponges are species-rich ecological key players on coral reefs, but their species diversity is still poorly understood. This is particularly true for the demosponge order Haplosclerida, whose systematic relationships are contentious due to the incongruencies between morphological and molecular phylogenetic hypotheses. The single gene markers applied in previous studies did not resolve these discrepancies. Hence, there is a high need for a genome-wide approach to derive a phylogenetically robust classification and understand this group's evolutionary relationships. To this end, we developed a target enrichment-based multilocus probe assay for the order Haplosclerida using transcriptomic data. This probe assay consists of 20,000 enrichment probes targeting 2956 ultraconserved elements in coding (i.e. exon) regions across the genome and was tested on 26 haplosclerid specimens from the Red Sea. Our target-enrichment approach correctly placed our samples in a well-supported phylogeny, in agreement with previous haplosclerid molecular phylogenies. Our results demonstrate the applicability of high-resolution genomic methods in a systematically complex marine invertebrate group and provide a promising approach for robust phylogenies of Haplosclerida. Subsequently, this will lead to biologically unambiguous taxonomic revisions, better interpretations of biological and ecological observations and new avenues for applied research, conservation and managing declining marine diversity.

KEYWORDS

bait design, exon, phylogenetic markers, target capture, ultraconserved element

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1 | INTRODUCTION

Worldwide biodiversity is declining at an unprecedented rate; thus, there is an urgent need for novel approaches to characterise biodiversity and tools for faster biomonitoring that could aid conservation efforts (Formenti et al., 2022). Sponges (Phylum Porifera) are a diverse animal taxon with wide abundance in almost all aquatic habitats wherein they fulfil a multitude of ecological functions, such as providing nutrients to higher trophic levels and contributing to the habitat heterogeneity of coral reefs (Bell, 2008; De Goeij et al., 2013). Despite their ecological importance, the biodiversity of sponges is still poorly understood but essential for a better interpretation of biological and ecological observations.

Here, we develop a multilocus probe assay based on target enrichment of ultraconserved elements (UCEs) for the first time for sponges. This assay can capture genome-wide markers for sponges to allow phylogenetic reconstructions at different taxonomic levels and has high potential to aid in (rapid) species identification and discovery in biodiversity surveys. UCEs are highly conserved regions in the genome that can easily be 'captured' using complementary synthetic DNA or RNA baits and can provide phylogenetically informative sites at the flanking regions of the UCEs but also within the UCEs (Faircloth et al., 2012). These informative sites can be used to infer the phylogenetic histories of systematically challenging groups across shallow and deep timescales (Faircloth et al., 2012; Faircloth et al., 2013; McCormack et al., 2012; Quattrini et al., 2018). UCEs have successfully resolved molecular phylogenetic relationships in taxonomically complex marine invertebrate taxa, such as molluscs (Goulding et al., 2023; Moles & Giribet, 2020), anthozoans (Cowman et al., 2020; Quattrini et al., 2018) and echinoderms (Hugall et al., 2015). The phylogenetic relationships of various groups within the phylum Porifera, particularly those belonging to the order Haplosclerida (Class Demospongiae), are still poorly understood due to the discrepancy between the morphological and molecular hypotheses proposed for this group (McCormack et al., 2002; Redmond et al., 2011, 2013). For taxonomically complex groups such as Haplosclerida, UCEs are potentially more informative for reconstructing molecular phylogenies than a single or handful of gene markers and are a promising tool to resolve the intra-order phylogenetic relationships. In addition, UCEs provide enough information for further downstream processing and can be leveraged for unambiguous species identification, delimitation and discovery in biodiversity surveys (Erickson et al., 2021). For complex holobionts like sponges, UCEs are preferred over other methods, such as restriction-site associated DNA (RAD) sequencing. This is due to the high abundance of sponge-associated microbes and other commensal DNA co-extracted with the sponge's DNA (Vargas et al., 2012), where the enzymatically produced DNA fragments are often of unknown identity. Importantly, in contrast to RAD-based methods, target capturing provides access to non-anonymous genomic markers of known homology.

We test our newly developed multilocus probe assay, specifically designed to capture the broad diversity of sponges belonging

to the demosponge order Haplosclerida, with specimens collected from the Red Sea. To date, very little has been documented on the sponge diversity of the Red Sea, which limits our understanding of the sponge communities present, their ecology, and how they will adapt under future climate conditions (Wooster et al., 2019). Haplosclerida is one of the most speciose sponge orders, with >800 species described to date (Van Soest et al., 2012). Furthermore, they are highly abundant in many tropical coral reef systems (e.g. Erpenbeck et al., 2016), which are under increasing pressure due to anthropogenic stressors and climate change. Currently, haplosclerids form a yet unresolved taxonomic conundrum, one of the largest and most challenging in poriferan taxonomy and systematics (Van Soest et al., 2012). First, because the lack of diagnostic characters makes unambiguous species identification and distinction very challenging in Haplosclerida (McCormack et al., 2002), and second, because molecular phylogenies demonstrate para- or polyphyly of all morphologically defined families and most genera (McCormack et al., 2002; Redmond et al., 2011, 2013), and in a few cases also the non-monophyly of species (Redmond et al., 2011, 2013). However, although incongruent with supra-specific morphological definitions, these molecular phylogenies converge on robust clades named A-E (Redmond et al., 2011, 2013).

With access to a broad range of taxa belonging to four families within Red Sea haplosclerids, we have a test case to assess the order-wide applicability of our newly developed multilocus probe assay. We demonstrate that the multilocus probe assay successfully captures genome-wide loci of the Red Sea sponges and that those loci have sufficient phylogenetic resolution to recover the clades previously established in molecular phylogenies of the order Haplosclerida. Furthermore, we illustrate the broad applicability of the designed probe assay, as it also captured sufficient loci of other demosponges to construct a phylogeny reflecting the ordinal relationships of this diverse group.

2 | MATERIALS AND METHODS

2.1 | Sampling and identification of Red Sea haplosclerids

In the scope of the Red Sea Biodiversity Project (Senckenberg Research Institute; King Abdulaziz University (KAU), Jeddah, Saudi Arabia) and sampling campaigns led by the King Abdullah University of Science and Technology (KAUST), Thuwal, Saudi Arabia, sponge specimens were collected in the northern, central and southern regions of Saudi Arabia's Red Sea area between 2012 and 2017 (Figure 1; Table 1). The sponges were collected between 1 and 43 m depth using SCUBA or by hand-picking in shallow waters, preliminarily sorted, photographed and preserved in 95% EtOH until further processing (Erpenbeck et al., 2016).

DNA was extracted from a small piece of sponge tissue for each sample using a NucleoSpin® Tissue kit (Macherey-Nagel GmbH & Co. KG, Germany) following the manufacturer's protocol for

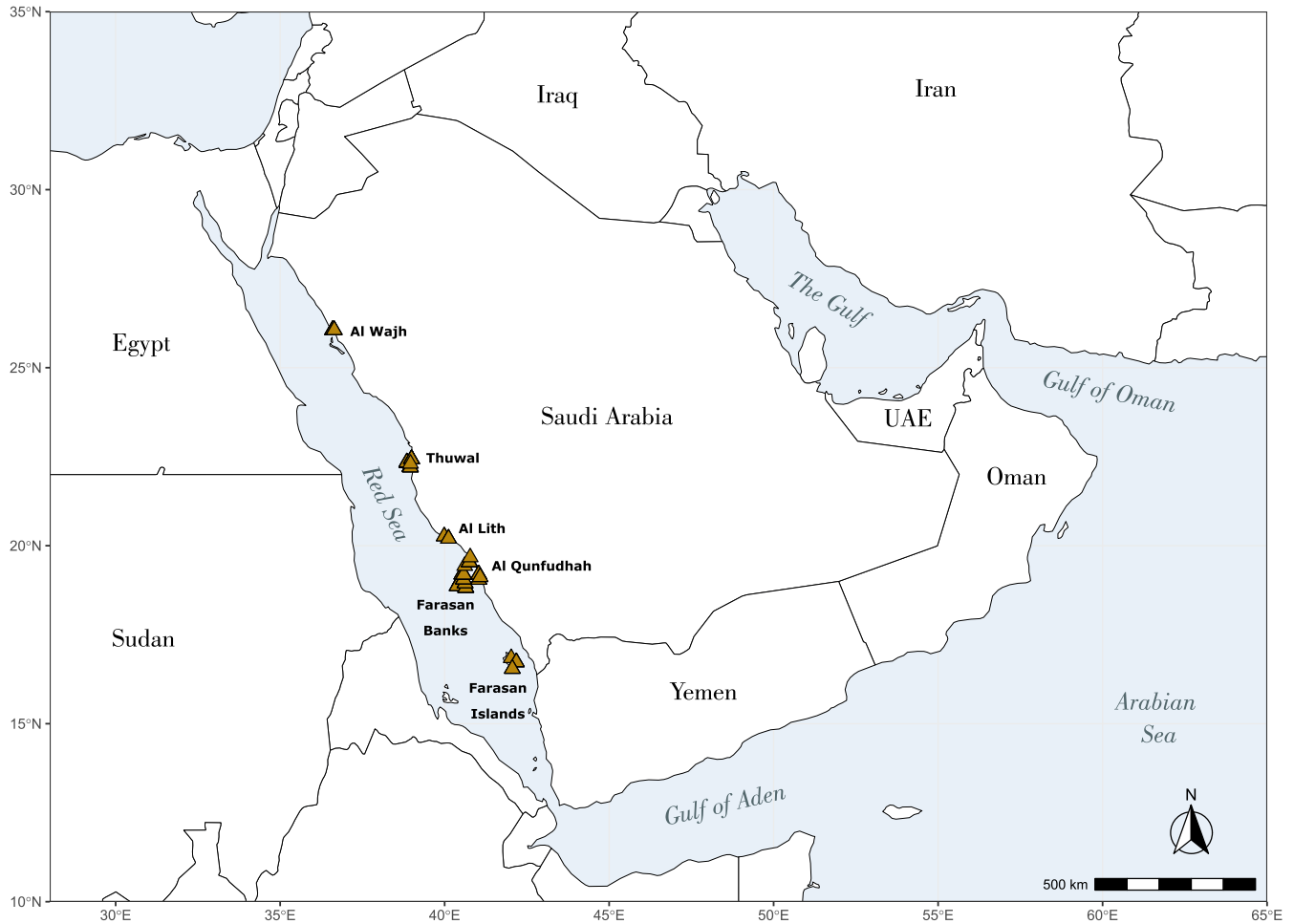


FIGURE 1 Localities (triangles) from which the 26 haplosclerid specimens were collected between 2012 and 2017 in the Red Sea.

genomic DNA from tissues. To screen for haplosclerid samples in the Red Sea Porifera collection, the V9 region of the 18S rRNA was amplified using Illumina compatible primers 18S-1391F (5' - AATGA TACGGCGACCACCGAGATCTACAC, NNNNNNNN, TATGGTAATT, GT, GTACACACCGCCCGTC - 3') and 18S-EukBr (5' - CAAGC AGAAGACGGCATAACGAGAT, NNNNNNNN, AGTCAGTCAG, CC, TGATCCTTCTGCAGTTACCTAC - 3') (see Pichler et al., 2018). Briefly, the primers contained an Illumina flow cell adapter (either i5: AATGATACGGCGACCACCGAGATCTACAC for Fwd or i7: CAAGCAGAAGACGGCATAACGAGAT for Rev) followed by an 8-nt unique index (*N* region; Table S1) to enable identification of individual samples, a 10-nt pad sequence, a 2-nt linker (GT, CC) and the primer for the 18S V9 region (underlined). PCR was performed in aliquots with a final reaction volume of 15 μ L containing 7.5 μ L GoTaq® G2 hot start master mix (Promega), 4.5 μ L dH₂O, 1 μ L 18S Barcoded-Fwd-Primer (5 μ M), 1 μ L 18S Barcoded-Rev-Primer (5 μ M) and 1 μ L sponge DNA (10 ng μ L⁻¹). The thermocycling conditions were 95°C for 3 min denaturation followed by 34 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 30 s and a final extension of 72°C for 5 min and performed on the Biometra TProfessional Thermocycler (Biometra, Göttingen, Germany). The PCR products were loaded on 1% agarose gels, and products of the expected

size (~270-bp) were purified using a NucleoSpin® Gel and PCR Clean-up kit, subsequently quantified using the Qubit dsDNA high-sensitivity (HS) kit (ThermoFisher), diluted to 1 nM, pooled and sequenced (150PE) on an Illumina MiniSeq. The resulting demultiplexed Illumina reads were further processed in VSEARCH version 2.5.0 (Rognes et al., 2016) to obtain $\geq 97\%$ similarity 18S V9 rRNA operational taxonomic units (OTUs) for each specimen. Because the sponge samples include commensal DNA, and the primers used are eukaryotic universal primers, the retrieved OTUs were pre-sorted for each of the haplosclerid specimens, followed by pre-screening the five representative sequences of the most abundant OTUs as a query for a BLAST search (blastn, <http://www.ncbi.nlm.nih.gov/BLAST/>) against NCBI GenBank. The most abundant 18S OTUs were blasted against the non-redundant nucleotide database, and the taxonomy of the most similar sequence to the query was used as a taxonomic proxy for the query sequence. Eventually, 26 haplosclerid specimens were selected and morphologically identified (up to species level, if possible). Briefly, the tissue and spicules of the specimens were prepared for light and scanning electron microscopy (SEM), respectively, using the methods described in Van Soest & Hooper (2005). Spicule measurements were carried out ($n=25$) and the range and mean of

TABLE 1 Specimens included for the *in silico* test and phylogenetic analysis of this study.

Family	Species	ID	SMF ID	Latitude	Longitude	Region	Location
Chalinidae	<i>Chalinula aff. milnei</i>	SNSB-BSPG.GW4433		19°32'26" N	40°45'06" E	Farasan Banks	Safiq Island
	<i>Chalinula aff. milnei</i>	SNSB-BSPG.GW6134	SMF.12227	20°14'44" N	40°00'03" E	Al Lith, NE	RSS1-2012St.51
	<i>Chalinula sp.</i>	SNSB-BSPG.GW3005		22°12'59" N	38°56'55" E	Thuwal reefs	Shi'b Esfenj
	<i>Chalinula sp.</i>	SNSB-BSPG.GW4226		18°49'05" N	40°38'32" E	Farasan Banks	Miskah Island
	<i>Chalinula sp.</i>	SNSB-BSPG.GW4148		19°00'60" N	40°28'08" E	Farasan Banks	Murabit Al Khali
	<i>Chalinula sp.</i>	SNSB-BSPG.GW4285		19°03'23" N	40°34'26" E	Farasan Banks	Awol Marah
	<i>Cladocroce sp.</i>	SNSB-BSPG.GW5878	SMF.12229	16°43'08" N	42°03'54" E	Farasan Islands	RSS1-2012St.10
	<i>Haliclona sp.</i>	SNSB-BSPG.GW3018		22°12'59" N	38°56'55" E	Thuwal reefs	Shi'b Esfenj
Callyspongiidae	<i>Haliclona sp.</i>	SNSB-BSPG.GW3108		22°20'31" N	38°51'15" E	Thuwal reefs	Shi'b Nazar, NE
	<i>Arenosciera arabica</i>	SNSB-BSPG.GW4246		18°56'42" N	40°37'11" E	Farasan Banks	Tidhkar Island
	<i>Arenosciera arabica</i>	SNSB-BSPG.GW4146		19°00'60" N	40°28'08" E	Farasan Banks	Murabit Al Khali
	<i>Arenosciera arabica</i>	SNSB-BSPG.GW6018	SMF.12222	19°02'41" N	41°02'13" E	Qunfudhah	RSS1-2012St. 46
	<i>Callyspongia (Callyspongia) siphonella</i>	SNSB-BSPG.GW3123		22°25'58" N	38°59'36" E	Thuwal reefs	Qita-al-Kirsh, W
	<i>Callyspongia (Callyspongia) siphonella</i>	SNSB-BSPG.GW3010		22°12'59" N	38°56'55" E	Thuwal reefs	Shi'b Esfenj
	<i>Callyspongia (Euplaccella) sp.</i>	SNSB-BSPG.GW4436		19°32'26" N	40°45'06" E	Farasan Banks	Safiq Island
	<i>Callyspongia (Euplaccella) sp.</i>	SNSB-BSPG.GW6059	SMF.12228	19°11'41" N	41°02'19" E	Qunfudhah, N	RSS1-2012St.47
	<i>Amphimedon aff. jalae</i>	SNSB-BSPG.GW5936	SMF.12223	19°43'43" N	42°10'46" E	Farasan Islands	RSS1-2012St. 28
	<i>Amphimedon aff. jalae</i>	SNSB-BSPG.GW5998	SMF.12224	16°37'08" N	41°56'02" E	Farasan Islands	RSS1-2012St.37
Niphatidae	<i>Amphimedon chloros</i>	SNSB-BSPG.GW4507	SMF.12230	19°42'00" N	40°42'23" E	Farasan Banks	Ghubbat Channel, N
	<i>Amphimedon dinae</i>	SNSB-BSPG.GW6114	SMF.12226	20°15'49" N	39°59'07" E	Al Lith, NE	RSS1-2012St.50
	<i>Amphimedon sp.</i>	SNSB-BSPG.GW5894	SMF.12225	16°43'08" N	42°03'54" E	Farasan Islands	RSS1-2012St.10
	<i>Neopetrosia sp.</i>	SNSB-BSPG.GW3257		26°03'30" N	36°35'41" E	Saudi-Arabia	Al-Wajh
	<i>Petrosia (Petrosia) aff. spheroida</i>	SNSB-BSPG.GW6015	SMF.12221	19°02'41" N	41°02'13" E	Qunfudhah	RSS1-2012St.46
Petrosiidae	<i>Xestospongia testudinaria</i>	SNSB-BSPG.GW3122		22°25'58" N	38°59'36" E	Thuwal reefs	Qita-al-Kirsh, W
	<i>Xestospongia testudinaria</i>	SNSB-BSPG.GW4191		18°52'04" N	40°22'55" E	Farasan Banks	Shib Maras
	<i>Xestospongia testudinaria</i>	SNSB-BSPG.GW4374		19°17'48" N	40°41'04" E	Farasan Banks	Two Wall

Note: Ten specimens were collected in the framework of the Red Sea Biodiversity Project (Senckenberg Research Institute; King Abdulaziz University, KAU, Saudi Arabia) and are part of the collection of Senckenberg Research Institute in Frankfurt (SMF), Germany. The other 16 specimens were collected under the campaigns led by the King Abdullah University of Science and Technology (KAUST) Thuwal, Saudi Arabia, in the Red Sea. Samples were collected between 2012 and 2017.

the spicule's length and width were calculated. The systematic assignment of the haplosclerid specimens followed the 'Systema Porifera' (Hooper & Van Soest, 2002). In addition, haplosclerid species descriptions of the Red Sea were consulted (Helmy & Van Soest, 2005; Ilan et al., 2004; Keller, 1889; Lévi, 1965; Vacelet et al., 2001).

2.2 | Preparation of transcriptome data prior bait design

Raw transcriptome sequence data were obtained from public databases (Table 2). These data were quality-filtered and assembled using TransPi (Rivera-Vicéns et al., 2022). The transcriptome assemblies were ranked based on the number of contigs, mean contig length (bp), N50 value (bp) and completeness as judged by searches against the Benchmarking Universal Single-Copy Orthologs (BUSCOs) using rnaQuast v2.0.1 and BUSCO v3 and v4 respectively. In addition, to ensure that the assemblies fell within the correct demosponge order, the full-length 18S rRNA gene was searched for in each transcriptome and used to infer a maximum likelihood (ML) phylogeny (Figure S1) using RAxML 8.2.12 (Stamatakis, 2014).

2.3 | Conserved locus identification and bait design

For bait design, a modified workflow based on the online tutorial IV of the open-source software PHYLUCE version 1.7.1 (Faircloth, 2016) (<https://phyluce.readthedocs.io/en/latest/tutorials/tutorial-4.html>) and Quattrini et al. (2018) was applied to the transcriptomic data. The programs and parameters used for bait design are deposited in our project repository (<https://github.com/PalMuc/HaploTC>). The final number of retrieved probes depends on the selected base transcriptome and the number of taxa in which candidate loci can be found (e.g. Gustafson et al., 2019). Therefore, each design was considered a separate bait set and given a version number to keep track of the specificity of the retrieved probes during downstream analysis (Figure S2; Table S2). This resulted in a total of 137 bait sets, including 48 bait sets using only transcriptome data of haplosclerid sponges and 99 bait sets that included transcriptomes of haplosclerid sponges and other demosponges as outgroups. For the bait sets, including transcriptome data of haplosclerid sponges (from here further referred to as 'haplo-only bait sets'), we used transcriptomes from haplosclerids representing the four clades previously identified within this group. Specifically, we used transcriptomic resources of *Haliclona (Reniera) cinerea*, *Haliclona (Reniera) tubifera* and *Haliclona (Haliclona) oculata* (Clade A; Guzman & Conaco, 2016; Redmond et al., 2011, 2013), *Amphimedon queenslandica* and *Haliclona (Haliclona) simulans* (Clade B; Redmond et al., 2011, 2013), *Haliclona (Rhizoniera) indistincta* and *Haliclona (Rhizoniera) viscosa* (Clade C; Sandoval & McCormack, 2022) and *Neopetrosia*

compacta (Clade E; Erpenbeck et al., 2023). Because the available transcriptome data of the Haplosclerida are limited to a few species from four clades, we designed additional bait sets including transcriptome data of other demosponges as outgroups (from here further referred to as 'haplo+outgroup bait sets') to broaden the applicability of the multilocus probe assay. For each of the haplo+outgroup bait sets, we used the eight aforementioned haplosclerid transcriptomes plus one non-haplosclerid demosponge transcriptome as an outgroup, with a single outgroup transcriptome included from the following list for bait design, and then bait design repeated with the other outgroups individually: the pocilosclerids *Crella (Crella) elegans*, *Latrunculia (Latrunculia) apicalis*, *Mycale (Carmia) phyllophila* and *Tedania (Tedania) anhelans*, the scopalinid *Scopalina* sp., the tethyid *Tethya wilhelma*, the tetractinellid *Geodia atlantica*, (all Heteroscleromorpha, that is, the same subclass as Order Haplosclerida) and the dictyoceratids *Pleraplysilla spinifera*, *Lendenfeldia chondrodes*, *Phyllospongia foliascens* and *Vaceletia* sp. from Subclass Keratosa.

To search for ultraconserved element (UCE) loci across different subsets of transcriptomes, 100-bp paired reads were simulated for each taxon using ART_ILLUMINA (Huang et al., 2012) and aligned to a reference or 'base' transcriptome using stampy version 1.0.32 (Lunter & Goodson, 2011) with a sequence divergence of ≤ 5 and the resulting BAM output files were filtered for unmapped reads using samtools (Li et al., 2009). This was followed by converting the BAM files into BED format using bedtools (Quinlan & Hall, 2010), sorting by contig and position along that contig and merging proximate or overlapping putative conserved regions (of the simulated reads). Any putative conserved intervals shared between the taxa and the selected base transcriptome and/or repetitive regions were removed. Also, intervals shorter than 80-bp, $>25\%$ of the regions were masked, and/or that had ambiguous (N or X) bases were removed using the command `phyluce_probe_strip_masked_loci_from_set`. The identified UCes were extracted with a buffer region of 120-bp using `phyluce_probe_get_genome_sequences_from_bed`, and temporary bait sets targeting the respective UCes were designed with `phyluce_probe_get_tiled_probes` with the tiling density set to two. Potentially problematic baits—those with a $>25\%$ repeat content and a GC content $>70\%$ or $<30\%$ —and duplicates (i.e. sequences that were $\geq 50\%$ identical over $\geq 50\%$ of their length) were removed from the temporary bait set using `phyluce_probe_easy_lastz` followed by `phyluce_probe_remove_duplicate_hits_from_probes_using_lastz`. To confirm whether the UCes of the temporary bait sets could be located in the exemplar taxa, the baits were aligned against each of the transcriptomes using `phyluce_probe_run_multiple_lastzs_sqlite`, with an identity value of 70% and a default value for minimum coverage of 83% (Quattrini et al., 2018). Baits matching multiple contigs were removed. The resulting regions were extracted from each transcriptome sequence, buffering each locus to 140-bp, using `phyluce_probe_slice_sequence_from_genomes`.

The final bait set to target haplosclerid UCes was designed using `phyluce_probe_get_tiled_probe_from_multiple_inputs`. The baits were designed to be 80-bp long with a two-times tiling density and

TABLE 2 Transcriptome data of demosponges belonging to six orders used for bait design and *in-silico* testing. Included are the values obtained after quality control obtained from Transcriptome Analysis Pipeline (TransPi) (Rivera-Vicéns et al., 2022).

Order	Species	# Contigs	# mean contig length (bp)	# C: BUSCOs (%)	# Sc: BUSCOs (%)	N50	Reference
Haplosclerida	<i>Haliclona (Reniera) cinerea</i>	59,469	822	90.0	62.9	1857	Sandoval & McCormack (2022)
Haplosclerida	<i>Haliclona (Rhizoniera) indistincta</i>	42,407	935	90.3	64.5	2170	Sandoval & McCormack (2022)
Haplosclerida	<i>Haliclona (Haliclona) oculata</i>	65,478	777	89.1	65.2	1658	Sandoval & McCormack (2022)
Haplosclerida	<i>Haliclona (Haliclona) simulans</i>	51,464	844	90.4	56.1	1717	Sandoval & McCormack (2022)
Haplosclerida	<i>Haliclona (Rhizoniera) viscosa</i>	42,122	910	87.6	64.4	1998	Sandoval & McCormack (2022)
Haplosclerida	<i>Haliclona (Reniera) tubifera</i>	90,149	806	88.5	57.5	2125	(Guzman & Conaco, 2016)
Haplosclerida	<i>Amphimedon queenslandica</i>	83,671	725	88.2	56.6	1487	Srivastava et al. (2010)
Haplosclerida	<i>Neopetrosia compacta</i>	184,473	627	92.3	58.4	1069	Posadas et al. (2022)
Poecilosclerida	<i>Crella (Crella) elegans</i>	139,190	683	81.7	64.8	1223	(Pérez-Porro et al., 2013)
Poecilosclerida	<i>Tedania (Tedania) anhelans</i>	40,426	985	85.9	63.6	1973	Simion et al. (2017)
Poecilosclerida	<i>Mycale (Carmia) phyllophila</i>	182,491	557	84.5	71.9	894	Qiu et al. (2015)
Poecilosclerida	<i>Latrunculia (Latrunculia) apicalis</i>	19,393	1066	79.1	74	1612	Whelan et al. (2015)
Tethyida	<i>Tethya wilhelma</i>	53,470	894	89.9	71.2	2072	Francis et al. (2017)
Tetractinellida	<i>Geodia atlantica</i>	215,865	521	76.9	62.1	659	Koutsouveli et al. (2020)
Scopalinida	<i>Scopalina</i> sp.	39,282	839	83.7	72.6	1660	Diéz-Vives et al. (2017)
Dictyoceratida	<i>Vaceletia</i> sp.	72,796	585	78.2	63.4	940	Germer et al. (2017)
Dictyoceratida	<i>Plerapsylla spinifera</i>	60,078	788	90.7	64.8	1958	Simion et al. (2017)
Dictyoceratida	<i>Phyllospongia foliascens</i>	138,582	659	89.1	62.8	1433	Strehlow et al. (2021)
Dictyoceratida	<i>Lendenfeldia chondroides</i>	36,210	989	90.8	75.3	2030	Vargas et al. (2023)

40-bp overlap in the middle (two baits), GC content between 30 and 70% range and <25% masked bases. Duplicate baits (i.e. those that are $\geq 50\%$ identical over $\geq 50\%$ of their length) were removed using `phyluce_probe_easy_lastz` and `phyluce_probe_remove_duplicate_hits_from_probes_using_lastz` and assigned a UCE-code to uniquely identify the bait sets generated by the base transcriptome used and taxonomic specificity (i.e. haplo-only versus haplo+outgroup). The probes retrieved from the different bait set versions were merged, followed by filtering for duplicates. Eventually, from this final bait set, 20,000 baits were selected randomly for synthesis (from here on, further referred to as 'multilocus probe assay').

2.4 | *In silico* test of the bait set

The designed baits were mapped against 39 transcriptomes from various demosponges (Table S3) to assess whether the multilocus probe assay could successfully and consistently capture haplosclerid loci. We used `phyluce_assembly_match_contigs_to_probes` with the minimum coverage and identity set to 85%. This was followed by extracting the UCE loci using `phyluce_assembly_get_match_counts` to create the initial list of targeted loci. The targeted loci were then aligned with MAFFT (Katoh et al., 2002) using `phyluce_align_seqcap_align`, including 39 taxa. The conserved locus alignments were trimmed with GBlocks (Castresana, 2000; Talavera & Castresana, 2007) using `phyluce_align_remove_locus_name_from_files` with default parameters. This was followed by creating a data matrix with 35% completeness using `phyluce_align_get_only_loci_with_min_taxa`. The resulting alignments were concatenated into separate supermatrices using `phyluce_align_concatenate_alignments`. The resulting concatenated alignments were analysed with RAxML 8.2.12, with rapid bootstrapping (GTRGAMMAX model, 1000 bootstrap replicates) and RevBayes version 1.2.1 (Höhna et al., 2016) with 200,000 generations and 25% burn-in, using the same model settings as the RAxML analysis. A detailed overview of the steps and assemblies can be found in our project repository (<https://github.com/PalMuc/HaploTC>).

2.5 | *In vitro* test of the bait set

Following the *in silico* test, biotinylated RNA baits were synthesised using the myBaits® Custom (1–20,000) target capture kit by Daicel Arbor BioSciences (Ann Arbor, MI, USA). Prior to the *in vitro* test, the initial concentration of each sample was measured with a Qubit 2.0 fluorometer, and libraries were prepared using a modified protocol of the xGen™ ssDNA & Low-Input DNA Prep (96 rxn) library kit (Supplementary Information I). Briefly, we used half the reaction mix for the adaptase, extension, ligation and dual indexing PCR steps of the library preparation protocol. Following the manufacturer's protocol, we quantified and quality-controlled the libraries using a Qubit 2.0 fluorometer and a Bioanalyzer 2100 in High Sensitivity DNA mode and amplified

the genomic DNA libraries to obtain the required 250ng for hybridisation. Hybridisation and target capture enrichment were performed using pools of eight libraries and the MyBaits target enrichment standard protocol. The target-enriched libraries were 150PE sequenced in an Illumina MiniSeq in high-throughput mode. We sequenced up to 32 libraries (i.e. four target capture pools) per high-throughput MiniSeq run.

2.6 | Analysis of the sequenced enriched libraries

Upon sequencing, the demultiplexed Illumina raw reads were checked for adapter contamination and low-quality bases and trimmed using `fastp` (Chen et al., 2018). The cleaned reads were then further processed using a modified workflow of PHYLUCE: Tutorial I: UCE Phylogenomics (<https://phyluce.readthedocs.io/en/latest/tutorials/tutorial-1.html>) (Faircloth, 2016; Faircloth et al., 2012). The data were assembled using the `phyluce_assembly_assembly_spades` program, followed by looking for matches between the assembled contigs and UCE bait sequences (85% identity, 85% coverage) using `phyluce_assembly_match_contigs_to_probes`. Then, the loci were extracted using `phyluce_assembly_get_match_counts`, followed by `phyluce_assembly_get_fastas_from_match_counts`. Before aligning the UCE loci, we performed GBlocks internal trimming of the alignments using `phyluce_align_seqcap_align` and `phyluce_align_get_gblocks_trimmed_alignments_from_untrimmed` with default parameters. The alignments were cleaned using `phyluce_align_remove_locus_name_from_files`, and 35% complete data matrices were created (i.e. each locus had 35% of the total specimens' occupancy) for the entire data set and the individual clades A, B and C. The alignments were concatenated for further downstream processing. The number of phylogenetic informative sites was calculated using PAUP* 4.0a169 (Swofford & Sullivan, 2009). Maximum likelihood (ML) inference was performed with RAxML version 8.2.12, using the options for rapid bootstrapping (model GTRGAMMAX, 1000 bootstrap replicates). A Bayesian inference (200,000 generations, 25% burn-in) was conducted using RevBayes version 1.2.1 using the same model settings as the ML analysis.

3 | RESULTS

3.1 | Multilocus probe assay and targeted loci

Depending on the bait set and the universality of the selected UCEs, the number of recovered probes varied between 195 and 476 for $n_{\text{taxa}}=7$ and 5974 and 12,002 for $n_{\text{taxa}}=2$ for the haplo-only bait sets. These baits target 14 to 1790 loci (Table 3a). For the Haplo+outgroup bait sets, the number of recovered probes varied between 16 and 270 (opting for the most stringent option) and targeted one to 17 loci (Table 3b). After merging the probes retrieved from the different bait sets and filtering for duplicates, we obtained 21,463 unique baits. For the multilocus probe assay, we selected

TABLE 3 The number of recovered probes (left) and targeted loci (right) for (a) the haplo-only bait sets depending on the selected base transcriptome and the stringency level during the bait design (i.e. the number of taxa aligned against the base transcriptome) and (b) the haplo+outgroup bait sets including a demosponge outgroup in the bait design. Note that for the haplo+outgroup bait set, only the most stringent option (i.e. $n_{\text{taxa}} = 8$) was opted, except for two bait sets, namely the bait set with *Haliclona (Haliclona) oculata* as base transcriptome and *Crella (Crella) elegans* as demosponge outgroup and the bait set with *Neopetrosia compacta* with *Vaceletia sp.* as demosponge outgroup, for which the second most stringent option was selected (i.e. $n_{\text{taxa}} = 7$).

a. Haplo-only	Stringency level																					
	7	6	5	4	3	2																
Base transcriptome	7	6	5	4	3	2																
<i>Amphimedon queenslandica</i>	476	1680	3372	5522	7213	8270																
<i>Haliclona (Reniera) cinerea</i>	404	1612	3357	5565	7382	8485																
<i>Haliclona (Reniera) tubifera</i>	446	1592	3401	5712	7735	9032																
<i>Haliclona (Rhizoniera) indistincta</i>	221	1083	2687	4902	7149	8544																
<i>Haliclona (Rhizoniera) viscosa</i>	334	1463	3839	6817	9794	12,002																
<i>Haliclona (Haliclona) oculata</i>	210	1141	2725	4947	6868	8257																
<i>Haliclona (Haliclona) simulans</i>	248	1164	2437	3967	5214	6082																
<i>Neopetrosia compacta</i>	195	851	2020	3559	5027	5974																
b. Haplo+outgroup	Demosponge outgroup																					
Base transcriptome	Scopallina sp.	Geodia atlantica	Mycale (Carmia) phyllophila	Latrunculia (Latrunculia) apicalis	Tedania (Tedania) anhelans	Crella (Crella) elegans	Tethya wilhelma	Pleraplysilla spinifera	Vaceletia sp.	Lendenfeldia chondrodes	Phyllospongia foliascens											
<i>A. queenslandica</i>	191	12	144	9	187	12	156	10	108	7	139	9	174	11	96	6	16	1	63	4	127	8
<i>H. (Reniera) cinerea</i>	160	10	172	11	95	6	140	9	80	5	77	5	112	7	157	10	80	5	157	10	222	14
<i>H. (Reniera) tubifera</i>	239	15	96	6	127	8	94	6	80	5	111	7	95	6	93	6	96	6	156	10	128	8
<i>H. (Rhizoniera) indistincta</i>	187	12	270	17	80	5	80	5	48	3	64	4	111	7	80	5	32	2	108	7	47	3
<i>H. (Rhizoniera) viscosa</i>	124	8	191	12	96	6	128	8	128	8	47	3	48	3	96	6	80	5	78	5	175	11
<i>H. (Haliclona) oculata</i>	223	14	32	2	48	3	64	4	32	2	194 ^a	14	32	2	128	8	80	5	160	10	80	5
<i>H. (Haliclona) simulans</i>	233	15	76	5	63	4	48	3	63	4	16	1	128	8	128	8	16	1	111	7	144	9
<i>N. compacta</i>	48	3	16	1	48	3	16	1	64	4	48	3	32	2	32	2	140 ^a	10	48	3	32	2
Corresponding demosponge outgroup	61	4	16	1	16	1	16	1	16	1	23	2	64	4	16	1	16	1	95	7	48	3

^aFor these bait sets, the second most stringent option (i.e. $n = 7$) was opted to avoid an output of 0.

20,000 probes randomly, designed to capture 2956 exon loci from demosponges, particularly from haplosclerid sponges.

3.2 | Target-enrichment multilocus phylogeny of Red Sea Haplosclerida

The total number of reads obtained from the target-captured libraries ranged from 379,886 to 10,444,808 (mean: $2,258,719 \pm 2,102,803$ SD reads). Between 4.1% and 11.6% of reads were removed after quality filtering and adapter trimming from each sample, resulting in a mean of $2,133,464 \pm 2,012,006$ SD trimmed reads per target-captured sample (Table S4). Using SPAdes version 3.15.4 (Bankevich et al., 2012), we obtained between 10,512 and 144,337 contigs per sample (mean: $42,379 \pm 43,896$ SD contigs) with an average length of 301 ± 25 bp (range: 49 to 16,929 bp) (Table S5). PHYLUCE retrieved 269–1158 UCE loci (mean: 652 ± 268 SD UCE loci) from the assembled contigs. The average length of the UCE contigs varied between 345 and 859 bp (mean: 590 ± 137 SD bp) (Table S6). The 35% concatenated-alignment matrix with $n_{\text{taxa}}=40$ (i.e. 26 Red Sea specimens, 11 haplosclerid transcriptomes and 3 transcriptomes of other demosponges as an outgroup) was based on 446 loci and had a total length of 118,775 bp; 47% of the sites were phylogenetically informative (PI) (Table 4). The 35% matrices of the separate clades retained more loci (506–1241 UCEs) and had longer alignment lengths between 213,023 and 578,762 bp but fewer phylogenetic informative sites (6%–22%). The ML and Bayesian inference tree topologies obtained from analysing the 446 loci of the 35% data matrix of the Red Sea haplosclerids ($n_{\text{taxa}}=40$) were identical. Most nodes had moderate to high bootstrap and posterior probability values at shallow and deeper nodes (>70 and ≥ 0.99 , respectively), except for three nodes for which bootstrap values ranging between 61 and 68 were found (Figure 2; Figure S3). Both analyses recovered the four widely accepted clades within the Haplosclerida with high support (Figure 2). The 26 specimens, morphologically identified to genus or species level, covered nine different genera belonging to the families Chalinidae, Callyspongiidae, Niphatidae and Petrosiidae (Supplementary Information II). Accordingly, in the phylogeny, the species' transcriptomes used for bait design fell in the expected clades A, B, C and E, as shown by previous studies. Furthermore, we observed that morphologically identical specimens had sister relationships in the tree.

3.3 | Target-capture approach captures sufficient loci across Demospongiae

The designed probes 'enriched' between 234 and 798 loci in haplosclerids (572 ± 185 SD) and between 46 and 195 loci in the other demosponges (132 ± 32 SD) (Table 5). The concatenated 35% occupancy alignment matrix consisted of 72 conserved loci and had a trimmed mean locus length of $1196 \text{ bp} \pm 846$ SD, a total

TABLE 4 Alignment matrix statistics for the different data sets.

Data set	# taxa	Matrix (%)	# UCEs	Alignment length (bp)	Mean locus length \pm SD (bp)	Locus length range (bp)	# constant sites (bp)	# PI sites (bp)	PI sites (%)
<i>In-silico</i> test demosponges	39	35	72	86,118	1196 ± 846	222–3635	31,554	45,805	53
<i>In-vitro</i> test Red Sea haplosclerids	40	35	446	118,775	226 ± 115	40–784	49,787	55,802	47
Red Sea haplosclerids – Clade A	16	35	506	213,023	421 ± 382	117–3453	116,203	46,145	22
Red Sea haplosclerids – Clade B	8	35	988	560,743	568 ± 338	56–6376	441,193	31,056	6
Red Sea haplosclerids – Clade C	12	35	1241	578,762	466 ± 183	77–1503	376,801	81,468	14

Note: The percentage matrix value (%) equals the percentage of the total number of specimens (# taxa) that occupy a specific locus. The phylogenetic informative (PI) sites were calculated for the different data sets.

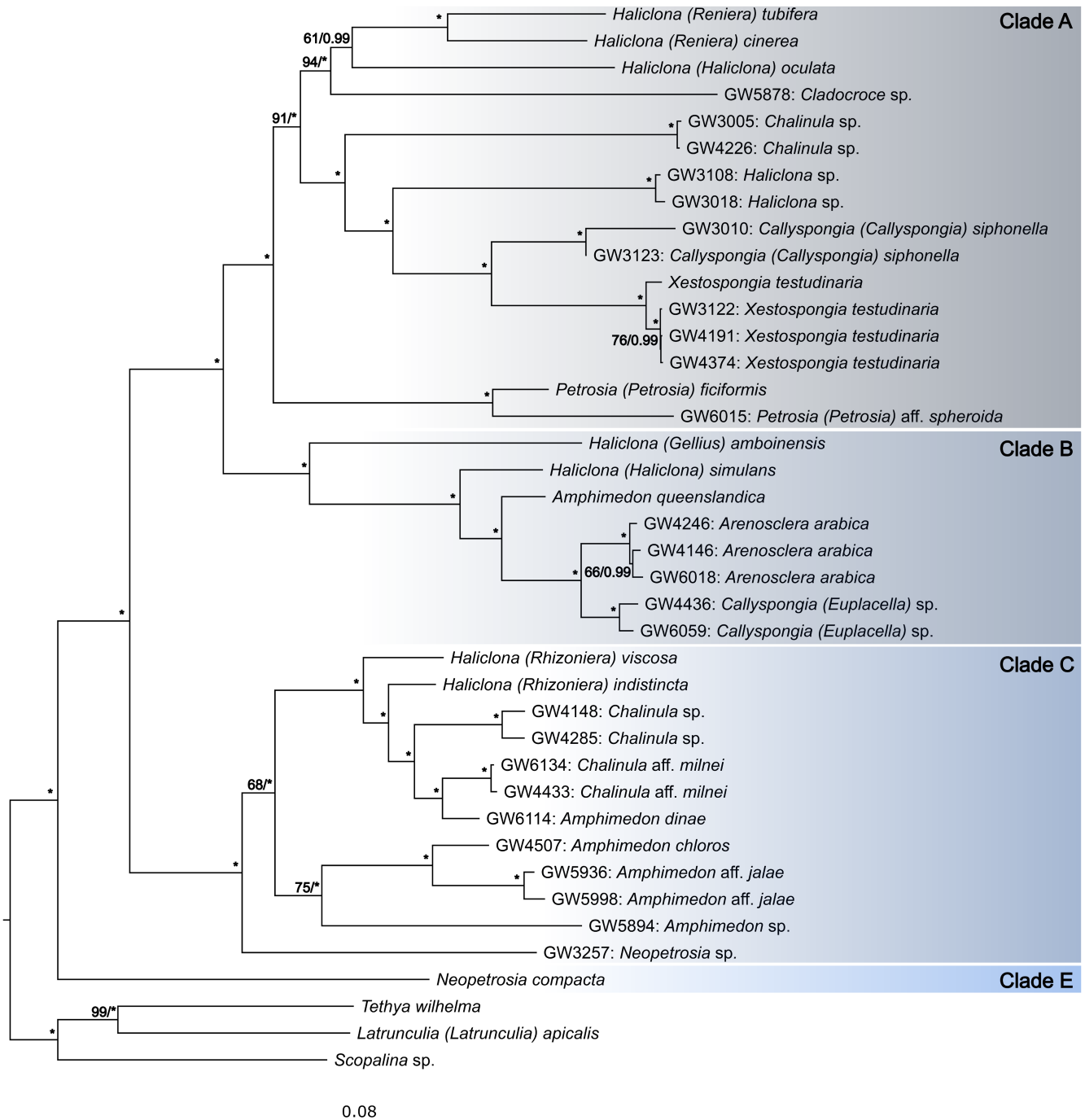


FIGURE 2 Maximum-likelihood phylogeny on a 35% concatenated alignment matrix of the 446 retrieved loci ($n_{\text{taxa}} = 40$, $n_{\text{char}} = 118,775$ bp). Branches denote bootstrap (BS) values/posterior probabilities (PP) from Bayesian analyses, with BS = 100% and PP = 1.0 indicated with an asterisk (*). The Red Sea samples are indicated with a GW-ID number. The other haplosclerids are derived from transcriptome data. The tethyid *Tethya wilhelma*, the poecilosclerid *Latrunculia (Latrunculia) apicalis* and scopallinid *Scopalina* sp. were used as outgroups. The scale is 0.08 substitutions/site.

length of 86,118 bp and 45,642 (53%) phylogenetically informative (PI) sites (Table 4). The ML phylogeny generated from the alignment had overall well-supported nodes. For the Red Sea haplosclerids, in particular, bootstrap values were moderate to high (74–100 respectively) (Figure 3). The Bayesian phylogeny was mostly congruent with the ML phylogeny, except for the placement of *Aptos* sp., which had a sister relationship with

Tentorium papillatum instead of forming a clade together with *Halichondria (Halichondria) panicea* and *Pseudospongosorites suberitoides* (Suberitida) (Figure S4). The transcriptome of *Aptos* sp. had the lowest number of contigs and complete and single-copy BUSCO values (10.4% and 8.4% respectively) compared to the other transcriptome data used for the analysis (Table S3). The posterior probability values in the Bayesian phylogeny indicated

TABLE 5 The number of UCE loci recovered from the targeted 20,000 baits from the 39 demosponge transcriptomes.

Order	Family	Species	# UCEs	# contigs	# UCE removed	# contigs removed
Axinellida	Axinellidae	<i>Cymbastela stipitata</i>	174	41,036	37	23
Axinellida	Stelligeridae	<i>Stelligera</i> sp.	139	27,047	13	8
Clionaida	Clionidae	<i>Cliona varians</i>	195	165,430	73	41
Haplosclerida	Chalinidae	<i>Haliclona (Gellius) amboinensis</i>	340	110,248	478	272
Haplosclerida	Chalinidae	<i>Haliclona (Reniera) cinerea</i>	761	59,469	588	621
Haplosclerida	Chalinidae	<i>Haliclona (Rhizoniera) indistincta</i>	627	42,407	435	606
Haplosclerida	Chalinidae	<i>Haliclona (Haliclona) oculata</i>	692	65,478	202	430
Haplosclerida	Chalinidae	<i>Haliclona (Haliclona) simulans</i>	798	51,464	554	536
Haplosclerida	Chalinidae	<i>Haliclona (Reniera) tubifera</i>	533	90,149	494	512
Haplosclerida	Chalinidae	<i>Haliclona (Rhizoniera) viscosa</i>	703	42,122	408	602
Haplosclerida	Niphatidae	<i>Amphimedon queenslandica</i>	621	83,671	386	381
Haplosclerida	Petrosiidae	<i>Neopetrosia compacta</i>	627	184,473	152	349
Haplosclerida	Petrosiidae	<i>Petrosia (Petrosia) ficiformis</i>	359	51,234	107	115
Haplosclerida	Petrosiidae	<i>Xestospongia testudinaria</i>	234	247,222	190	96
Poecilosclerida	Coelosphaeridae	<i>Forcepia</i> sp.	162	33,966	27	12
Poecilosclerida	Crellidae	<i>Crella (Crella) elegans</i>	108	139,190	64	29
Poecilosclerida	Hymedesmiidae	<i>Kirkpatrickia variolosa</i>	143	23,668	22	15
Poecilosclerida	Isodictyidae	<i>Isodictya</i> sp.	130	63,316	48	21
Poecilosclerida	Lantrunculiidae	<i>Latrunculia (Latrunculia) apicalis</i>	137	19,393	24	18
Poecilosclerida	Mycalidae	<i>Mycale (Carmia) cecilia</i>	156	195,334	95	49
Poecilosclerida	Mycalidae	<i>Mycale (Carmia) phyllophila</i>	143	182,491	60	46
Poecilosclerida	Myxillidae	<i>Myxilla</i> sp.	135	27,753	10	17
Poecilosclerida	Tedaniidae	<i>Tedania (Tedania) anhelens</i>	114	40,426	66	29
Poecilosclerida	Tedaniidae	<i>Tedania</i> sp.	157	26,944	14	15
Polymastiida	Polymastiidae	<i>Tentorium papillatum</i>	150	26,267	20	14
Scopalina	Scopalinidae	<i>Scopalina</i> sp.	159	39,282	26	25
Scopalina	Scopalinidae	<i>Stylissa carteri</i>	148	17,425	25	16
Suberitida	Halichondriidae	<i>Halichondria (Halichondria) panicea</i>	141	496,418	161	42
Suberitida	Suberitidae	<i>Aaptos</i> sp.	46	10,356	3	3
Suberitida	Suberitidae	<i>Pseudospongisorites suberitoides</i>	131	17,425	25	16
Tethyida	Tethyidae	<i>Tethya wilhelma</i>	171	53,470	60	41
Tetractinellida	Geodiidae	<i>Geodia atlantica</i>	140	216,528	50	27
Dendroceratida	Darwinellidae	<i>Dendrilla antarctica</i>	93	51,693	14	12
Dictyoceratida	Dysideidae	<i>Pleraplysilla spinifera</i>	91	60,078	35	23
Dictyoceratida	Irciniidae	<i>Sarcotragus fasciculatus</i>	109	37,032	22	7
Dictyoceratida	Spongiidae	<i>Spongia (Spongia) officinalis</i>	136	321,679	26	25
Dictyoceratida	Thorectidae	<i>Lendenfeldia chondrodes</i>	115	36,210	34	20
Dictyoceratida	Thorectidae	<i>Phyllospongia foliascens</i>	87	138,582	57	26
Dictyoceratida	Verticillitidae	<i>Vaceletia</i> sp.	85	73,029	25	11

Note: Additionally shown are the total number of contigs, the number of UCEs removed for matching multiple contigs and the number of contigs removed for matching multiple UCEs.

high support (>0.99). In both phylogenies, the haplosclerid transcriptomes grouped in clades A, B, C and E, congruent with the earlier single-marker phylogenies. In the demosponge phylogeny, the two *Tedania* species were not a sister group. However, both

fell within the order Poecilosclerida. Overall, we recovered a phylogeny that is mainly consistent with accepted relationships of Demospongiae (Erpenbeck et al., 2004; Redmond et al., 2013; Thacker et al., 2013).

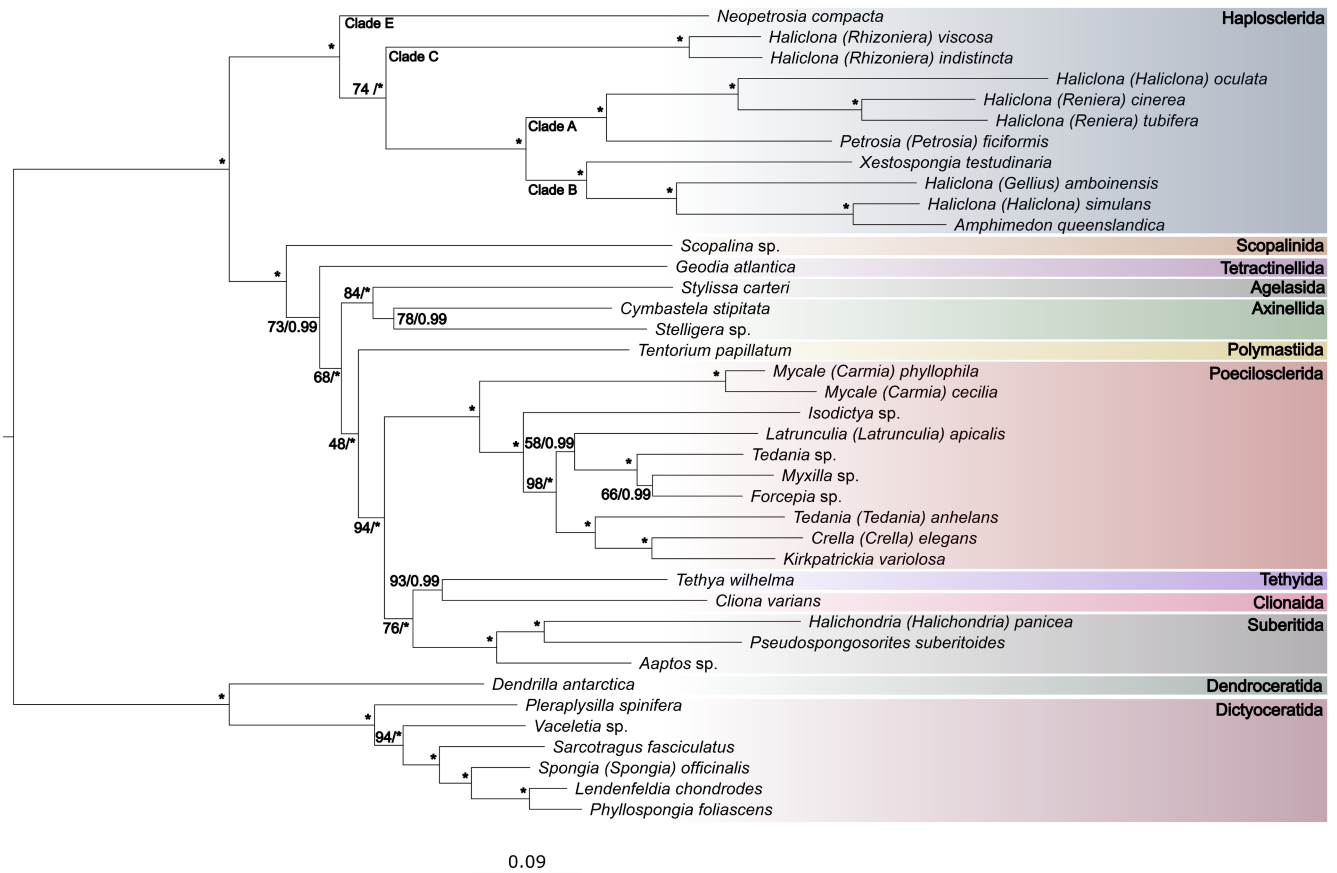


FIGURE 3 Maximum-Likelihood phylogeny on the demosponges based on a 35% concatenated alignment matrix ($n_{\text{taxa}} = 39$; 86,118 bp, 72 loci) rooted to the subclass Keratosa (i.e. Dendroceratida and Dictyoceratida). The ML phylogeny was computed using RAXML 8.2.12 with rapid bootstrapping (1000 replicates, indicated at the nodes) using the GTRGAMMAX model. Branches denote bootstrap (BS) values/posterior probabilities (PP) from Bayesian analyses, with BS = 100% and PP = 1.0 indicated with an asterisk (*). The scale is 0.09 substitutions/sites.

4 | DISCUSSION

To our knowledge, this is the first time a multilocus probe assay has been designed to capture hundreds of genome-wide loci from one of the systemically most challenging groups of non-model organisms, namely sponges (Phylum Porifera). Here, we demonstrate the applicability of target-capture enrichment using ultraconserved element (UCE) loci as an effective and robust approach for resolving phylogenetic relationships on both deep and shallow evolutionary timescales from species across one of the most diverse demosponge orders, the Haplosclerida. Thus far, target-capturing of UCES has been successfully applied to other marine invertebrates, such as echinoderms (Hugall et al., 2015), anthozoans (Cowman et al., 2020; Quattrini et al., 2018), arthropods (Ballesteros et al., 2021), annelids (Petersen et al., 2022) and recently molluscs (Goulding et al., 2023; Moles & Giribet, 2020), where they successfully resolved phylogenetic relationships at both high (class) and low (species) taxonomic levels, and provided insights into evolutionary dynamics between species.

Using our custom-made multilocus probe assay, we reconstructed a supra-specific phylogeny of a broad range of Red Sea

Haplosclerida based on the target-captured loci and additional transcriptome data of 11 taxa. This phylogeny was well-resolved, congruent with previous results (McCormack et al., 2002; Raleigh et al., 2007; Redmond et al., 2011, 2013) and had high branch support on both deeper and shallow nodes; thus, we demonstrate the high potential of the probe assay for demosponge taxonomy, systematics and phylogeny. We propose future studies to tailor the application of this target-capture enrichment approach for (rapid) species identification to specific sponge taxa to benefit the development of biomonitoring tools to aid biodiversity surveys. To this end, Erickson et al. (2021) demonstrated that sufficient SNPs could be extracted from the UCE loci to differentiate between populations of corals. A similar approach would be highly desirable for a broad variety of sponge taxa.

The performance of a bait set is inherently dependent on the quality of the input data used for its design. In the case of sponges, obtaining high-quality transcriptomes or genomes that can be used for probe design is challenging because of the potential inhibition of enzymatic reactions during library production by co-extracted biochemical compounds produced by the sponge holobiont (Chelossi et al., 2004) or co-amplification of commensal organisms

that reside in sponges (Vargas et al., 2012). At the time of probe design, only one haplosclerid genome (*Amphimedon queenslandica*) was published (Srivastava et al., 2010), which constrained us to work mainly with transcriptomic data and, as a result, the designed baits all fall in coding (exon) regions. Although highly conserved elements are more likely to be found in coding regions and are also expected to capture loci across species more efficiently, they are argued to be more suitable for resolving at low-moderate phylogenetic distances (Bi et al., 2012). However, when combined with target capture, previous studies have demonstrated the utility of exon-based loci for inferring and resolving phylogenetic relationships at both deeper and shallow nodes (Ballesteros et al., 2021; Quattrini et al., 2018). Transcriptomes of the haplosclerids *Petrosia* (*Petrosia*) *ficiformis*, *Haliclona* (*Gellius*) *amboinensis* and *Xestospongia testudinaria* that were available to us were not included in our bait design due to transcriptome incompleteness and questionable identification of the species due to their placement in the 18S rDNA phylogeny (Figure S1). However, these species were placed within the Haplosclerida in our in silico and in vitro tests based on the loci retrieved from these transcriptomes. Hence, despite the ambiguous placement of these haplosclerids using the 18S rDNA marker, our probes captured sufficient information to place them accurately in the UCE-based phylogeny, which is also in agreement with previous results (Guzman & Conaco, 2016; McCormack et al., 2002; Redmond et al., 2011, 2013). In addition, we observed inconsistencies in the transcriptomic data in the 18S-based phylogeny that were not present in UCE-based phylogenies. First, some of the other (non-haplosclerid) demosponges (*Cliona varians*, *Stelligera* sp., *Tedania* (*Tedania*) *anhelans* and *Kirkpatrickia variolosa*) were incorrectly placed in the 18S phylogeny, while they were correctly placed in our UCE-based phylogeny (Figure 3). Second, we observed long branches for some of the species (e.g. *Cliona varians*) or entire clades (e.g. *Mycale* (*Carmia*) *cecilia* and *Halichondria* (*Halichondria*) *panicea*) in the 18S phylogeny, which were, in turn, not detected in our UCE-based phylogenies. Although we cannot exclude the possibility of artificial errors occurring in the 18S sequences as a result of multiplexing PCR or the selected sequencing approach, we argue that the choice of 18S as a single marker is potentially to blame for the misplacement of certain species and the possible long branch attraction (LBA) observed. Another reason for such artefacts could be high substitution rates in the rRNA evolution of Haplosclerida (Lavrov et al., 2008; Simion et al., 2017). Here, we demonstrate that our UCE-based approach is likely less prone to these limitations and results in more reliable phylogenies compared to 18S or, generally, single marker-based phylogenetic reconstructions.

Between the different bait set versions, we observed considerable differences in the number of retrieved loci from essentially the same input data (Table 2). For example, we obtained a relatively high number of probes from bait sets with lower stringency levels (e.g. level 2–5) and with *H. (Rhizoniera)* *viscosa* set as the base transcriptome, while for the most stringent options (e.g. level 6–7) more probes were retrieved when *A. queenslandica* was set as the base

transcriptome. This is not trivial since the specificity of the bait set likely depends on the quality and completeness of the input data and the phylogenetic distance between the input species. Therefore, future studies focussing on specific families or clades could optimise our bait set to maximise the enrichment probability of a subset of loci in the taxon of interest. Although the ratio of probes may not be directly proportional to the different clades within the Haplosclerida, our bait set successfully enriched numerous loci from our Red Sea haplosclerids and resulted in a phylogeny that recovered almost all previously molecularly defined clades (i.e. Clade A–C, and E) of the Haplosclerida.

Ambiguities regarding the placement of species in the phylogenetic trees were only noticed in a few cases, which is likely the result of the incompleteness of the transcriptome data used for the analysis (Table S3). For example, when comparing the haplosclerid and the demosponge phylogenies, the placement of *Xestospongia testudinaria* remained ambiguous. Namely, in the Red Sea analysis, *X. testudinaria* is nested within Clade A, while in the demosponge phylogeny, it was found to be a sister group to Clade B. The alternating position of *X. testudinaria* between trees with different taxon samplings could be explained by the lower number of haplosclerid taxa in the demosponge phylogeny, which is also based on less retrieved loci compared to the phylogeny containing largely haplosclerid taxa. Another reason for the different placement can be the result of incomplete taxon sampling (see discussion in Quattrini et al., 2018; Wiens, 2005) because our Red Sea phylogeny contains a more reduced taxon set compared with both McCormack et al. (2002) and our larger demosponge phylogeny. Adding more haplosclerid representatives, and in particular type material that functions as a taxonomic reference point, will certainly lead to a more accurate placement of the species and genera in this group and an improved phylogeny-based classification of Haplosclerida.

The target-capture enrichment approach uses relatively short bait sequences and, therefore, is suitable for generating genome-scale data from older and possibly poorly preserved or formalin-fixed museum material (Agne et al., 2022; McCormack et al., 2016). Thus, it can be leveraged for 'museomics' to serve as the baseline for re-evaluating systematics and taxonomy in sponges and other groups. In this vein, a new phylogenetic classification of Haplosclerida should implement a 'bottom-up strategy', applying target-capture enrichment to each haplosclerid genus type if possible or to taxonomically validated material if types are not available to derive a phylogenetically consistent classification of this order.

This study demonstrates that target-capture enrichment combined with our newly designed multilocus probe assay is a valuable genomic resource for understanding evolutionary relationships among demosponges, particularly haplosclerid sponges. We confirmed the broad applicability of the bait set as ordinal relationships of species were revealed across the class Demospongiae. Future studies are recommended to test and possibly broaden the applicability of the bait set to other demosponge groups. For example, studies could select the markers of our multilocus probe assay that successfully captured loci from these groups and enrich the subset

with more taxon-specific information to design additional baits and derive better phylogenies of other demosponge orders. Based on our observations, we conclude that target-capture enrichment is highly suitable for gathering phylogenetic markers of known homology across sponges and is a promising tool for resolving evolutionary relationships within Porifera. With the high number of variable sites found for the retrieved loci, our study opens many possibilities, including supra-specific phylogenies and population-level studies to discover species boundaries, population structures and new species. Ultimately, this will contribute to the development of better biomonitoring tools, aid in conservation efforts and lead to more reliable interpretations of the evolutionary history, the ecology, and, in general, the biology of sponges.

AUTHOR CONTRIBUTIONS

JS, SV, DE and GW are part of the DFG-funded promotion project, with the latter three responsible for the project's design and supervising the research, assisting in interpreting the data and writing the paper. JS designed the multilocus probe assay, contributed to the laboratory work, performed bioinformatic analyses of the data and wrote the initial draft of the manuscript with significant contributions of SV. SS conducted the library preparation and target enrichment. The sponge specimens were collected between 2012 and 2017 under the Red Sea Biodiversity Project framework by OV, DE and GW. JS and NJV conducted morphological taxonomic identification of the Red Sea specimens at the Naturalis Biodiversity Center, Leiden, the Netherlands. GPM and KS provided the project with transcriptomic data for bait design. All authors revised the manuscript.

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CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Tree and alignment files, multilocus probe assay and PHYLUCe modified workflow: <https://github.com/PalMuc/HaploTC>. Raw data are deposited to the European Nucleotide Archive (ENA) under project PRJEB60480.

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SUPPORTING INFORMATION

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