Molecular Phylogenetics and Evolution 213 (2025) 108436

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Target-enriched multilocus assay on poriferan museum collections unsettles tethyid classification

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ARTICLE INFO

Keywords:
Target-enriched multilocus assay
Sponges
Demospongiae
Tethya
Archival DNA

ABSTRACT

Marine biodiversity collections are critical resources for understanding biodiversity and evolutionary patterns. However, their taxonomic utility is limited due to challenges in morphological identification and cryptic speciation, particularly in sponges (Porifera). This study applied a novel target-enriched multilocus assay to a decades-old unidentified collection of Tethyidae from the Queensland Museum. Target-specific baits were designed based on *Tethya* genome data, enabling the sequencing of nuclear ultraconserved elements (UCEs) and mitochondrial genomes, even from degraded samples. In the study, 40 specimens were sequenced, and we found up to 4,440 UCEs per sample. The phylogenies were based on up to 2,788 loci and demonstrated overall high bootstrap support. Results include robust support for earlier findings on the non-monophyly of *Tethya*, with lineages linking to other genera (e.g., *Stellitethya*, *Tethycometes*), suggesting that *Tethya* is a paraphyletic group foundational to Tethyidae. Morphological subgroupings, previously hypothesized, were validated genetically, though several novel relationships emerged. This approach highlights the suitability of target-enriched multilocus assays for archival collections, enabling detailed biodiversity assessments using collection material initially not sampled for molecular work. Our *Tethya* case study underlines that this methodology provides a framework for broader applications in marine biodiversity conservation, facilitating the understanding and preservation of past and present biodiversity to guide future efforts.

1. Introduction

In the face of declining marine biodiversity worldwide, there is an urgent need for novel and innovative approaches to rapidly assess and monitor both current and future biodiversity. Without a clear understanding of the existing status, it is impossible to adequately evaluate any degree of biodiversity loss or implement appropriate conservation measures. Natural history museums and other biodiversity collections worldwide are recognised as valuable repositories of specimens, capable of documenting the dynamic changes in biodiversity, and revealing evolutionary patterns across time and space (Bi et al., 2013; Wandeler et al., 2007). However, the utility of these valuable data relies heavily on the availability of experienced taxonomists to identify the specimens, which form the foundation for all subsequent biodiversity assessments. Unfortunately, this resource is highly limited, and the prospects of improvements in the near future are bleak, particularly for marine organisms (Armbrust and Palumbi, 2015; Drew, 2011; Khuroo et al., 2007). Consequently, large marine biodiversity collections have often

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https://doi.org/10.1016/j.ympev.2025.108436
Received 30 January 2025; Received in revised form 23 June 2025; Accepted 10 August 2025
Available online 13 August 2025

been superficially or only partially identified (Hooper et al. 2013). At most, specimens may be superficially assigned to an operational taxonomic unit (or morphospecies) as a species surrogate with crude characteristics documented in a summary description (e.g., "mudmaps", in Hall et al., 2013). Here, intrinsic phenomena such as cryptic speciation and excessive morphological plasticity lead to incorrect estimations of biodiversity with consequences for conservation and management strategies and our overall understanding of speciation (e.g., Gómez et al., 2002; Pöppe et al., 2010). Therefore, molecular genetic, and more recently, genomic tools are employed to "unlock the vault" of museum biodiversity collections, with limited dependency on morphological expertise (Bi et al., 2013). Notably, the sequencing of museum material benefits from next-generation sequencing approaches that work with shorter and more fragmented DNA sequences, which are common in historical DNA (Raxworthy and Smith, 2021). A preferred method for biodiversity surveys is the target enrichment and sequencing of ultraconserved elements (UCEs), i.e. highly conserved regions with phylogenetically informative flanking regions that can be selectively 'captured' by specific baits and assembled to a multilocus assay (Faircloth et al., 2012). This technique is used for species discovery, identification and delimitation of marine organisms (Erickson et al., 2021).

Particularly, biodiversity collections of sponges (Porifera) could benefit from target-enriched multilocus assays, as recently demonstrated in the case study by Van der Sprong et al. (2024). Sponges are notoriously challenging for non-experts to identify due to the limited diagnostic characters available, homoplasy, secondary loss, and environmental plasticity (e.g., Erpenbeck et al., 2006a). Likewise, molecular genetic approaches are hindered by the high abundance of sponge-associates, microbes and other commensal DNA co-extracted with their hosts (Vargas et al., 2012). Such co-sequencing of foreign DNA is minimised in a target enrichment assay due to the taxon specific custom-developed gene markers of known homology, whereas other methods like genome skimming may suffer from high proportions of non-sponge reads (Derkarabetian et al., 2019).

In this study, we are applying a target-enriched multilocus assay for the first time on a decades-old yet unidentified museum collection of demosponges of the genus *Tethya*. *Tethya* is a conspicuous sponge genus that currently includes over 100 accepted species. Several *Tethya species* are described from aquaria systems, where they became popular model organisms for sponge physiology and development (Hammel et al., 2009; Mills et al., 2018; Nickel, 2006, e.g., Nickel, 2004; Nickel et al., 2006; Steinmetz et al., 2012). Moreover, the sequenced genome of *Tethya wilhelma* (Wörheide et al., 2024) has proven fundamental for studies on nerve system evolution, hypoxia adaptation, silica biomineralization, and body plan evolution (Francis et al. 2017; Renard et al. 2018; Mills et al. 2018; Wörheide et al. 2024). Despite *Tethya* possessing a broader diversity of skeletal elements to guide classical taxonomy compared to most other demosponge genera, species identification remains challenging due to high phenotypic plasticity and cryptic diversity, which have been observed in several *Tethya* lineages (Sarà et al., 1993; Shaffer et al., 2019). The non-monophyly of *Tethya* has also been hypothesized but yet awaits further testing (Sorokin et al. 2019; see also Santodomingo et al. 2024 for the currently most comprehensive phylogenetic analysis of this genus). As a result, *Tethya* is among the most studied sponge genera. The Queensland Museum Brisbane (Australia) possesses a substantial collection of *Tethya* samples, predominantly collected from the Southern Hemisphere, although these have only been initially classified into morphospecies. In the present study, we utilise an yet unidentified museum collection of sponges to apply a target-enriched multilocus assay in order to assess the evolutionary histories and genetic diversity of the collection. This will facilitate subsequent identification, and phylogenetic analysis. The collection serves as a test case for broader applications on the many, yet unidentified, sponge biodiversity collections, with the aim of facilitating biodiversity assessments of the present and the past – thereby contributing to the preservation of biodiversity for the future.

2. Material and methods

2.1. Bait design

Tethyid specific baits were designed based on proteome data predicted from several *Tethya* genomes. These comprised the *Tethya wilhelma* and *Tethya minuta* genome drafts (Wörheide et al., 2024) and an unpublished low pass assembly of *Tethya citrina*. The proteomes from all three genome assemblies were extracted and translated in Geneious Prime R 2019.2 (Kearse et al., 2012). Using OrthoFinder (Emms and Kelly, 2019; Stanke et al., 2008), we identified putative single orthologs in a two-step approach. This was necessitated by the significant differences in the quality of the three *Tethya* assemblages. We first ran OrthoFinder for *T. wilhelma* and *T. minuta*, and then for all three *Tethya* species. The results of both approaches were merged, and duplicates were removed, the single copy genes yielded were aligned using a custom wrapper Perl script ("mafft_wrapper.pl", available with all other scripts and alignments of this study from https://github.com/PalMuc/Tethya-MuseOMICS/) and MAFFT-7.453 (Katoh, 2005). For mitochondrial genome baits, the published mitochondrial genome sequences of *T. wilhelma* (INSDC Genbank accession number LR862379), *T. minuta* (LR877180), *T. citrina* (LR862354), and *T. actinia* (ANC_006991) were used.

The resulting alignments were used for bait design in BaitFisher-v1.2.8 (Mayer et al., 2016) using *T. wilhelma* as reference with the bait offset set to 80 bp. BaitFilter v1.0.6 (Mayer et al., 2016) further reduced the set of bait sequences by 1) removing unspecific hits, 2) removing bait sequences with multiple hits on the genome, and 3) minimising the number of required baits per target region. The resulting bait sequences were mapped back to the *T. wilhelma* exome BWA-MEM (Li and Durbin, 2009). Finally, an additional Perl Script filtered only the baits that mapped to their assigned genes ("baitsorter.pl"). This final bait set was synthesised by Ann Arbor Bioscience (USA) as biotinylated in solution RNA fragments.

2.2. Samples

The *Tethya* collection investigated in this study is part of the collection of the Queensland Museum Brisbane, Australia, (QM). A further sample was collected by the Coral Reef Research Foundation (CRRF) as part of the Rhodes University and United States National Cancer Institute's shallow-water collection program, and now part of the TS collection at the Iziko Museum of South Africa. Additionally, specimens have been deposited in the Bavarian State Collection of Palaeontology and Geology (SNSB-BSPG). The sample set comprises specimens of *Tethya*, as well as from other genera of its family Tethyidae (*Stellitethya*) or other Tethyida (*Liosina paradoxa*, *Axos flabelliformis* (Hemiasterellidae)), which were used as outgroup taxa. Samples were collected between 1988 and 2009 at various locations around Australia, furthermore Fiji and South Africa (see Supplementary Table 1). After collection they were preserved in 70 % Ethanol and subsequently classified into morphospecies, as documented as "mudmaps" (see Hall et al., 2013). In 2010, small sections of the samples were transferred to 96 well plates and shipped to Munich in the course of the Barcode of Life initiative (MarBol), funded by the Alfred P. Sloan Foundation. Data were complemented with *T. wilhelma* and *T. minuta* of our aquaria system and additional *Tethya* specimens from reef sites near Magoodhoo, Faafu Atoll, Maldives, collected with help and support from staff and scientists of MaRHE Center of University of Milano-Bicocca, Magoodhoo, and local collaborators (Agreement (AGR)438-ENV/PRIV/2024/83 incl. enclosed permits).

2.3. DNA extraction and library preparation, target capturing and sequencing

Genomic DNA of the 40 tethyid specimens (plus one technical replicate [QM G319612]) was extracted using the CTAB extraction for mucus-rich invertebrates (Vargas et al., 2021). Double indexed paired end libraries were prepared using Accel-NGS™ 1S Plus DNA Library Kit (Swift Biosciences), quantified using Qubit dsDNA High Sensitivity Assay Kit (Invitrogen), and quality controlled on a Bioanalyzer 2100 (Agilent). Target capture was performed using the myBaits Hybridization Capture for Targeted NGS protocol version 4.01 (hybridization temperature: 62 °C). Four multiplexed equimolar libraries, each containing 125 ng of DNA, were used for each hybridization reaction. After hybridization, the captured libraries were amplified using KAPA HiFi (Roche) and quality controlled on the Bioanalyzer 2100 (Agilent). We combined and sequenced equimolar target captured pools in an Illumina MiniSeq in pair-end mode (PE) using a 300 cycles Mid-Output Kit (Illumina).

2.4. Sequence post-processing, alignment, and phylogenetic inference

Reads were demultiplexed with Je demultiplex (Girardot et al., 2016), quality checked with FastQC (Andrews, 2015), and trimmed using Trimmomatic (Bolger et al., 2014) and cutadapt (Martin, 2011). Reads were subsequently processed with the PHYLUCE v1.7.3 program package (Faircloth, 2016), following the workflows by Van der Sprong et al. (2024). Reads were assembled with SPADES (Nurk et al., 2013) with PHYLUCE's phyluce_assembly_assemblo_spades program under default settings. Matches between the assembled contigs and the UCE bait sequences were searched (settings: minimum 85 % identity, minimum 85 % coverage) using phyluce_assembly_match_contigs_to_probes. Baits targeting regions of the mitochondrial genome were disregarded, leaving exclusively nuclear data for the following steps. Matching loci were extracted as fasta files using phyluce_assembly_get_match_counts (settings: incomplete matrix) and phyluce_assembly_get_fastas_from_match_counts. For each locus mafft-alignments were obtained with phyluce_align_seqcap_align, and edges trimmed with mafft-nexus-edge-trimmed under default settings. Internal trimming of the alignments was performed with phyluce_align_seqcap_align and edges trimmed (setting: mafft-nexus-edge-trimmed) followed by GBlocks using trimming using phyluce_align_get_gblocks_trimmed_alignments_from_untrimmed. Alignments were cleaned using phyluce_align_remove_locus_name_from_files. Finally, alignments with different minimum taxonomic completeness were selected, i.e., for each locus, a minimum percentage of the total sample occupancy had to be present. This was performed with phyluce_align_get_only_loci_with_min_taxa with settings of 25–95 % (using increments of 10 %). Based on these, we created alignments with 25 %-95 % taxon completeness per locus for subsequent phylogenetic inference with phyluce_align_concatenate_alignments. Maximum Likelihood (ML) inference was performed with RAxML version 8.2.12 (Stamatakis, 2014) under the GTRGAMMAX model and 1000 rapid bootstraps.

Additionally, we compared our phylogenetic results to the findings of Sorokin et al (2019), who reconstructed the phylogeny of tethyids with 28S rRNA data and a broader taxonomic range than our approach. For this, full 28S rRNA data were extracted from our samples and combined with 28S rRNA data of published non-*Tethya* tethyids, given that sequences were at least 50 % complete and included the variable D1-D5 regions. The data were aligned with MAFFT (Katoh, 2005) as implemented in Geneious Prime 2019.2.3 (Kearse et al., 2012) and analysed using RAxML with 100 rapid bootstrap replicates after removing ambiguous alignable sites from the alignment with Gblocks (Castresana, 2000), allowing "smaller final blocks," "gap positions within final blocks," and "less strict flanking positions." All data were submitted to the European Nucleotide Archive (https://www.ebi.ac.uk/ena, BioProject PRJEB85411) and all alignments are deposited at https://github.com/PalMuc/Tethya-MuseOMICS.

2.5. Morphological analyses

Subsequent morphological analyses of the phylogenomic results were conducted in the Queensland Museum, Brisbane, Australia. Preserved specimens were photographed with a Canon G5X. Surface characteristics were examined using an Olympus SZ60 dissection microscope with a Tucsen 3.0 camera. Thin sections through the ectosome and choanosome of the tissue were cut with a scalpel. The sections were cleared in phenol-xylene overnight and embedded in Fluka Durcopan™ (Sigma-Aldrich Co., St. Louis, MO, USA). Additional preparations of the spicules were made for light microscopy by digesting small portions of sponge in nitric acid over a flame and the remaining spicules were mounted in Canada balsam. Separate preparations of fibers were conducted by the addition of 12.5 % sodium hypochlorite to remove soft tissue. The dissolution of tissue was monitored and facilitated by the removal of excess collagen with forceps, with subsequent neutralization of the reaction with distilled water, and finally two-fold rinsing in 70 % ethanol and then 100 % ethanol. The fixed section and spicule microscope slides were examined using an Olympus BH2 with an optical stage micrometer and photographed with a Nikon CoolPix 5400 mounted camera. Spicule preparations for Scanning Electron Microscope (SEM) were made by dissolving the tissue in 12.5 % sodium hypochlorite for the removal of soft tissue, followed by neutralization in distilled water, rinsing twice in 70 % ethanol, and finally rinsing twice in 98 % ethanol and air-drying the preparations. SEM preparations were sputter-coated in gold to improve resolution. The scanning electron micrograph photos and measurements were made using a Hitachi TM-1000 SEM. In situ, section, and spicule images of the identified species are provided in Supplementary Fig. 1.

3. Results

3.1. Bait design results

OrthoFinder analyses of the *Tethya* spp. proteomes recovered 9,866 single copy genes for *T. wilhelma* and *T. minuta*, and 2,206 single copy genes in a run with all three *Tethya* species, resulting after merging and removal of duplicates in 8,126 single copy genes. Out of these 5,735,400 bait sequences were found, of which 21,024 remained after removing baits with unspecific and multiple hits and minimising the number of required baits per target region. Of these, 20,999 bait sequences rematched successfully with the *T. wilhelma* exome, of which 19,134 could be mapped back to their dedicated genes. These 19,134 bait sequences were combined with similarly identified 343 bait sequences for mitochondrial genomes.

3.2. Sequencing results

In the first run, including 23 pooled samples, a total of 11,757,890 reads were gained from sequencing with reads ranging from 1,134,576 to 129,126 per sample (mean: 511,213 ± 311,209 SD). After trimming 10,892,442 reads remained (ranging from 119,400 to 1,056,462 per sample, mean: 473,584 ± 289,315 SD). In the second run, including 21 pooled samples, we recovered a total of 17,921,160 reads (ranging between 542,918 to 2,714,576 for each sample; mean: 853,389 ± 583,434 SD). After trimming, 16,376,070 reads remained (between 497,140 and 2,416,966 for each sample; mean: 779,813 ± 525,809 SD). For each museum sample, trimmed reads were assembled ranging from 6,326 to 192,675 contigs (mean: 66,542.35 ± 48,921.89 SD) with a total of 1,837,057 to 54,435,909 bp (mean: 21,478,102 ± 15,305,396 bp SD) (Table 1). Museum samples were complemented with target capturing

Table 1
Dimensions of the different taxon per locus alignments. Character increase is the factor compared to the next 10% coverage step.

Coverage (min taxa/locus)	Loci	Characters in matrix (bp)	Samples	Character increase
25 %	2,788	640,778	41	2.06 x
35 %	1,525	310,807	41	1.65 x
45 %	926	188,214	41	1.71 x
55 %	545	110,250	41	1.84 x
65 %	294	59,847	41	2.17 x
75 %	131	27,589	41	2.95 x
85 %	42	9,355	41	38.03 x
95 %	1	246	38	

data from recent material, including *T. wilhelma*, *T. minuta* and a yet unidentified *Tethya* sp. resulting in 74,083 to 211,477 contigs ranging from 50,044,420 to 97,152,515 bp (see all values in Supplementary Table 1).

Re-mapping the bait sequences against the contigs recovered 19 to 4,440 UCEs (mean 1378 ± 1234 SD) in the taxon set. Due to the large differences, we omitted taxa with less than 400 UCEs recovered, leading to a final taxon set of 41 samples. The data of these 41 samples comprised a total of 6,629 UCE loci shared between at least three taxa. The reconstruction of the 8 datasets of 25–95 % taxon coverage per locus resulted in differences in dataset sizes (Table 1), by factor 1.71——2.95 (mean 2.03) for the 41 samples data sets. The 95 % taxon coverage dataset comprised only 38 taxa and was omitted from phylogenetic analyses due to insufficient genomic loci. Correlation analysis indicated that for the present sample set higher collection age had no negative influence on the loci number yielded in the approach (R = -0.250, Fig. 1).

3.3. Phylogenetic results of UCE data

We reconstructed UCE-phylogenetic trees for seven of the eight coverage sets (25 %, 35 %, 45 %, 55 %, 65 %, 75 %, and 85 %). All seven trees share a high degree of congruence and displayed high bootstrap for basal but also derived nodes (Fig. 2). Node support mostly decreases with a lower number of UCE data in the data set (i.e., a higher number of minimum taxa per locus), despite the subsequent decrease of missing data. All trees displayed a well supported deep split with a clade of *Stellitethya extensa* and *Tethycometes radicosa* with *Tethya samaaii* and other *Tethya* spp., resulting in nonmonophyletic *Tethya*. The genus *Tethycometes* has been successfully sequenced for the first time. Sister group to this *Stellitethya* + *Tethycometes* + *Tethya* spp clade is a large clade comprising several *Tethya* species: The largest clade consists of *T. bergquistae* that resemble (isolate BB128, Shaffer et al., 2019) in Shaffer et al. (2019) based on mitochondrial 16S rRNA and cytochrome *c* oxidase subunit I (COI) (I3M11 partition sensu Erpenbeck et al., 2006b). The other, previously undetermined, *Tethya* samples fell in highly supported clades that could subsequently be determined as *T.* cf. *ingalli*, *T. seychellensis*, *T. burtoni*, *T. robusta*, *T. communis*, *T.* (cf.) *fissurata*, and *T.* cf. *levii* (Fig. 2).

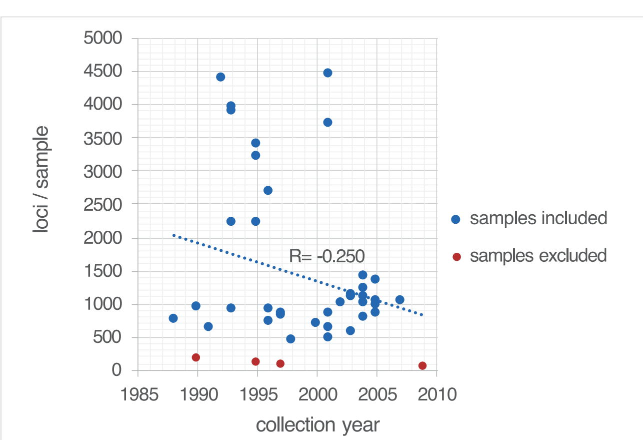

Fig. 1. Collection year of the Queensland Museum samples plotted against the subsequent UCE yield. The samples in red dots were not included in the phylogenetic analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Comparison with full-length 28S rRNA data

Full-length 28S rRNA data could be extracted from out of 25 samples, besides shorter 28S rRNA regions, covering the phylogenetic particularly informative D1-D5 regions of 28S rRNA. The INSDC-published 28S rRNA data of the non-*Tethya* tethyids *Xenospongia patelliformis* (KC869650), *Stellitethya ingens* (AY561899), and *Tectitethya keyensis* (KC869616) possessed more than 50 % of the complete 28S rRNA data, and were included in the data set (Nichols, 2005; Thacker et al., 2013). The sequence of *Laxotethya dampierensis* (AY561905 and AY626307) appeared only incompletely published in INSDC Genbank and was not included in the data. The final data set comprised 34 taxa and 2,732 characters (after Gblocks). Phylogenetic reconstruction strongly supports our UCE data, confirming the non-monophyly of *Tethya*, and recovers the other non-*Tethya* tethyid genera as sister group to different *Tethya* lineages consistent with the findings of Sorokin et al. (2019) and Santodomingo et al. (2024), but now with high bootstrap support at all relevant nodes (Fig. 3).

3.5. Mitochondrial data

Besides the nuclear UCEs, the present approach enabled us to capture mitochondrial genomic data that partially could be assembled to full mitochondrial genomes for 22 taxa, including the hemiasterellid outgroups *Liosina paradoxa* (QM G312681), and *Axos flabelliformis* (QM G303324), furthermore *Stellitethya extensa*, *Tethycometes radicos*, and 18 *Tethya* species. The full mitochondrial genomes displayed for all taxa possess the same gene order, including tRNA genes, as published earlier for Tethyidae (Lavrov et al., 2005) (Fig. 4). The mitochondrial genomes have size ranges between 19,339 bp (*T. minuta*) and 24,052 bp (*Stellitethya extensa*). A maximum-likelihood tree of the protein-coding regions is provided in Supplementary Fig. 2. The full mitochondrial tree differs from the UCE and full-length 28S rRNA phylogenies in the position of *T. bergquistiae* and shows lower support at deeper nodes compared to the UCE tree.

In four taxa COI group I intron sequences of the class IB were detected, encoding a homing endonuclease gene (HEG) of the LAGLI-DADG family. Several taxa were found to contain multiple COI introns: We found three introns in *Stellitethya extensa* (ORF 387, ORF 723, ORF 870 sensu Erpenbeck et al., 2015; Schuster et al., 2017) and two in *Axos flabelliformis* (ORF 387, ORF 870) and one in *Liosina paradoxa* (ORF870). We also detected in the COI sequence of *Tethya communis* (QM G307066) of an ORF 870 intron, however this intron could only be recovered partially (588 bp).

4. Discussion

4.1. Taxonomic screening of sponge collections with target-enriched multilocus assays

We present here a successful target-enriched multilocus assay attempted on a largely unidentified museum collection of sponges. Our results confirm that target capturing multilocus approaches are well-applicable on decades-old sponge sample collections, which were not necessarily collected for molecular genetic or genomic purposes. These collections represent an unparalleled source of data collected during the

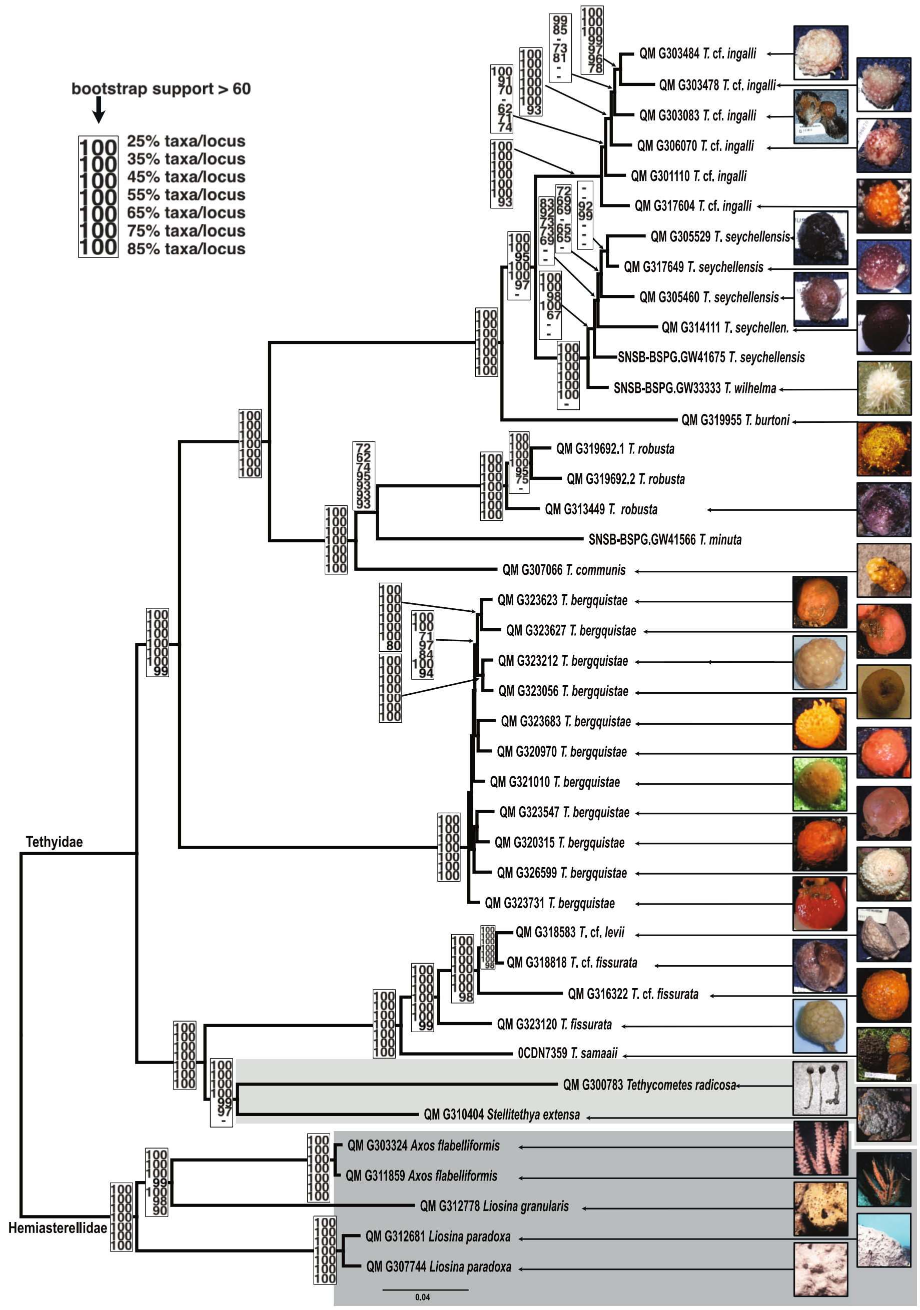

Fig. 2. Maximum likelihood UCE tree with min. 55 % taxa / locus. Numbers on branches are bootstrap values > 60 for the phylogenetic reconstructions of the other min. taxa / locus data sets with 25 % (top) to 85 % (bottom). Shaded taxa are Hemiasterellidae outgroups (dark grey) or non-*Tethya* Tethyidae (light grey). Only Genus *Tethya* is abbreviated with "*T*.". Pictures on the right are *in situ* or deck photos of the specimens after collection. Scale bar depicts substitutions per site.

last 200 years and can be leveraged in biodiversity and taxonomic research (Bi et al., 2013; Wandeler et al., 2007). Target-enriched multilocus assays comparably outcompete approaches on collection material in sponges that rely on only a single or few loci and are subsequently more prone to missing data or consist of difficult amplifiable material (Erpenbeck et al., 2016; Vargas et al., 2012). The bait design intentionally minimises co-sequencing of sponge associated symbionts and commensals, in contrast to genome skimming methods, which do not discriminate among DNA sources. Although the present bait set was developed as a test case primarily designed for *Tethya* and closely related tethyid outgroups, it is not necessarily restricted to Tethyidae and may also be applicable to other demosponge groups. However, broader taxonomically designed bait sets (e.g., Van der Sprong et al. 2024) would be more suitable for non-tethyid demosponge collections.

On average we target-enriched almost 1,400 loci per museum sample (mean 1,378 ± 1234 SD) collected within the last 36 years. This range parallels the yield reached in similar approaches on the anthozoans *Sinularia* (1,946 ± 227 SD) and *Alcyonium* (1,863 ± 177 SD) collected 1989 and 1996 from locations in the North Atlantic and Mediterranean, however with a smaller standard deviation than our approach (Erickson et al., 2021). This difference might be due to the considerably larger number of baits used in the Erickson et al. study (29,181) compared to 19,134 baits in our study (65 % of Erickson et al. total). In contrast, our bait set was designed to target a considerably more restricted number of loci (3,023 loci in Erickson et al.) compared to 7,755 loci (256 %) – which may explain the variability in enrichment success across samples..

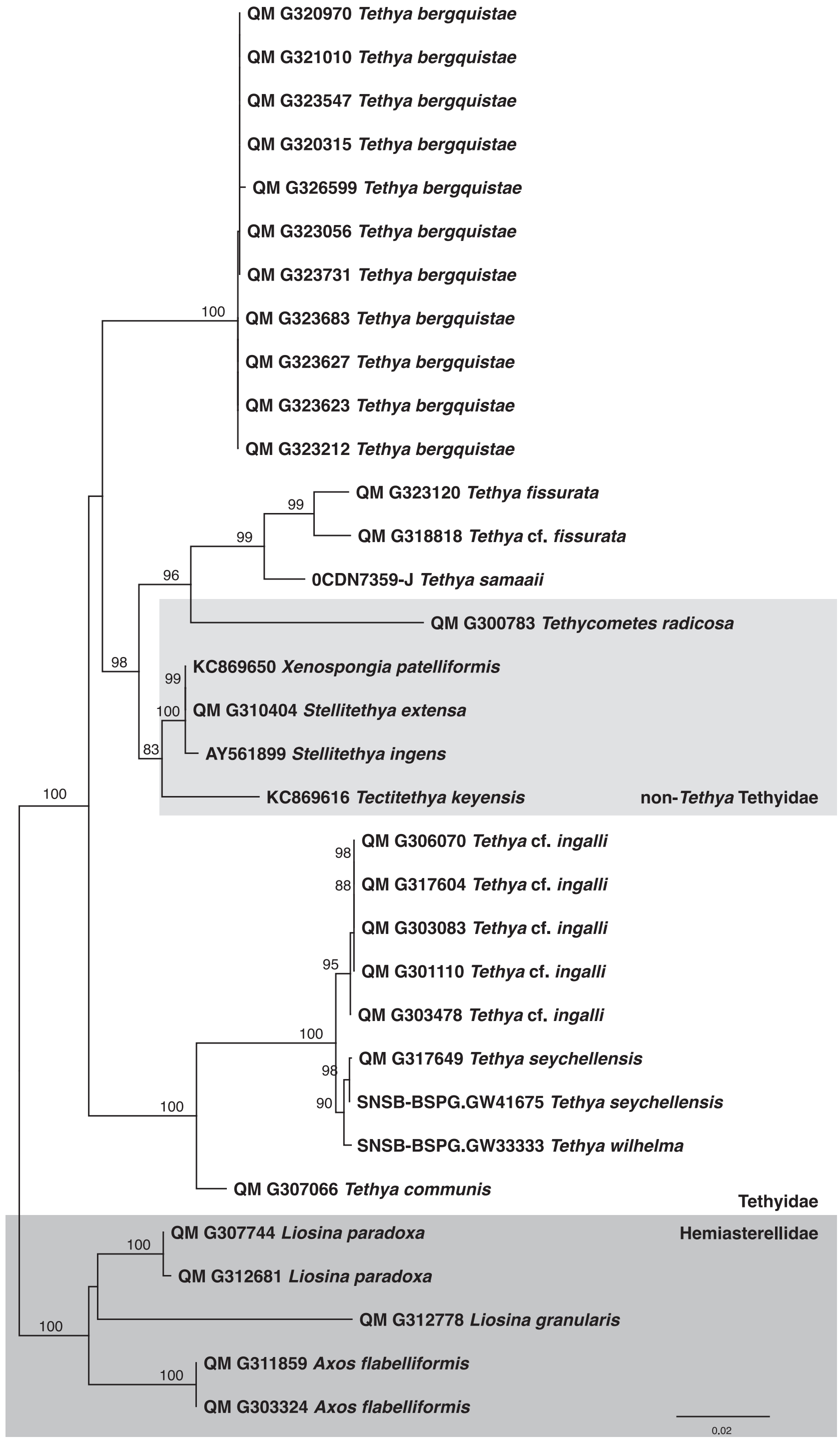

Fig. 3. Maximum likelihood tree of nearly complete 28S rRNA. Numbers on branches are bootstrap values > 80. Shaded taxa are Hemiasterellidae outgroups (dark grey) or non-*Tethya* Tethyidae (light grey). Scale bar depicts substitutions per site.

4.2. *Phylogenetic robustness increases compared to single locus approaches*

While other, particularly single or few marker analyses struggle to gain support in the deeper nodes, the phylogenies based on target enrichment data displays high levels of support throughout the topology. We observe that the node support is higher with a higher number of UCE data in the data set (= higher number of minimum taxa per locus), despite the subsequent increase of missing data. The resulting increase of characters and loci by factor 2.03 contributes more to the robustness of the branch than the 10 % increase of missing data hampers the support, which is beneficial when equal data coverage across the taxon set is difficult, e.g., archival DNA (Raxworthy and Smith, 2021).

4.3. *Genetic data is indispensable even in character-rich demosponge taxa*

Our data demonstrated that superficial screening based on morphospecies is insufficient to classify tethyid sponges. For example, recent suggestions to discriminate *Tethya* clades by colour (e.g., Sorokin et al., 2019) are clearly refuted by our findings (Fig. 1) (see also Santodomingo

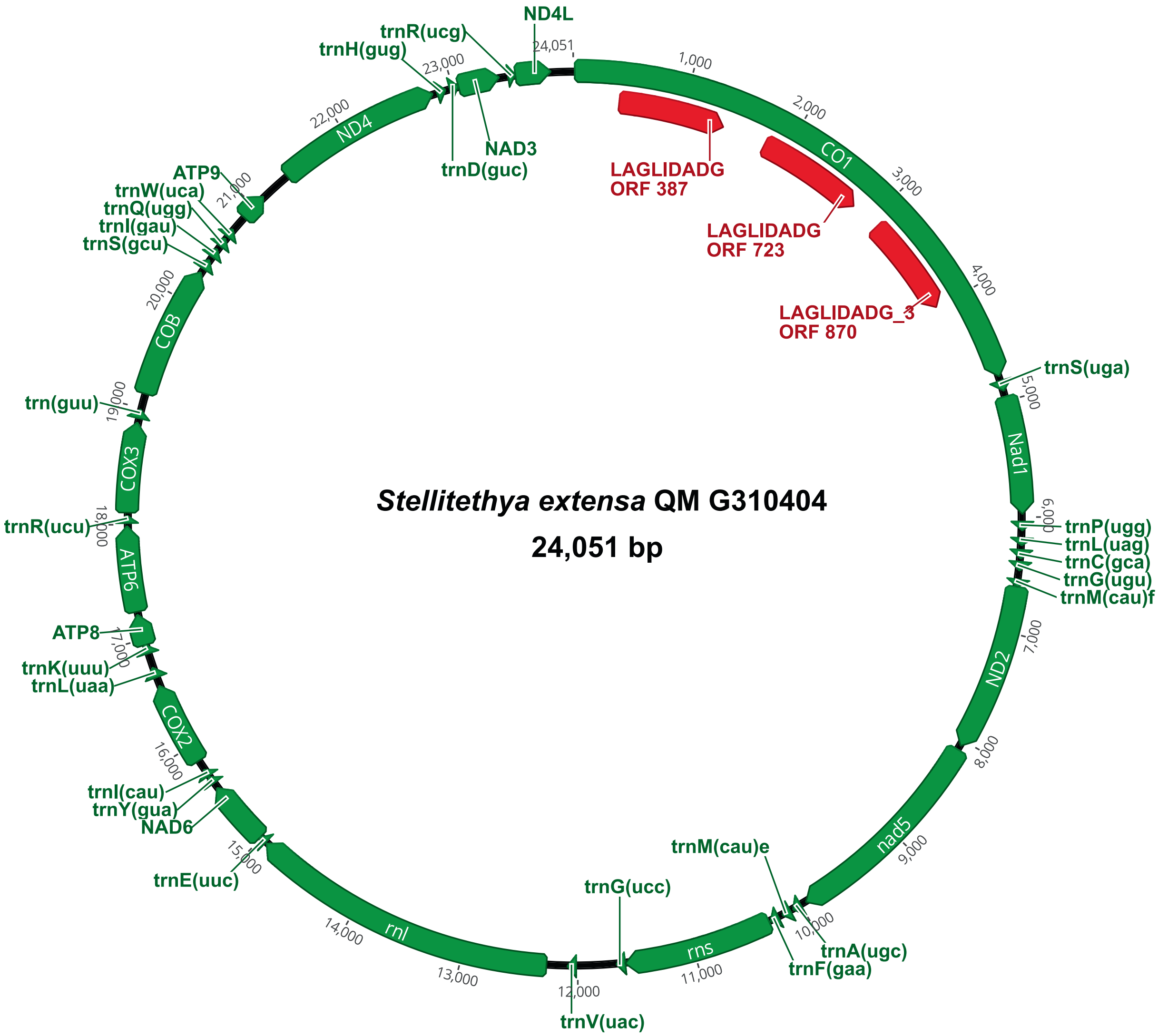

Fig. 4. The mitochondrial genome of *Stellitethya extensa*. Green annotations are genes (protein coding, ribosomal, and tRNA genes). In red the position of the CO1 group I intron sequences of the class IB, encoding a homing endonuclease gene (HEG) of the LAGLIDADG family. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al. 2024 for a more detailed discussion). While *Tethya* is using different carotenoids that facilitate the characteristic ectosomal coloration—partially self-synthesised and independent of symbionts (Liaaen-Jensen et al., 1982; Tanaka et al., 1982)—our data underline the heterogeneity in the coloration as discriminatory factor, and support the findings on colour inconsistencies in *Tethya bergquistae* and *T. burtoni* (Shaffer et al., 2019), which prompt for further investigation of the genetic control of coloration (Santodomingo et al. 2024). Additionally seasonal morphological changes of *Tethya*, e.g., due to interaction with coralline algae change the surface appearance of the specimens considerably within few weeks (Bergquist and Kelly Borges, 1991), and are in combination with the detection of cryptic species [e.g.,*T. burtoni* (Shaffer et al., 2019)] sufficient indication to refrain from morphology-only taxonomy of *Tethya*, or sponges in general.

The phylogenetic relationships reconstructed from the present samples are in line with some earlier, morphology-based hypotheses. For example, the morphology-based studies of Sarà (1988), and Sarà and Sarà (2004) suggested a *Tethya* “subgroup” consisting of *T. seychellensis* and *T. ingalli.* This morphological subgroup is recovered with high support in our current target-enriched multilocus assay. *Tethya seychellensis* is a widespread species reported from Indo-West Pacific and Central West Atlantic localities, and likely forms a species complex (Van Soest and Beglinger, 2008). In this respect, *T. wilhelma* was morphologically regarded as a part of this “*T. seychellensis* species group” (cf. Sará et al., 2001). This was supported by Heim et al. (2007) based on partial mtDNA COI gene sequences, and here in our current approach. Likewise, *T.* (cf.) *levii's* similarity to *T. fissurata* (see Sarà, 1988) is reflected in our phylogenetic reconstruction. However, several other groupings that are strongly supported in our trees (e.g., *T. communis*-(*T. minuta*)-*T. robusta*), are divergent from previous morphological predictions, which underlines that genetic data in sponge phylogenetics are indispensable, even in character-rich demosponge taxa such as Tethyidae.

4.4. *Tethya* is a founder genus of (many) Tethyidae

Tethyidae are currently classified into 14 genera, several of which possess a stalk that can exceed the body diameter by several times, including *Tethycometes* (Lim and Tan, 2008; Sarà, 2002). The presence, and particularly the metrics of the stalk, are important discriminatory characteristics between several Tethyidae genera (Sarà, 2002; Sara, 1994). Sorokin et al. (2019) criticised that the development of a stalk in Tethyidae was acknowledged as adaptation to deep-water, soft sediment habitat (Sara and Burlando, 1994), but was nevertheless regarded as discriminatory character for genus-level classification. As ecological adaptations in *Tethya* appear to have convergently evolved (Heim et al., 2007), the many different modes of *Tethya* attachment, i.e., basal roots, rooting processes, basal stolons, curved peduncles, attachment discs and narrow skirts of tissue to name a few, could have given rise to those stalked forms, which are currently classified as separate genera (Sorokin et al., 2019). In their 28S rRNA phylogenetic analysis Sorokin et al. (2019) and Santodomingo et al. (2024) revealed paraphyletic *Tethya* grouping with *Xenospongia*, *Stellitethya*, *Tectitethya* and *Tethytimea*, however support values were mostly low. Our UCE data analysis strongly corroborates this hypothesis by recovering *Tethya* as paraphyletic with respect to *Stellitethya* and *Tethycometes radicosa* (Fig. 3), which has been sequenced here for the first time. Also, the full-length 28S rRNA data yielded from our target capturing approach, now support *Xenospongia*, *Tectitethya* and *Tethytimea* descending from a *Tethya* ancestor. *Tethya* can therefore be regarded as the founder for *Xenospongia*, *Tectitethya, Stellitethya* and *Tethycometes,* prompting the need of reclassification of this family, and the re-evaluation of the morphological traits.

4.5. Mitochondrial introns in sponges: Haphazard occurrence, but not rare at all

A further by-product of our approach is the discovery of COI group I intron sequences of the class IB, encoding a homing endonuclease gene (HEG) of the LAGLIDADG family. These introns have yet speculative origin and modes of distribution and have been reported from a haphazard distribution of sponges. After their discovery in present-day Tetractinellida (Szitenberg et al., 2010), they have been sporadically reported from Homoscleromorpha and the demosponge orders Axinellida, Agelasida, Bubarida, Clionaida, Verongida, and recently from Tethyida (Lavrov et al., 2023). Within Tethyida we have now detected these introns in two further genera of Hemiasterellidae (*Liosina* and *Axos*) but also for the first time in Tethyidae (*Tethya* and *Stellitethya*). Also, the presence of three group I introns in a single species is documented for the first time. So far, only up to two group I introns were reported, or in combination with group II introns (Huchon et al., 2015; Schuster et al., 2017)—now we find the three group I introns ORF 387, ORF 723 and ORF 870 simultaneously in *Stellitethya extensa*. The ongoing (phylo)mitogenomic techniques, including our target capturing approach will undoubtedly reveal many additional occurrences of mitochondrial introns in COI and other mitochondrial genes (Jenkins et al., 2022; Lavrov et al., 2023), unmasking them still as haphazard, but rather common occurrences.

CRediT authorship contribution statement

Dirk Erpenbeck: Writing – original draft, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Fabian Deister:** Methodology, Investigation, Formal analysis. **Merrick Ekins:** Writing – review & editing, Resources, Investigation. **Nora Dotzler:** Formal analysis. **John N.A. Hooper:** Resources. **Toufiek Samaai:** Writing – review & editing, Resources. **Simone Schätzle:** Methodology. **Joëlle van der Sprong:** Writing – review & editing, Methodology. **Sergio Vargas:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Conceptualization. **Gert Wörheide:** Writing – review & editing, Resources, Methodology, Investigation, Funding acquisition, Conceptualization.

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.

Acknowledgements

We are grateful to Stefanie Ries for help in the Lab, Swee-Cheng Lim for help with morphological questions, and Megan Shaffer for fruitful discussions. This work was funded by the German Research Foundation (DFG, to G.W. [project WO896/19-1], SV [project VA1146/3-1] and DE [project ER611/5-1] within the DFG SPP 1991 “Taxon-Omics”). This work includes contributions from the Coral Reef Research Foundation, Koror, Palau (CRRF) and we thank Lori Bell and Patrick Colin for the collection of specimens made in South Africa and donation of specimens by Prof. Micheal Davis-Coleman, Rhodes University, Chemistry Department to Toufiek Samaai (TS collection). Collections by the Coral Reef Research Foundation were made under contract to the United States National Cancer Institute Contract no. N01–CM–27704. The Government of South Africa granted CRRF permits for collection, and they were made through the co-operation of Prof. Mike Davies-Coleman, then at Rhodes University.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2025.108436.

Data availability

All data are available at the European Nucleotide Archive (https://www.ebi.ac.uk/ena, BioProject PRJEB85411), and all alignments are deposited at https://github.com/PalMuc/Tethya-MuseOMICS.

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