



# Hidden diversity in the genus *Tethya*: comparing molecular and morphological techniques for species identification

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## Abstract

Correctly determining species' identity is critical for estimating biodiversity and effectively managing marine populations, but is difficult for species that have few morphological traits or are highly plastic. Sponges are considered a taxonomically difficult group because they lack multiple consistent diagnostic features, which coupled with their common phenotypic plasticity, makes the presence of species complexes likely, but difficult to detect. Here, we investigated the evolutionary relationship of *Tethya* spp. in central New Zealand using both molecular and morphological techniques to highlight the potential for cryptic speciation in sponges. Phylogenetic reconstructions based on two mitochondrial markers (rnl, COI-ext) and one nuclear marker (18S) revealed three genetic clades, with one clade representing *Tethya bergquistae* and two clades belonging to what was a priori thought to be a single species, *Tethya burtoni*. Eleven microsatellite markers were also used to further resolve the *T. burtoni* group, revealing a division consistent with the 18S and rnl data. Morphological analysis based on spicule characteristics allowed *T. bergquistae* to be distinguished from *T. burtoni*, but revealed no apparent differences between the *T. burtoni* clades. Here, we highlight hidden genetic diversity within *T. burtoni*, likely representing a group consisting of incipient species that have undergone speciation but have yet to express clear morphological differences. Our study supports the notion that cryptic speciation in sponges may go undetected and diversity underestimated when using only morphology-based taxonomy, which has broad scale implications for conservation and management of marine systems.

## Introduction

Correctly identifying species units is critical for estimating biodiversity, defining species boundaries and measuring connectivity patterns (Hey et al. 2003; Frankham et al. 2012). Methods for species identification have been so hotly debated in the literature that the phrase 'species problem' has emerged to describe the conundrum that biologists face when defining a species (Mayden 1997; De Queiroz 2007). Mayr (1942) was the first to address this problem by conceiving the biological species concept, where he defined a species as a group of interbreeding individuals which are

reproductively isolated from other groups. Since then, other concepts have emerged based on: an organism's functional role/ecological niche (ecological species concept, Van Valen 1976); historical ancestry lineages (evolutionary species concept, Simpson 1951); and the sharing of common ancestors/alleles (phylogenetic species concept, Hennig 1966; Rosen 1979; Baum and Shaw 1995; reviewed in De Queiroz 2007). While many concepts exist, the most traditional, widespread and consistently employed method for taxonomy is based on comparative morphology (Padiál et al. 2010).

The recent advancement and accessibility of molecular tools has begun to reveal conflicting evolutionary histories for many species previously defined by morphological characteristics (Wendel and Doyle 1998; Knowlton 2000; Lecompte et al. 2005; Lihová et al. 2007; Wallis et al. 2017). Distinguishing between intraspecific plasticity and interspecific cryptic diversity can be problematic when only using morphology-based taxonomic methods (Knowlton 1993; Vrijenhoek 2009; Capa et al. 2013, Czarna et al. 2016). Additionally, congeneric species that live in sympatry often challenge the notion of species boundaries

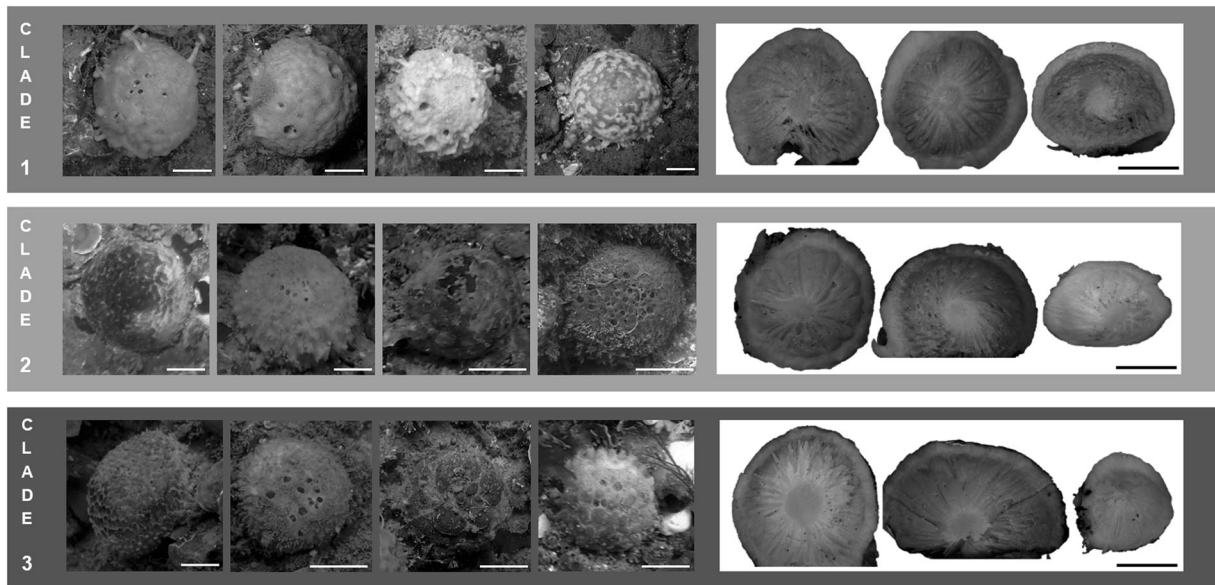
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**Fig. 1** Examples of sponges collected belonging to clades identified in Fig. 3, to allow comparison of macromorphological features. For each clade, cross sections that encompass the ectosome and choanosome are included for a subsample ( $n = 3$ ) of genetically identical individuals.

Clade 1 (top) are examples of *Tethya bergquistae*, while Clades 2 and 3 (middle and bottom, respectively) are examples of *T. burtoni*. Scale bars in each photo = 10 mm

because introgression (i.e. where alleles from one species enter the gene pool of another species via hybridization and backcrossing) is common (Harrison and Larson 2014). Failure to correctly identify species boundaries has consequences for implementing effective conservation and management strategies and for our overall understanding of important evolutionary processes like speciation (Gómez et al. 2002), bioinvasions (Knapp et al. 2015) and hybridization (Kausserud et al. 2006).

Organisms that have only a few defining traits available for comparison are particularly prone to identification problems (Knowlton 1993; Bickford et al. 2007; DeBiasse and Hellberg 2015). In such groups, cryptic species often emerge during molecular studies, as sequences provide more characteristics (bases) with which to differentiate organisms, which allows a finer resolution of relatedness between individuals to be determined (Hillis 1987; Hebet et al. 2004; Bickford et al. 2007). Sponges have relatively few morphological traits, yet a rich taxonomic literature exists delineating species boundaries within sponges based on phenotypic characteristics (Hooper and Van Soest 2002). Skeletal composition, arrangement and spicule morphology are key features used to classify and identify sponges (Hooper and Van Soest 2002), despite the fact that structural components of sponges can be highly dynamic (Bond 1992; Nakayama et al. 2015) and spicules can exhibit variability under different environmental conditions (Bavestrello et al. 1993; Bell et al. 2002; McDonald et al. 2002). Furthermore, recent studies on sponge taxonomy

have highlighted problems with using morphology as a sole defining trait and have used phenotypic traits combined with molecular analyses to resolve evolutionary relationships (Alvarez et al. 2000; Erwin and Thacker 2007; Morrow and Cárdenas 2015; Plotkin et al. 2017). As a result, phylogenetic analyses for many sponges have begun to reveal cryptic speciation throughout the phylum, suggesting that diversity within the phylum is even higher than previously thought (see Table 1 in Xavier et al. (2010) for reported cases of cryptic speciation in sponges, plus Andreakis et al. 2012; De Paula et al. 2012; DeBiasse and Hellberg 2015; Knapp et al. 2015; Uriz et al. 2017).

In this study, we used ‘golf ball sponges’ belonging to the genus *Tethya* in New Zealand as a model to investigate cryptic speciation and to better understand how evolutionary processes occur for species living in sympatry. *Tethya* is a ubiquitous genus, with 92 described species worldwide (Van Soest 2008), and has a particularly high diversity (31 species) recorded in coastal waters of Australia and New Zealand (Bergquist and Kelly-Borges 1991; Sarà 1998; Sarà and Sarà 2004; Heim et al. 2007). While members of the genus are easily distinguishable by their globular form, identifying *Tethya* to the species level can be difficult because they exhibit a high degree of morphological plasticity, and have only a few defining traits available for species differentiation. The challenge in species identification is reflected in the dynamic taxonomic literature, where new species are constantly being described (Corriero et al. 2015), and previously described species are regularly

reconsidered and reclassified (Sarà 1987; Bergquist and Kelly-Borges 1991; Hooper and Wiedenmayer 1994; Sarà and Sarà 2004; Heim et al. 2007). In New Zealand, taxonomy has been based solely on morphology with no genetic phylogenies constructed to date (Bergquist and Kelly-Borges 1991; Sarà and Sarà 2004). Here, we used two common species of *Tethya* in New Zealand (*Tethya bergquistae* and *Tethya burtoni*) as models to investigate evolutionary relationships and cryptic speciation using both genetic (COI-ext, rnl, 18S and 11 microsatellite markers) and morphological traits (spicules) in order to understand the advantages and limitations of both methods in determining species boundaries.

## Materials and methods

### Study species

*Tethya bergquistae* and *T. burtoni* are two of the most common species of *Tethya* found in the waters of central New Zealand, and both occur in the waters around Wellington, New Zealand (Fig. 1). *Tethya bergquistae* is described as rose-pink in colour, firm to the touch and with an irregular surface with grouped oscula (Bergquist and Kelly-Borges 1991; Hooper and Wiedenmayer 1994; Battershill et al. 2010). *Tethya burtoni* is bright orange to yellow and is described as having a warty surface with an inflated, soft texture (Sarà and Sarà 2004; Battershill et al. 2010). *Tethya bergquistae* is generally found in well-lit areas with moderate flow, whereas *T. burtoni* tends to prefer more shaded, sedimented areas (Battershill et al. 2010). Spicules for both include megascleres, which are strongyloxeas; and microscleres, which are megasters and micrasters of similar sizes (Bergquist and Kelly-Borges 1991; Sarà and Sarà 2004). A key distinguishing feature between both species relies on their spicular composition with respect to the location/density of megasters, as *T. burtoni* contains fewer megasters, which are mainly found in the cortex (Bergquist and Kelly-Borges 1991; Sarà and Sarà 2004).

Tracking the description of both species through the literature reveals a complex taxonomic classification history within New Zealand, highlighting the complexity in species identification common to Porifera. For instance, *T. bergquistae* was incorrectly identified as *Tethya ingalli* by Pritchard et al. (1984) and formerly described as *Tethya australis* by Bergquist and Kelly-Borges (1991), and *T. burtoni* has been also incorrectly referred to as *Tethya aurantium* by Pritchard et al. (1984). Overall in New Zealand, there are ten species of *Tethya* recorded, but two of these are also thought to exist in Australian waters

(Ledenfield 1888; Bergquist 1961; Bergquist and Kelly-Borges 1991; Sarà and Sarà 2004). Many of these ten species are thought to be endemic to certain regions (e.g. *Tethya compacta* to the Chatham Islands and *Tethya fastigata* to the Poor Knight Islands), but this has been based on observations from only one to a few sponges (e.g. *T. compacta*), or on descriptions dating from the 19th Century (e.g. *Tethya multistella*). In the literature, pictures identifying New Zealand *Tethya* are often inconsistent and classification by comparative morphology is therefore challenging. Descriptions of species within *Tethya* are based on cross sections and spicule compliments, but differences can be often subtle and difficult to detect, especially for non-specialists. Around New Zealand, sympatric *Tethya* spp. display a wide range of colours and textures, making them difficult to confidently match to descriptions in the current taxonomic literature. *Tethya* also appear to undergo seasonal morphological changes (M. Shaffer, personal observations from monthly monitoring for 2+ years). For example, the same sponge's appearance may go from porous and soft to very firm over a period of a month. In addition, during the summer a sponge may be covered more extensively by crustose coralline algae (CCA), rendering it more reminiscent of *Tethya amplexa* (Bergquist and Kelly-Borges 1991), but in the winter be completely free of CCA. Because morphology can be very variable and temporally dependent, assessing species based solely on morphology has the potential to lead to erroneous conclusions. To date, molecular differences have allowed at least one other instance of sibling species within the genus *Tethya* to be uncovered. Sarà et al. (1993) reported cryptic speciation in two populations of *Tethya robusta*, which were morphologically similar but genetically different. The taxonomic history, phenotypic plasticity and previous report of a species complex within the *Tethya* genus makes other occurrences of cryptic speciation within this group likely.

### Collection

Sponges which were a priori thought to be *T. bergquistae* and *T. burtoni* were collected using SCUBA from two different locations in waters around Wellington, New Zealand (Breaker Bay: 41°19'53.3"S 174°49'52.6"E; Kapiti Island: 40°53'23.6"S 174°52'40.3"E). Additional specimens of *T. burtoni* were collected from two further locations (Somes Island: 41°15'36.9"S 174°43'54.4"E; Red Rocks: 41°21'04.5"S 174°43'54.4"E). Sponges were collected 3–5 m apart to avoid collecting clones, at depths from 5 to 10 m. Three sponges of unknown identity were collected from Breaker Bay ( $n = 2$ ) and Kapiti Island ( $n = 1$ ), which were texturally similar to *T. bergquistae* but the same colour as *T. burtoni*. A picture of each specimen was taken

**Table 1** Primer information for markers used: 18S, rnl and COI-ext. Forward and reverse sequences presented 5' → 3'. Product sizes are in base pairs. Accession numbers refer to distinct haplotypes and genotypes that were deposited to GenBank for each marker.

| Region  | Primers   | Size (bp) | Ref.               | Accession number of products |
|---------|---|-----------|--------------------|------------------------------|
| 18S     | 18S_1-600_F:<br>GCCAGTAGTCATATGCTTGTCTCA<br>18S_1-600_R:<br>GACTTGCCCTCCAATTGTTTC     | 425       | Knapp et al. 2015  | MH180010-MH180012            |
| rnl     | diplo-rnl-f1:<br>TCGACTGTTTACCAAAAACATAGC<br>diplo-rnl-r1:<br>AATTCAACATCGAGGTSGGAAAC | 570       | Lavrov et al. 2008 | MH180013-MH180019            |
| COI-ext | COX1-R1:<br>TGTTGRGGAAAAARGTTAAATT<br>COX1-D2:<br>AATACTGCTTTTTTGTATCTGCCGG           | 425       | Rot et al. 2006    | MH180020-MH180023            |

to aid in later identification. Tissue was extracted for both genetic and spicule analysis, and the rest of the specimen was preserved in ethanol. In total, 71 sponges of various morphologies were collected for phylogenetic and morphological analyses. For those sponges assumed to be *T. bergquistae*, samples were collected from Breaker Bay ( $n = 10$ ) and Kapiti Island ( $n = 5$ ). For those sponges assumed to be *T. burtoni*, samples were collected from Breaker Bay ( $n = 17$ ), Kapiti Island ( $n = 20$ ), Red Rocks ( $n = 5$ ) and Somes Island ( $n = 12$ ). For information on specimens collected for this study, see Table S1 in the Supplementary Information.

### DNA extraction, PCR amplification, sequencing and fragment analysis

Tissue used for genetic analysis was taken from the inside (choanosome) of the sponge and rinsed thoroughly to reduce the risk of contamination from epibionts or other associated organisms. DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen), following the protocol of the manufacturer. We tried five primer sets, but two regions failed to consistently give readable sequences across all samples (ITS, primer set: ITS2.2/ITSRA2, as in Adlard and Lester 1995; and COI, primer set: dgLCO1490/dgHCO2198; Folmer et al. 1994), so were discarded for further analysis. Three markers were amplified in total: 18S, rnl and COI-ext. 18S is a nuclear gene that codes ribosomal DNA (rDNA), and rnl is a mitochondrial partition that codes the large ribosomal subunit RNA (Boore 1999). COI-ext is a region downstream of COI, which is a mitochondrial coding region for the cytochrome *c* oxidase subunit used in cellular metabolism (Folmer et al. 1994). This extended region is thought to be more informative for closely related species because its substitution pattern (mainly consisting of transversions) reveals a more progressive stage of character evolution (Erpenbeck et al. 2006a). As

such, this region exhibits variation during early stages of species divergence compared to the traditionally conserved COI region (by Folmer et al. 1994) that is commonly used (Erpenbeck et al. 2006a). Primer information and product sizes are shown in Table 1.

Reaction and cycling conditions were similar for all three markers. Reactions were carried out in 30  $\mu$ l volumes consisting of: 15  $\mu$ l MyTaq Red Mix (Bioline), 0.6  $\mu$ l of each primer (10  $\mu$ M), ~25 ng template DNA and a volume of distilled water to reach 30  $\mu$ l. Amplification profiles were as follows: an initial denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 30 s,  $T_a$  for 55 s ( $T_a = 50$  °C for 18S;  $T_a = 58$  °C for COI-ext and rnl), 72 °C for 45 s; followed by a final extension at 72 °C for 7 min. Products were visually checked on a 1.5% agarose gel stained with RedSafe Nucleic Acid Staining Solution (20,000 $\times$ ) and sequenced using automated DNA sequencing services provided by Macrogen, Inc. (Seoul, South Korea). Sequences were deposited into GenBank, with accession numbers for haplotypes of all individuals provided in Table S1 in the Supplementary Information.

Eleven microsatellite markers were developed for *T. burtoni* (see Supplementary Information for microsatellite development and Table S2 for microsatellite information). These markers failed to amplify for *T. bergquistae*. Fragment analysis was performed on sponges belonging to the *T. burtoni* clades. Reactions were carried out in 30  $\mu$ l reactions, with 15  $\mu$ l MyTaq Red Mix (Bioline), 0.6  $\mu$ l of each primer (10  $\mu$ M), ~25 ng template DNA and a volume of distilled water to reach 30  $\mu$ l. Amplification profiles were as follows: an initial denaturation at 94 °C for 10 min; 35 cycles of 94 °C for 45 s, 60 °C for 55 s, 72 °C for 60 s; followed by a final extension at 72 °C for 10 min. Fluorescently labelled products were visually checked, pooled, purified and then genotyped using the 3730xl DNA Analyzer (Applied Biosystems) from GeneScan Services for fragment analysis by Macrogen Inc. (Seoul, South Korea).

Alleles were visualized in GeneMarker v2.2 (Hulce et al. 2011) and scored manually relative to a size standard (LIZ500). Some sponges that were thought to be *T. burtoni* had a lower amplification success for some loci, even after redoing the DNA extraction and fragment analysis two to three times. Because of this, missing information was considered informative in analyses.

## Genetic analysis

All sequences were checked for contaminants and verified as being poriferan using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences were edited in Geneious 11.0.2 (Kearse et al. 2012) and aligned using CLUSTALW2.0 (Larkin et al. 2007), as implemented in Geneious 11.0.2 (Kearse et al. 2012). Phylogenetic analyses were applied to 18S, rnl and COI-ext separately, as well as to the mitochondrial partitions concatenated (COI-ext + rnl), creating four data sets. MrModeltest v2.3 (Nylander 2004) was used to select the best-fitting model of nucleotide substitution for each data set under the Akaike information criterion, and it was determined that the SYM + G model was the best fitting model for all data sets. The closely related *Tethya actinia* was chosen as an outgroup because both the 18S region and its complete mitochondrion genome have been sequenced and made available on GenBank, making it an ideal candidate to root all three markers (accession numbers: AY320033 for rnl and COI-ext; AY878079 for 18S).

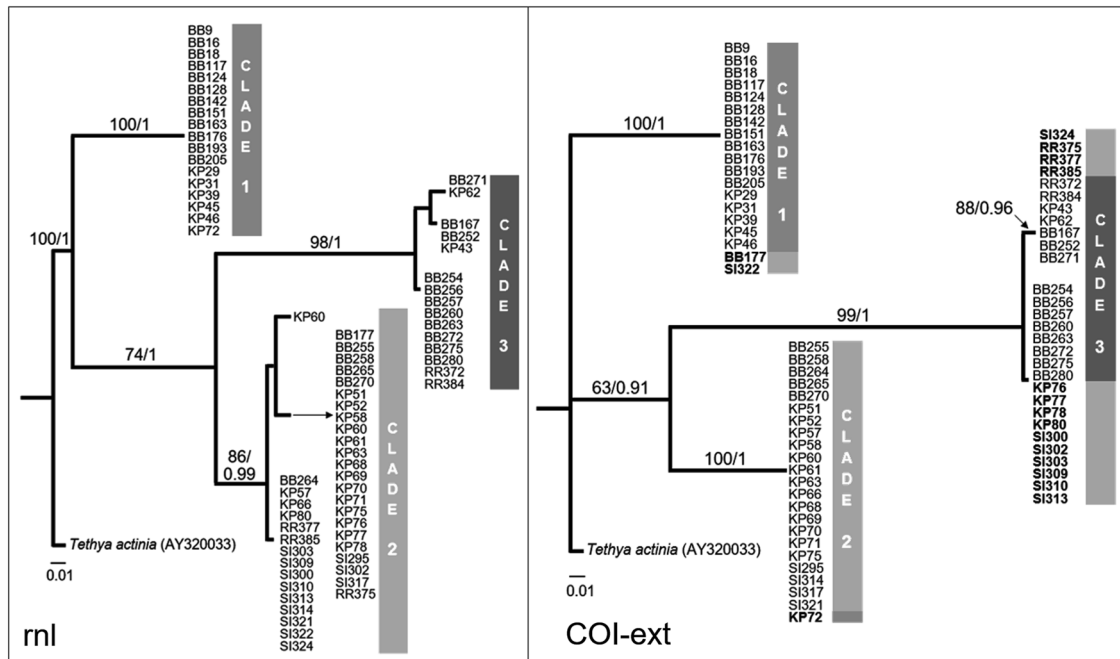
Phylogenetic trees were constructed using both Maximum Likelihood (ML) and Bayesian Inference (BI) methods for each data set. ML constructions were performed in PAUP\* 4.0 (Swofford 2002), and BI constructions were performed in MrBayes 3.2.6 (Ronquist et al. 2012), using the model criterion generated from MrModeltest v2.3 (Nylander 2004). For ML analyses, a bootstrap analysis using a tree-bisection-reconnection (TBR) heuristic search with 1000 replicates was performed to determine topological confidence of the tree. For BI analyses, posterior probability of trees was estimated using Monte Carlo Markov Chain (MCMC) analyses, where one million generations were run sampling four chains every 100 generations, with a burn-in of 0.25. For each marker, *p* distances were calculated within and between clades as a measure of divergence using MEGA 7 (Kumar et al. 2015). In addition, *p* distances were calculated between and within *T. bergquistae* (Clade 1) and *T. burtoni* (Clades 2 + 3, combined).

For microsatellite data from sponges belonging to the *T. burtoni* clades, a principal coordinate analysis (PCA) based on genetic distances from allele frequencies was performed in the *adegenet* package (Jombart 2008) in R (<https://www.r-project.org/>). Assignment of individuals into genetic clusters was also assessed using STRUCTURE, which is a Bayesian clustering analysis for multilocus genotype data

which probabilistically assigns individuals to clusters (Pritchard et al. 2000). Analyses were carried out in STRUCTURE v 2.3.2 (Pritchard et al. 2000), using 100,000 Markov Chain Monte Carlo (MCMC) iterations, a burn in of 10,000 iterations, 10 replicates per run and with the *K* value set from 1 to 8. The optimal *K* was determined in Structure Harvester (Earl and vonHoldt 2012) to be *K* = 4, and the 10 replications for *K* = 4 were merged in CLUMPP (Jakobsson and Rosenberg 2007). Plots were visualized in MS Excel (2016). Clusters within *T. burtoni* were consistent with phylogenetic clades produced by 18S and rnl, and the degree of differentiation between these two groups was assessed using *F*<sub>st</sub>. Because individuals belonging to Clade 3 contained loci that failed to amplify (missing data), these loci were removed for calculations of *F*<sub>st</sub>. A pairwise *F*<sub>st</sub> value was calculated from the remaining 5 loci with significance determined by performing 20,000 permutations in Arlequin 3.1 (Excoffier et al. 2005). In addition, because measures of differentiation based on distance are sensitive to deviations from Hardy Weinberg Equilibrium (HWE; Waples 2015), *F*<sub>st</sub> was calculated again removing one (out of five) loci that was not in HWE (Supplementary Information).

## Morphological analysis

To determine if morphological characteristics were consistent with the observed genetic clades, a subsample of six sponges from each clade was selected for spicule analysis. We chose genetically identical individuals that fell consistently into the same clade for all three markers. Samples were collected from the same location and at the same time in order to reduce potential environmental and seasonal variation in spicule morphology (Bell et al. 2002). Each sponge was cut in half to photograph its cross section. Further, a small piece of tissue was embedded in paraffin wax and sectioned to 20 µm using a rotary microtome (Leica Biosystems RM2235) to examine the location, arrangement and density of spicules across the ectosome and choanosome. A ~5 mm<sup>3</sup> tissue sample was then removed from each specimen and dissolved in bleach at 50 °C for 48 h and the remaining spicules were washed with distilled water and rinsed 3× with absolute ethanol, following the recommendations of Hooper (2003). One millilitre of the remaining spicule solution was mounted onto a slide using DPX and examined at a magnification of ×100–200, using a compound light microscope (Leica Microsystems DM LB) with attached digital camera (Canon EOS 70D). Spicule type (see Supplementary Information for definitions of spicule types) and location (defined as region of tissue in the sponge—cortex versus choanosome) was recorded for a qualitative analysis. For a quantitative comparison, the sizes of 30 of each spicule type which were



**Fig. 2** Phylograms showing the relationship between *Tethya bergquistae* (Clade 1) and *T. burtoni* (Clades 2 and 3) based on individual mitochondrial markers (rnl and COI-ext). Topology generated from Maximum Likelihood (ML) analysis. ML bootstrap confidence values/Bayesian Inference (BI) posterior probabilities given on each branch.

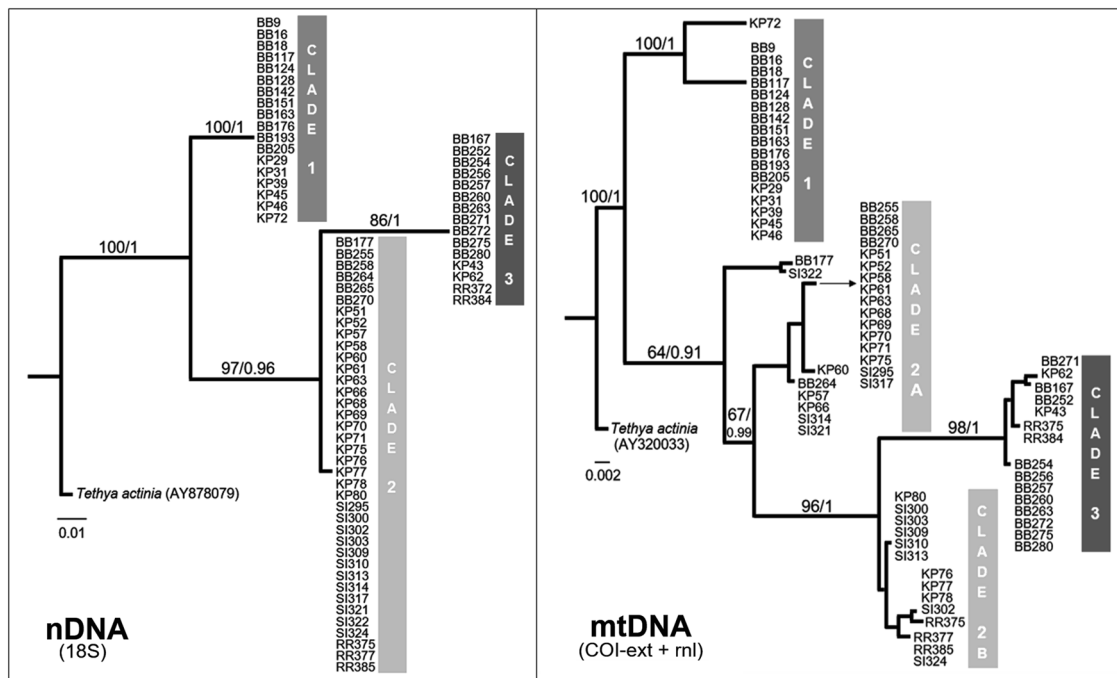
*T. actinia* was selected as an outgroup. Inconsistencies in the COI-ext tree versus rnl are highlighted by having sponges retain the colour of the rnl placement and by bolding individuals. Scale bar = substitutions per site

present in all sponges (strongyloxea, megasters and micrasters) were recorded per sponge. For megascleres (strongyloxea), the length and width were recorded for both thicker and thinner auxiliary types (strongyloxea and styles, respectively). For both megasters and micrasters, the diameter and R:C ratio (ray length to centre length ratio) were recorded, following Bergquist and Kelly-Borges (1991) and Sàra and Sàra (2004). Size measurements for all megasters (spherasters and oxyspherasters) were grouped, as well as micrasters (oxyasters, chiasters, stronglyasters and tylasters), because they could not be differentiated using light microscopy. Sizes were measured using ImageJ (Schneider et al. 2012). Non-parametric multidimensional scaling (nMDS) ordinations were generated using Euclidean distance on Primer v7 (Clarke 1993) to determine any clustering based on average, minimum and maximum sizes. Spicules from all 18 sponges that were subsampled for spicule analysis were also observed using Scanning Electron Microscopy (SEM), to visualize the megasters and micrasters at higher resolution and magnification. Spicules preserved in ethanol were mounted on stubs, air dried and coated with a gold/palladium alloy using a sputter coater. Scanning electron micrographs were taken with the Hitachi TM3000 Benchtop Scanning Electron Microscope in high vacuum mode.

## Results

### Molecular phylogeny

A total of 1420 bp (425 bp for 18S, 570 bp for rnl and 425 bp for COI-ext) were sequenced for 71 *Tethya* individuals. For all trees, branch confidence (via bootstrap/posterior probability values) ranged from 64 to 100%/0.91 to 1 for divergences between the main clades (Figs. 2 and 3). 18S resolved the clades above 86%/0.96 and rnl above 74%/0.99, but COI-ext exhibited slightly lower confidence from 63%/0.91. The nuclear 18S region grouped sponges into three genotypes, and for the mitochondrial rnl and COI-ext partitions there were seven and four haplotypes present, respectively. The concatenated data set for the mitochondrial markers revealed 15 different haplotypes (Fig. 3). For each individual locus, phylogenetic reconstructions revealed three divergent clades that each contained samples from all sampling locations; in other words, none of the clades contained individuals from strictly one location. For rnl and 18S, those sponges that were thought to be *T. bergquistae* all grouped together into one clade (Clade 1), but for COI-ext, one individual that was thought to be *T. bergquistae* grouped with Clade 2 (*T. burtoni* clade). For *T. burtoni*, rnl and 18S generated congruent placement of sponges into two



**Fig. 3** Phylograms showing the relationship between *Tethya bergquistae* (Clade 1) and *T. burtoni* (Clades 2A + B and 3) based on nuclear DNA (18S) and mitochondrial DNA (concatenated COI-ext + rnl). Topology generated from Maximum Likelihood (ML) analysis.

ML bootstrap confidence values/Bayesian Inference (BI) posterior probabilities given on each branch. *T. actinia* was selected as an outgroup. Scale bar = substitutions per site

separate groups. However, COI-ext placed sponges differently, where some sponges that belonged to Clade 2 for rnl and 18S were placed into Clade 3; and in addition, two sponges which were thought to be *T. burtoni* were placed into Clade 1 (*T. bergquistae* clade). The three uncertain yellow sponges (Fig. 1) grouped with *T. bergquistae* for all three markers.

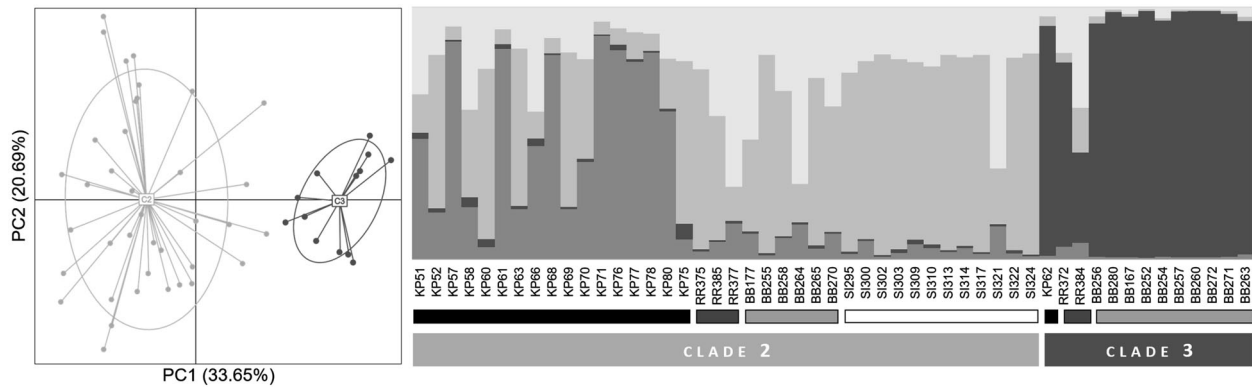
Divergence was calculated between and within all genetic clades using *p* distances (Table 2). Intraclade *p* distance across all three markers ranged from 0 to 0.13% (average = 0.04%, stdev = 0.06%), whereas interclade *p* distances for all pairwise clade comparisons across all markers ranged from 0.5 to 11.2% (average = 5.29%, stdev = 3.83%). For all pairwise clade comparisons, interclade *p* distances for 18S (0.5–1.2%) were lower than for the mt-markers rnl and COI-ext (4.8–8.6, 6.4–11.2%, respectively). For *T. bergquistae* (Clade 1), intraclade *p* distances for all markers was zero. For *T. burtoni* (Clades 2 + 3, combined), intraclade *p* distances were: zero for 18S, 2.1% for rnl and 4.2% for COI-ext. The divergence between *T. bergquistae* and *T. burtoni* was: 0.8% for 18S, 6.6% for rnl and 9.0% for COI-ext.

For *T. burtoni*, both PCA and STRUCTURE based on microsatellite allele frequencies revealed two distinct genetic clusters that were congruent with the *T. burtoni* clades generated from phylogenetic analyses for 18S and rnl (Fig. 4). The *T. burtoni* clades (Clades 2 and 3) were

**Table 2** Genetic divergence (*p* distances, %) for *Tethya* spp. for all gene markers, where Clade 1 is *T. bergquistae* and Clades 2 and 3 are *T. burtoni*. Intraclade *p* distances are reported for individuals within clades, while interclade values are pairwise comparisons between all clades.

| 18S        |       |            |         |         |   |
|------------|-------|------------|---------|---------|---|
| Intraclade |       | Interclade |         |         |   |
|            |       | Clade 1    | Clade 2 | Clade 3 |   |
| Clade 1    | 0     | Clade 1    | 0       |         |   |
| Clade 2    | 0     | Clade 2    | 0.7%    | 0       |   |
| Clade 3    | 0     | Clade 3    | 1.2%    | 0.5%    | 0 |
| rnl        |       |            |         |         |   |
| Intraclade |       | Interclade |         |         |   |
|            |       | Clade 1    | Clade 2 | Clade 3 |   |
| Clade 1    | 0     | Clade 1    | 0       |         |   |
| Clade 2    | 0     | Clade 2    | 5.9%    | 0       |   |
| Clade 3    | 0.13% | Clade 3    | 8.6%    | 4.8%    | 0 |
| COIext     |       |            |         |         |   |
| Intraclade |       | Interclade |         |         |   |
|            |       | Clade 1    | Clade 2 | Clade 3 |   |
| Clade 1    | 0     | Clade 1    | 0       |         |   |
| Clade 2    | 0.10% | Clade 2    | 6.4%    | 0       |   |
| Clade 3    | 0.13% | Clade 3    | 11.2%   | 8.3%    | 0 |

significantly different with respect to their genetic structure ( $F_{st} = 0.39$ ,  $P < 0.0001$ ). When using only those loci in



**Fig. 4** Genetic differentiation within *Tethya burtoni* based on microsatellite data. On left: Principal coordinate analysis (PCA) based on genetic distances from microsatellite allele frequencies for the *T. burtoni* clades (Clades 2 and 3 from Fig. 3). Each point represents a *T. burtoni* genotype, and the two groups (C2 and C3) are labelled inside of their 67% inertia ellipses. On right: Group assignment of *T. burtoni* individuals into four genetic clusters ( $K = 4$ ) based on STRUCTURE.

Each vertical line represents an individual, and the coloration is the proportion of that individual's estimated membership into each of the four genetic clusters. Labels along the horizontal refer to individual IDs (see Supplementary Table S1 for more information). Horizontal bars under individual IDs correspond to sampling locations, which are: Kapiti Island (black), Red Rocks (dark grey), Breaker Bay (light grey) and Somes Island (white)

**Table 3** Mean lengths for spicules in *Tethya bergquistae* (Clade 1) and *T. burtoni* (Clades 2 + 3) for three genetic clades identified by 18S and rnl (Figs. 2 and 3) and for all samples combined (overall). Strongyloxea are presented as length  $\times$  width. R:C refers to ray length:diameter-to-centre ratios. All sizes are in microns ( $\mu\text{m}$ )

|         | Strongyloxea ( $l \times w$ ) ( $\mu\text{m}$ ) | Style ( $l \times w$ ) ( $\mu\text{m}$ )  | Megaster diameter ( $\mu\text{m}$ ) | Megaster R:C    | Micraster diameter ( $\mu\text{m}$ ) | Micraster R:C   |
|---------|---|---|-------------------------------------|-----------------|--------------------------------------|-----------------|
| Clade 1 | 1032.0 $\pm$ 219.4 $\times$ 22.7 $\pm$ 5.7      | 356.4 $\pm$ 103.6 $\times$ 14.4 $\pm$ 3.3 | 42.9 $\pm$ 12.5                     | 0.75 $\pm$ 0.15 | 13.7 $\pm$ 3.2                       | 0.81 $\pm$ 0.19 |
| Clade 2 | 1054.7 $\pm$ 320.4 $\times$ 21.6 $\pm$ 4.6      | 424.1 $\pm$ 108.1 $\times$ 13.6 $\pm$ 3.8 | 44.7 $\pm$ 6.4                      | 0.73 $\pm$ 0.15 | 15.2 $\pm$ 1.4                       | 0.79 $\pm$ 0.16 |
| Clade 3 | 1104.0 $\pm$ 273.5 $\times$ 16.7 $\pm$ 5.3      | 419.7 $\pm$ 124.7 $\times$ 9.9 $\pm$ 2.9  | 44.1 $\pm$ 9.0                      | 0.86 $\pm$ 0.15 | 12.4 $\pm$ 2.0                       | 1.07 $\pm$ 0.25 |
| Overall | 1061.2 $\pm$ 275.3 $\times$ 20.6 $\pm$ 5.8      | 399.0 $\pm$ 115.9 $\times$ 12.8 $\pm$ 3.9 | 43.9 $\pm$ 9.7                      | 0.77 $\pm$ 0.16 | 13.8 $\pm$ 2.6                       | 0.88 $\pm$ 0.23 |

HWE, differentiation between clades was also significant and strong ( $F_{st} = 0.47$ ,  $P < 0.0001$ ).

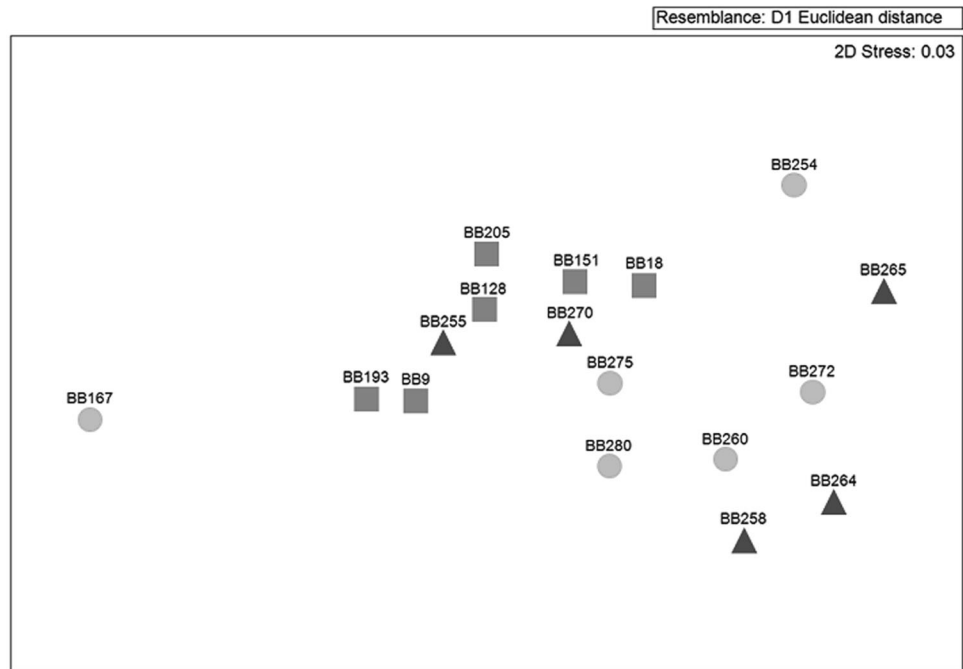
### Morphological analysis

All sponges contained megascleres that were strongyloxea and anisostrongyles, along with smaller, thinner auxiliary styles. Megasters were spherasters and oxyspherasters, and micrasters were tylasters, chiasters, stronglyasters and oxyasters. Spicule sizes are summarized in Table 3. Average strongyloxea and auxiliary style length  $\times$  width were  $1061 \pm 275 \times 21 \pm 6 \mu\text{m}$  and  $399 \pm 116 \times 13 \pm 4 \mu\text{m}$ , respectively. Respective megaster and micraster diameters were  $44 \pm 10$  and  $14 \pm 3 \mu\text{m}$ , and the average R:C ratio was  $\sim 0.8 \pm 0.2$  for all asters. n-MDS plots for spicule size (average, minimum and maximum sizes) revealed no evident clustering (Fig. 5). Qualitative differences were observed between the location and density of megasters

within the different genetic clades. Those sponges belonging to Clade 1 contained megasters throughout both the cortex and choanosome, whereas those sponges in Clades 2 and 3 had megasters in the cortex, but little to none in the choanosome. Within the *T. burtoni* clades (Clades 2 and 3), there were no differences in spicule composition detectable by light microscopy. Scanning electron micrographs of spicules from the three genetic clades also revealed no visible differences in megaster or micraster morphology (Fig. 6). However, sponges belonging to the *T. bergquistae* clade contained an additional spined oxyaster, which was absent in the *T. burtoni* clades. A qualitative assessment of cross sections from this subsample of sponges revealed high plasticity within each genetic clade, and no clear differences between clades (Fig. 1). Further,  $20 \mu\text{m}$  cross sections examining the spicule composition between the choanosome and ectosome revealed that *T. bergquistae* contained more asters in the choanosome than *T. burtoni* (Fig. 7).



**Fig. 5** Nonparametric-multidimensional (nMDS) scaling ordination of mean spicule sizes for *Tethya bergquistae* (pink squares, Clade 1) and *T. burtoni* (orange circles, Clade 2; and blue triangles, Clade 3). Clades are identified in Fig. 3. Spicules sizes are based on measurements from stronglyloxea, megasters and micrasters ( $n = 30$  per sponge). Codes above each sample refer to individual sample ID (refer to Supplementary Table S1 for more information on individual samples). A subset of all samples are presented in the nMDS, based on a Euclidean distance matrix



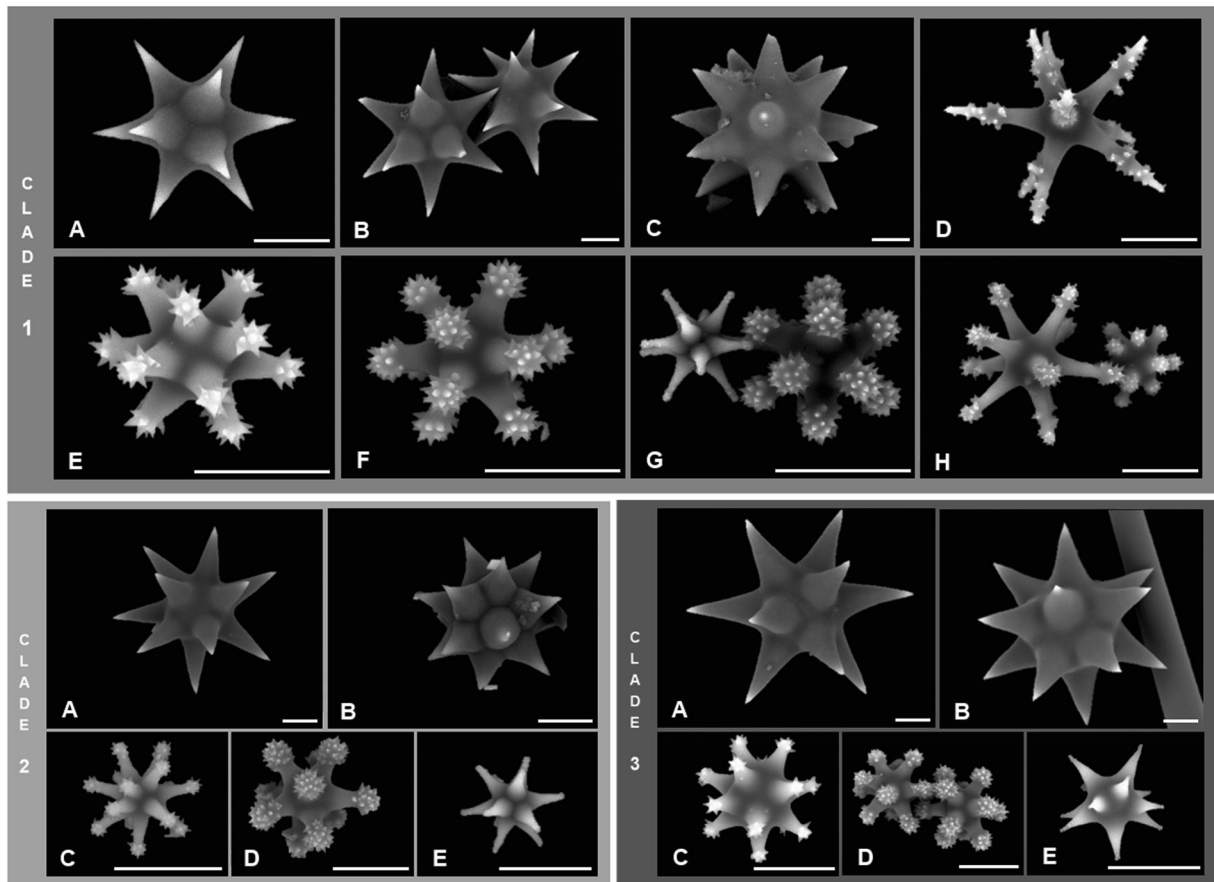
However, within *T. burtoni*, there were no evident differences between Clades 2 and 3. While some members of Clade 2 appeared to contain a slightly higher density of asters in the cortex, this was not consistent for all individuals within that clade.

## Discussion

Here, we document discordance between molecular and morphological descriptions of *Tethya* spp. in central New Zealand and highlight the potential for cryptic speciation to go undetected based on morphological features alone. Phylogenetic reconstructions based on 18S, rnl and COI-ext revealed three genetic clades, with a speciation event likely occurring in the group described as *T. burtoni*. This division within *T. burtoni* was further confirmed using novel microsatellite markers. Morphological analyses based on spicule size and composition allowed *T. bergquistae* to be differentiated from *T. burtoni* but failed to reveal differences between the *T. burtoni* clades. To our knowledge, this is only the second report of cryptic species on the basis of molecular differences within the group *Tethya* (with the first being *T. robusta*, Sarà et al. 1993). Understanding how organisms evolve is central to ecology and evolution, but can be particularly complex for phenotypically similar organisms living in sympatry with no obvious barriers to reproduction, reflected in *Tethya* spp. examined here. Overall, our study highlights the need to use more than one method to define sponge species boundaries in a reliable way.

## Species delineation based on genetics

The phylogenetic relationship of *Tethya* spp. was not congruent for all three markers, as COI-ext placed sponges into different clades with most discrepancies occurring in the *T. burtoni* group. Microsatellite markers also supported the *T. burtoni* clade division that was indicated in the 18S and rnl trees (Figs. 2 and 3). The use of COI to delineate species has had differential success among sponges (Erpenbeck et al. 2006b; Wörheide and Erpenbeck 2007; Erpenbeck et al. 2007; Poppe et al. 2010; Belinky et al. 2012) as well as other organisms (Shearer and Coffroth 2008; Derycke et al. 2010), resulting in debate around its use as an informative marker (Waugh 2007). The COI region often has low variability (Bucklin et al. 2011), and this conservativeness can fail to capture evolutionary relationships between closely related species. We instead employed COI-ext to avoid this drawback, as this region downstream of COI is thought to substitute earlier during species divergence (Erpenbeck et al. 2006a). We captured variation between *T. bergquistae* and *T. burtoni*, as well as within *T. burtoni*; however, the relationship produced from COI-ext was incongruent with that produced from 18S, rnl and the 11 microsatellite markers. For species living in sympatry, introgression can be common, resulting in semipermeable species boundaries (Rüber et al. 2001, Harrison and Larson 2014). Genes can introgress at different rates (called differential introgression), which can result in phylogenies from multiple markers in disagreement (Harrison and Larson 2014). It is possible that the inconsistency in the COI-ext phylogeny is a product of this, and if true, gives evidence of introgression



**Fig. 6** Scanning electron micrographs of spicules belonging to *Tethya bergquistae* (Clade 1) and *T. burtoni* (Clade 2 + 3), from sponges belonging to the three genetic clades of *Tethya* identified in Fig. 3. Clade 1: A–C = spherasters; D = spined oxyaster; E–H = tylasters; G

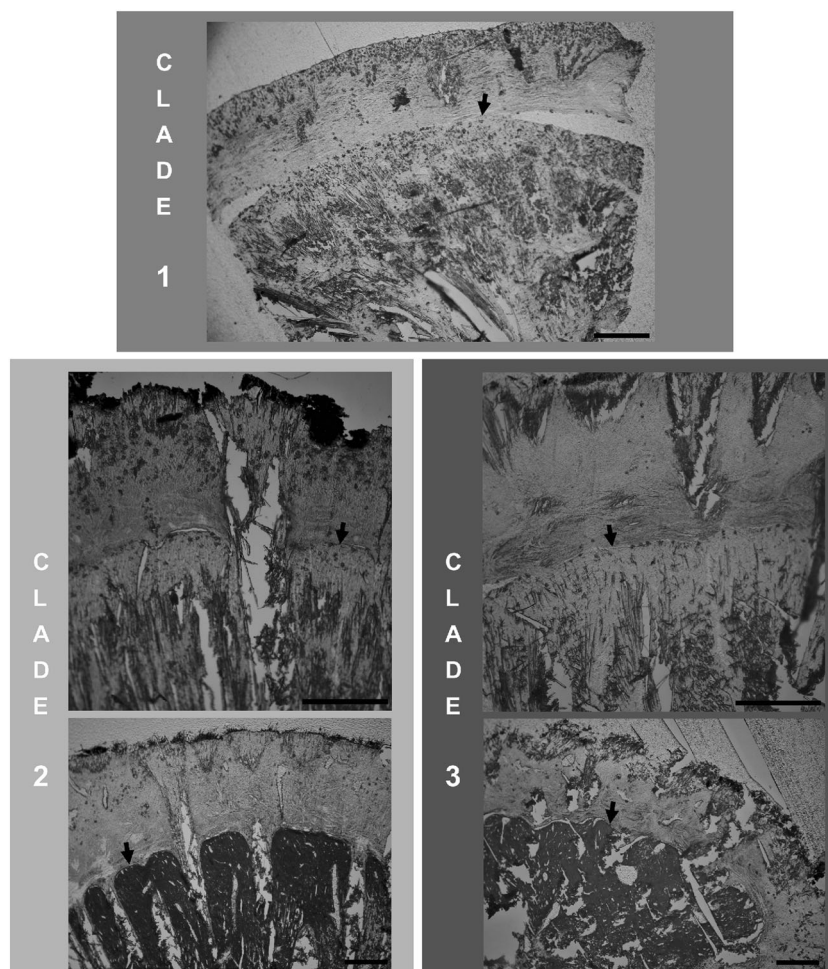
= oxyaster. Clade 2 and 3: A–B = spherasters; C–D = tylasters; E = oxyaster. Scale bars are located in the bottom right of each micrograph and represent 10  $\mu\text{m}$

between *T. bergquistae* and *T. burtoni*. For instance, one sponge (KP72) that was thought to be *T. bergquistae* (as evidenced by its placement into Clade 1 for 18S and rnl) was placed into the *T. burtoni* clade (Clade 2) for COI-ext. In addition, two sponges (BB177 and SI322), which were thought to be *T. burtoni* (from their placement into Clade 2 by 18S and rnl), were placed into the *T. bergquistae* clade (Clade 1) for COI-ext. Within *T. burtoni*, there is more disagreement between COI-ext and the other markers within the clades. For example, those sponges belonging to Clade 2B of the mitochondrial concatenated data set (Fig. 3) were placed into Clade 2 by 18S, rnl and the microsatellite markers, but Clade 3 for COI-ext. It is possible that this group of sponges, which possess genes common to two different putative species (discussed below), have also undergone introgressive hybridization. Some corals have also been found to exhibit high degrees of sympatry, cryptic speciation and introgression (Ladner and Palumbi 2012), and perhaps shared life-history traits between corals and sponges (i.e. sessile, sexual and asexual reproductive ecology) shape the evolutionary history of these populations.

While additional sequencing work would provide clarity on this topic, it appears highly probable that the species boundaries between *T. bergquistae* and *T. burtoni*, as well as between the putative *T. burtoni* cryptic species, are actually semipermeable.

The definition of a species continues to be debated, and when complex evolutionary processes like introgression can shape species, understanding species delineations becomes more complicated. The topology produced from 18S clearly differentiates our clades. 18S is a slowly evolving gene region (Berntson et al. 2001), and as such often captures interspecific variation rather than intraspecific variation. Three separate clades, despite the slow evolving nature of this gene, provides strong evidence that these three clades correspond to three different species. Most interestingly, two clades exist within *T. burtoni* and suggest cryptic speciation within the group. Other measures exist (e.g.  $p$  distances,  $F_{st}$ ) to more definitively set a quantitative value to define species divisions. Comparing the  $p$  distances obtained in this study to other observed divergence values for sponges (Table 4), both within- and between-clade

**Fig. 7** Cross sections of a small piece of tissue (20  $\mu\text{m}$  thick) from *Tethya* spp., where Clade 1 is *T. bergquistae* and Clades 2 and 3 are *T. burtoni*. Arrows are pointing to the line that divides the outer cortex and the inner choanosomal tissue. Black scale bars represent 500  $\mu\text{m}$



distances give further evidence for species-level relatedness. Our intraclade  $p$  distances were about ~5–50 times less than the interclade values for all pairwise comparisons of clades, which is consistent with intraspecific values for other sponge taxa. These intraclade distances suggest that sponges belonging to the same clade are conspecific. When comparing the individual markers used here to the same markers (or similarly evolving markers) in other studies, the interclade  $p$  distances calculated for all pairwise clade comparisons in this study compare to interclade  $p$  distances for other sponge species (Table 4). This further reveals our clades do in fact correspond to species. For example, *Scopalina lophyropoda* was reported as being comprised of a cryptic species complex (Blanquer and Uriz 2007), and the interclade  $p$  distances for 18S (0.3–2%) were on the same scale as for those clades we found within *T. burtoni* (0.5%). Considering *T. bergquistae* (Clade 1) versus *T. burtoni* (Clades 2 + 3, combined), divergence between the two species was: 0.8% for 18S, 6.6% for rnl and 9% for COI-ext. Divergence between the *T. burtoni* clades (Clade 2 versus Clade 3) were on the same order: 0.5% for 18S, 4.8% for rnl and 8.3% for COI-ext. Divergence within *T. burtoni*

that is on the same scale as between *T. burtoni* and *T. bergquistae* gives further evidence that the two *T. burtoni* clades correspond to different species. The microsatellite data also reveal strong differentiation within the *T. burtoni* group ( $F_{st} = 0.39$ ,  $P < 0001$ ). While microsatellite markers are traditionally not used as a tool for phylogenetic studies (but instead population structure analysis), used here in combination with the other genetic markers gives further evidence for strong genetic differentiation between clades. Interestingly, differentiation between both *T. burtoni* clades was not a product of location (population structure of *T. burtoni* discussed elsewhere), as evident from the STRUCTURE plot (Fig. 4). Instead, sponges that lived sympatrically were strongly genetically different, suggesting reproductive isolation for sponges in the same location. Cryptic speciation has been recorded throughout the Porifera phylum (Solé-Cava et al. 1991; Muricy et al. 1996; Nichols and Barnes 2005; Wörheide et al. 2008; Xavier et al. 2010; DeBiasse and Hellberg 2015; Knapp et al. 2015; Uriz et al. 2017), and is also likely for what's known as *T. burtoni*. We believe our genetic data combined gives strong evidence that *T. burtoni* is in fact be comprised of a species

**Table 4** Summary of genetic divergence (*p* distances, %) for various sponge species. Ranges given for intraclade and interclade *p* distances

| Species                                   | Marker        | Intraclade<br><i>p</i> distance | Interclade<br><i>p</i> distance | Reference                  |
|---|---------------|---------------------------------|---------------------------------|----------------------------|
| <i>Aplysina</i> spp.                      | COI           | 0                               | –                               | Cruz-Barraza et al. 2012   |
|   | ITS1-5.8-ITS2 | 0                               | 0.1–2.2                         |                            |
| <i>Astrosclela willeyana</i>              | COI           | –                               | 0.2–0.4                         | Wörheide 2006              |
| <i>Cliona</i> spp.                        | COI           | –                               | 2.7–8.7                         | De Paula et al. 2012       |
|   | ITS           | –                               | 5.9–14.8                        |                            |
| <i>Cliona celata</i> <sup>a</sup>         | COI           | –                               | 2.7–8.3                         |                            |
|   | ITS           | –                               | 8.1–11.6                        |                            |
| <i>Cliona celata</i> <sup>a</sup>         | COI           | 0.1–0.5                         | 6.2–8.4                         | Xavier et al. 2010         |
|   | Atp8          | 0–0.5                           | 7.9–16                          |                            |
|   | 28S           | 0                               | 2.8–5.4                         |                            |
| <i>Haliclona</i> sp.                      | ITS1          | 1.7–3.0                         | –                               | Redmond and McCormack 2009 |
|   | ITS2          | 0.7–1.8                         | –                               |                            |
| <i>Hemimycale</i> spp.                    | COI           | –                               | 8.4–8.7                         | Uriz et al. 2017           |
|   | 28S           | –                               | 1.8–2.2                         |                            |
|   | 18S           | –                               | 1.4–1.9                         |                            |
| <i>Hexadella</i> spp.                     | COI           | 0–3.5                           | 3.9–8.7                         | Reveillaud et al. 2010     |
|   | ATPS          | 0–6.3                           | 10–28.5                         |                            |
| <i>Ianthella basta</i> <sup>a</sup>       | COI           | –                               | 0.5–1.75                        | Andreakis et al. 2012      |
| <i>Plocamionida</i> spp.                  | COI           | 0.5                             | 1.3–20                          | Reveillaud et al. 2011     |
|   | 28S           | –                               | 0.1–3.3                         |                            |
| <i>Scopalina lophyropoda</i> <sup>a</sup> | COI           | 0–3                             | 13–22                           | Blanquer and Uriz 2007     |
|   | 28S           | 0–0.8                           | 2–19                            |                            |
|   | 18S           | 0.1                             | 0.3–2                           |                            |
| <i>Tethya</i> spp.                        | COI-ext       | 0–0.1                           | 6.4–11.2                        | This study                 |
|   | ml            | 0–0.1                           | 4.8–8.6                         |                            |
|   | 18S           | 0                               | 0.5–1.2                         |                            |
| <i>Tethya burtoni</i> <sup>a</sup>        | COI-ext       | 0.1                             | 8.3                             |                            |
|   | ml            | 0–0.1                           | 4.8                             |                            |
|   | 18S           | 0                               | 0.5                             |                            |

<sup>a</sup>Reported as cryptic species

complex, being more genetically diverse than previously thought based on morphology alone.

### Discordance between molecular and morphological characteristics

Our analysis of spicule size/type, in conjunction with our qualitative assessment of macromorphological features

(colour and cross sections), was inconsistent with the molecular phylogeny that we generated. Macromorphological features allowed sponges belonging to the *T. bergquistae* clade to be distinguished from sponges belonging to the *T. burtoni* clades; however, we were not able to identify sponges belonging to separate genetic clades within *T. burtoni* from morphology. The three uncertain, bright yellow specimens grouped together with those sponges belonging to *T. bergquistae*, which has not previously been described as yellow (Bergquist and Kelly-Borges 1991; Hooper and Wiedenmayer 1994; Battershill et al. 2010), indicating that colour is not a reliable distinguishing feature in this case. Qualitative observations revealed no obvious differences in cross sections between *T. burtoni* clades, and instead were highly variable within each clade. While generally cross sections are a key defining feature between species of *Tethya*, they did not provide clear resolution here. There was a marked difference between the spicule composition of *T. bergquistae* and the *T. burtoni* complex (more asters in the choanosome of the former), but there were no differences evident within the *T. burtoni* clades. Morphological characteristics therefore provided no clear evidence of speciation within *T. burtoni*. Because there is a lack of defining characteristics available for Porifera, it is common for genetic diversity (and cryptic speciation) to go undetected, where molecular and morphological relationships are commonly incongruent (McCormack et al. 2002; Erpenbeck et al. 2006b; Xavier et al. 2010). Determining which morphological traits are informative in species differentiation can also be complicated. For example, Erpenbeck et al. (2006b) documented that sponges containing highly divergent skeletal features are actually closely genetically related. In contrast, spicules within the same species may be highly variable due to environmental influences (Bell et al. 2002). We show here that morphological features may not be able to reveal species boundaries, as perhaps skeletal changes following divergence may be delayed. Mutations may accumulate without altering the phenotype when nucleotide changes have no effect on how its product (protein) folds and its function overall (Avisé 2012), which could explain delayed morphological expression in genetically different individuals. While it is possible that subtle morphological differences may exist, they were not able to be detected in this study. The high phenotypic plasticity observed across individuals of *T. burtoni* resulted in a lack of clear diagnostic features for sponges belonging the different genetic clades. Furthermore, the potential introgression events between cryptic species may make detecting morphological differences between such species more difficult. To avoid this potential confounding factor, we used sponges which showed no introgression based on our markers for the

morphological analysis presented here. However, we cannot confirm that these sponges have not undergone introgression, as we only surveyed three genes. A survey of other regions of the genome may reveal clearer relationships and show the scope of introgressive hybridization between the *T. burtoni* cryptic species. This may shed more light on differences between plasticity versus distinct morphological features that allow species to be characterized. With such incongruence between morphological and molecular data, it is imperative to use a combination of methods to correctly delineate species in order to fully understand the species evolutionary relationships.

## Conclusions

Our study highlights the complexity in delineating sympatric, morphologically similar species when disagreement between morphological and molecular traits exist, and the importance of using multiple taxonomic methods. While traditional morphology-based taxonomy can be the first step in species identification, we show that it can fail to reveal cryptic species. Being able to confidently identify a species is crucial to any ecological or evolutionary study, yet this remains a challenge. Organisms with few defining traits, like sponges, present an opportunity to examine potential morphological and molecular discord, and highlight the complexity in defining species boundaries. Failure to detect cryptic species may result in an underestimation of biodiversity and an incorrect interpretation of functional roles of an organism. In addition, it can mislead our understanding of connectivity patterns and evolutionary relationships, if interspecific diversity is confused for intraspecific variation. As such, we strongly recommend the use of integrative taxonomy in species identification.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Data availability** Sequence data have been submitted to GenBank, under the accession numbers MH180010–180023. Microsatellite

genotype data is available on Pangaea (<https://doi.org/10.1594/PANGAEA.889125>).

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