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Cryptic genetic lineages of a colonial ascidian host distinct microbiomes

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Abstract

Colour variation in marine invertebrates is typically attributed to intraspecific variability; however, the use of molecular tools to investigate its genetic basis has uncovered many instances of cryptic speciation. The colonial ascidian *Distaplia bermudensis* occurs in a wide array of colour morphs, ranging from all white to a dark blue tunic with yellow siphon apertures; yet careful observations of zooid and larval morphologies have not revealed any consistent difference among these colour morphs. In this study, we sampled the 5 main colour morph groupings of *D. bermudensis* from Wilmington, North Carolina (USA) and sequenced fragments of the mitochondrial cytochrome c oxidase subunit I (COI) and the nuclear 18S rRNA genes. Analyses of both genes revealed the presence of two distinct genetic lineages that corresponded to unique colour morphology groupings. We further characterized symbiotic microbial communities for four samples from each lineage by high-throughput sequencing of partial 16S rRNA genes. Each genetic lineage also harboured unique symbiont communities that differed significantly in diversity and structure. Thus, the two genetic lineages detected herein corresponded to unique colour morphs and microbial communities, indicating that two distinct species coexist under the same scientific name. Further, our study reports the utility of symbiont profiling in resolving the taxonomic status of phenotypically variable ascidian species.

KEYWORDS

ascidian, cytochrome c oxidase subunit I, cryptic species, microbial symbiosis, tunicate 18S rRNA

1 | INTRODUCTION

Ascidians, or sea squirts (Chordata; Tunicata), are sessile marine invertebrates that are common constituents of marine benthic communities worldwide. The largest of the tunicate classes, the ascidians, also represent a highly diverse lineage, with some 3,000 species currently described (Shenkar & Swalla, 2011). This diversity is reflected in substantial interspecific morphological differences, yet intraspecific differences are also common, with colour, morphometry (size, shape) and other anatomical differences (e.g. spicule form) among the most commonly reported (Dalby, 1997; López-Legentil & Turon, 2005). In particular, differences in coloration among ascidians have historically been attributed to intraspecific variation among individuals (Bancroft, 1903; Van Name, 1945). Bancroft (1903) showed that pigmentation in colonial ascidians could vary between parent and offspring, or even within the same colony over time in response to changes in environmental or physiological conditions. Coloration patterns in animals are typically linked to sexual selection or predation avoidance (Heinen-Kay et al., 2015; McQueen et al., 2019), but ascidians are broadcast spawners, without active sexual selection of mates and no known purpose

for this diversity of coloration. Further, coloration does not seem to assist in predation avoidance in ascidians (Kott, 1989), although some studies have suggested larval coloration is at least in some cases aposematic (Young & Bingham, 1987). Alternatively, pigmentation of the adult ascidian tunic, whether provided by microbial symbionts or ascidian pigmentary tunic cells, has been suggested to provide protection from solar radiation in some species (Hirose, 2009; Hirose et al., 2004), but the ecological significance of such a large diversity of colour morphologies in most species remains unclear.

With the increasing use of genetic sequencing technologies, numerous instances of cryptic speciation have been detected among morphologically indistinguishable organisms (Florio et al., 2012; Thongtam na Ayudhaya et al., 2017), including some native (Aron & Sole-Cava, 1991; Tarjuelo et al., 2004) and invasive (Turon et al., 2020) ascidian species. However, genetic analyses have simultaneously determined phenotypic colour variation may indeed represent intraspecific variability among some colour morphs of other ascidian species (e.g. *Cystodytes dellechiajei*; López-Legentil & Turon, 2005). The importance of identifying cryptic species extends beyond taxonomic classification, since ecological differences between morphologically indistinguishable organisms (e.g. prey or food source, habitat type, or ecological role) may be profound (De Meester et al., 2016; Duda et al., 2009; Hebert et al., 2004). The misidentification of species may also lead to an underestimation of overall biodiversity and an overestimation of individual species' abundance and geographic range, such that potentially vulnerable species may be overlooked with critical consequences for conservation (reviewed in Chenuil et al., 2019). Similarly, previously unrecognized species may also have important human implications, such as lost opportunities for new sources of undiscovered pharmaceuticals or new technological advancements (reviewed in Erwin et al., 2010; Skoracka et al., 2015). In addition, misidentification of species may further restrict our ability to identify biological invasions or reconstruct bioinvasion pathways (Evans et al., 1998; Geller et al., 1997; Sweijd et al., 2000).

Ascidians, like most living animals, harbour highly diverse symbiotic microbial communities (Dror et al., 2019; Erwin et al., 2014; Evans et al., 2017). These microbial communities inhabit both the tunic and the zooids of the animal (Blasiak et al., 2014; Erwin et al., 2014; Schreiber, Kjeldsen, Funch, et al., 2016), and despite the fact that sea squirts are filter feeders, remain structurally and compositionally distinct from the bacterioplankton community of the surrounding water (Dror et al., 2019; Erwin et al., 2014; Evans et al., 2017). Some of these symbionts appear to be horizontally sourced from the seawater microbial seed bank, and their numbers then amplified within optimal 'microniches' in the ascidian tunic (Behrendt et al., 2012; Casso et al., 2020; Erwin et al., 2014). Interestingly, ascidian microbial communities are also highly host-specific (Erwin et al., 2014; Evans et al., 2017; Tianero et al., 2015), with significant differences detectable even among congeneric and closely related species (Evans et al., 2017) and between native and introduced populations of invasive species (Casso et al., 2020; Goddard-Dwyer et al. 2021). Additionally, for many species, these microbial assemblages have been shown to remain consistent across broad timescales and geographic ranges (Cahill et al., 2016; López-Legentil et al., 2015; Martínez-García et al., 2008). This stability, in combination with bacterial presence in ascidian larvae and high host-specificity, suggests that vertical transmission from parent to offspring also plays an important role in the sourcing of ascidian microbial symbionts (Hirose, 2015; López-Legentil et al., 2015). Thus, in-depth characterization of symbiotic microbial assemblages could be a useful and largely unexplored mechanism for distinguishing between morphologically similar host ascidians of debated taxonomical classification.

The colonial ascidian *Distaplia bermudensis* (Van Name 1902) was first described in the Bermuda Islands and currently exhibits a worldwide distribution, with documented populations in the Mediterranean Sea, Brazil and the southeastern United States (Lezzi et al., 2018; Mastrototaro & Brunetti, 2006; Rocha & Bonnet, 2009; Villalobos et al., 2017; Zhan et al., 2015). In North Carolina, *D. bermudensis* is considered cryptogenic (Villalobos et al., 2017) and exists in a vast assortment of colours, with tunics in varying shades of purple, orange, pink, grey, red, tan, blue and white, combined with an equally diverse array of siphon opening colours. These colour differences have typically been considered the result of intraspecific variation as no morphological differences were observed in zooid anatomy (Van Name, 1945). Historically, taxonomic classification of species has depended on the identification of morphological differences between organisms at one or more life stages, but with the growing use of genetic sequencing, species identifications are often accompanied by genotypic information. The mitochondrial gene cytochrome c oxidase subunit I (COI) is commonly utilized to identify and barcode species, and has been consistently successful in detecting cryptic speciation in ascidians (Bock et al., 2012; Caputi et al., 2007; López-Legentil & Turon, 2006; Pérez-Portela & Turon, 2008). Here, we sequenced a fragment of the COI gene for 5 colour combinations of *Distaplia bermudensis* to determine whether colour variation had a genetic basis. To lend robustness to any observed divergence patterns, we also sequenced a fragment of the 18S rRNA gene, as this gene has proved similarly successful in detecting cryptic speciation in ascidians (Bock et al., 2012). Finally, we examined the microbial symbiont communities present within contrasting colour morphs in order to assess symbiont characterization's usefulness in discerning closely related ascidian species.

TABLE 1 List of samples analysed, including sample code, collection date and location, colour morph code with description of colour morphologies, and genetic lineage

Sample ID	Collection date	Location	Colour morph	Colour morph code	Genetic lineage	COI acc. num.	18S rRNA acc. Num.
24Jun15-D4	June 2015	Heide Trask Memorial Bridge (Bridge Tender Marina)	Dark blue-purple tunic with yellow-pink siphon openings	1	B (b)	MF034529[a]	N/A
24Jun15-D6	June 2015	Heide Trask Memorial Bridge (Bridge Tender Marina)	Brown, tunic with pink-orange siphon openings	2	B (b)	MF034530[a]	N/A
24Jun15-D7	June 2015	Heide Trask Memorial Bridge (Bridge Tender Marina)	Beige, light pink tunic with brown siphon openings	4	A (d)	MT572341	N/A
24Jun15-D8	June 2015	Heide Trask Memorial Bridge (Bridge Tender Marina)	Purple, dark pink tunic with brown siphon openings	5	A (e)	MT572342	N/A
24Jun15-D9	June 2015	Heide Trask Memorial Bridge (Bridge Tender Marina)	Brown tunic with white-cream siphon openings	3	B (b)	MF034531[a]	N/A
24Jun15-D10	June 2015	Heide Trask Memorial Bridge (Bridge Tender Marina)	Purple, dark pink tunic with brown siphon openings	5	A (d)	MT572343	N/A
24Jun15-D11[a]	June 2015	Heide Trask Memorial Bridge (Bridge Tender Marina)	Dark blue-purple tunic with yellow-pink siphon openings	1	B (b)	MF034532[a]	N/A
24Jun15-D12	June 2015	Heide Trask Memorial Bridge (Bridge Tender Marina)	Brown tunic with white-cream siphon openings	3	B (b)	MF034533[a]	N/A
1Mar16-3A-1	March 2016	5-Mile Ledge Reef	Dark blue-purple tunic with yellow-pink siphon openings	1	B (a)	MT572331	MT572319
1Mar16-3B-1	March 2016	5-Mile Ledge Reef	Dark blue-purple tunic with yellow-pink siphon openings	1	B (c)	KY111419[b]	MT572320
1Mar16-3C-1	March 2016	5-Mile Ledge Reef	Brown tunic with white-cream siphon openings	3	B (a)	MT572332	MT572321
1Mar16-3E	March 2016	5-Mile Ledge Reef	N/A	N/A	B (a)	MT572333	MT572322
8Mar16-1E	March 2016	Heide Trask Memorial Bridge (Bridge Tender Marina)	Dark blue-purple tunic with yellow-pink siphon openings	1	B (a)	MG525010[c]	MT572323
8Mar16-2B	March 2016	Heide Trask Memorial Bridge (Bridge Tender Marina)	Brown, tunic with pink-orange siphon openings	2	B (b)	MT572334	MT572324
8Mar16-3A	March 2016	Heide Trask Memorial Bridge (Bridge Tender Marina)	Brown, tunic with pink-orange siphon openings	2	B (a)	MT572335	MT572325
8Mar16-4A	March 2016	Heide Trask Memorial Bridge (Bridge Tender Marina)	Purple, dark pink tunic with brown siphon openings	5	A (d)	MT572336	MT572326
8Mar16-4B	March 2016	Heide Trask Memorial Bridge (Bridge Tender Marina)	Purple, dark pink tunic with brown siphon openings	5	A (d)	MT572337	MT572327

(Continues)

TABLE 1 (Continued)

Sample ID	Collection date	Location	Colour morph	Colour morph code	Genetic lineage	COI acc. num.	18S rRNA acc. Num.
8Mar16-5A	March 2016	Heide Trask Memorial Bridge (Bridge Tender Marina)	Purple, dark pink tunic with brown siphon openings	5	A (d)	MT572338	MT572328
8Mar16-8	March 2016	Heide Trask Memorial Bridge (Bridge Tender Marina)	Beige, light pink tunic with brown siphon openings	4	A (d)	MT572339	MT572329
8Mar16-9	March 2016	Heide Trask Memorial Bridge (Bridge Tender Marina)	Dark blue-purple tunic with yellow-pink siphon openings	1	B (a)	MT572340	MT572330

Note: Accession numbers (acc. num.) for genetic data from ascidians (COI and 18S rRNA genes) shown where applicable (N/A = not available). Lineage (A or B) is indicated, with COI haplotype codes (a, b, c, d or e) shown in parenthesis. No colour description was obtained for sample 1Mar16-3E. Superscript letters denote sources of previously published COI accession numbers.

[a]Evans et al. (2017).

[b]Villalobos et al. (2017).

[c]Evans et al. (2018).

2 | MATERIAL AND METHODS

2.1 | Sample collection and processing

Twenty colonies representing the 5 most common colour combinations of *D. bermudensis* were collected in June 2015 and March 2016 from two nearby sites (<15 km apart) off the coast of Wilmington, North Carolina, USA (Table 1, Figure S1): the pillars of the Heide Trask Memorial Bridge (34°13′05″N 77°48′45″W; accessed from the Bridge Tender Marina) and 5-Mile Ledge reef (34°06′08″N 77°45′03″W). Reef samples were obtained by SCUBA diving at <17 m in depth, while samples from the bridge pillars were collected by snorkelling at <5 m in depth. All samples were stored in an insulated cooler for transport and processed in the laboratory (<10 km away from sampling sites) within 2 hours of collection. Each sample was photographed (Table S1) and its coloration recorded prior to processing (Table 1). One half of each colony was then fixed in a 10% formalin and filtered seawater solution and stored at room temperature for morphological comparisons of the host ascidians, while the other half was fixed in absolute ethanol and stored at −20°C for molecular analysis. Morphological examinations of zooids fixed in formalin revealed no morphological differences among sampled colonies. Ethanol-preserved samples were subsequently dissected under a stereomicroscope into inner tunic (i.e., internal tunic tissue not in contact with either the zooids or ambient seawater) and zooid fractions for characterization of the microbiome and host-barcoding, respectively. DNA extractions were performed for both the zooid and tunic fractions using the DNeasy Blood and Tissue Kit (Qiagen), following manufacturer protocols.

2.2 | Genetic barcoding of ascidian hosts

Fragments of the mitochondrial cytochrome c oxidase subunit I (COI) and 18S rRNA genes (ca. 600-bp and 400-bp, respectively) were amplified using the 'universal' LCO1490 and HCO2198 primers (COI; Folmer et al. 1994) or the F16 and R497 primers (located near the end of the 18S rRNA gene; Price et al., 2005), respectively. PCR amplifications were performed in an Eppendorf Mastercycler nexus X2 using a total volume of 25 μl, consisting of 5 pmol of each primer, 12.5 μl of MyTaq HS Red Mix (Bioline) and 0.5–3 μl of template DNA. The COI thermocycler program consisted of an initial denaturation at 95°C for 1 min; 35 cycles of denaturation at 95°C for 15 s, annealing at 45°C for 15 s and extension at 72°C for 20 s; and a final extension at 72°C for 2 min. The 18S rRNA thermocycler program included an initial denaturation at 95°C for 1 min; 30 cycles of 95°C for 15 s, 50°C for 15 s and 72°C for 15 s; and a final extension at 72°C for 2 min. Subsequent sequence reactions were performed using BigDye Terminator

v. 3.1 (Applied Biosystems), and the same forward and reverse primers utilized during the initial amplifications. PCR products were purified with BigDye XTerminator (Applied Biosystems) and sequenced on an AB 3500 genetic analyzer (Applied Biosystems) located at the UNCW Center for Marine Science. Using Geneious version 8.02 (Kearse et al., 2012), raw forward and reverse sequences were assembled into consensus sequences. DNA sequences were obtained for all 20 individuals using the COI gene (with 13 of these sequences not previously published) and 12 individuals using the 18S rRNA gene, and all new sequences were archived in GenBank (Table 1). Note that one sample (1Mar16-3B-1) from Villalobos et al. (2017) was further analysed here. Villalobos et al. (2017) sequenced the COI gene; here, we sequenced the 18S rRNA gene (Table 1).

2.3 | Phylogenetic and species delimitation analyses

In order to compare relationships between haplotypes, independent neighbour-joining (NJ; Saitou & Nei, 1987) and maximum likelihood (ML) phylogenetic analyses were performed in MEGA v.7.0 (Kumar et al., 2016). Additional COI sequences for *Distaplia bermudensis* obtained by the authors in previous research (Evans et al., 2017, 2018; Villalobos et al., 2017) were retrieved from GenBank and added to the analyses together with colour morph information. For the COI gene, both NJ and ML trees were constructed with 35 sequences of *D. bermudensis* corresponding to the same 5 most common colour combinations (Table 1). Twenty-three additional sequences for other aplousobranchia ascidians and for the stolidobranch species *Styela plicata* (out-group) were retrieved from GenBank. Additional analyses performed with only *D. bermudensis* sequences and *Lissoclinum fragile* as an out-group resulted in identical tree topologies and are not shown here. NJ analyses were performed with the Tamura-Nei model (Tamura & Nei, 1993) and Gamma distribution (+G; Yang, 1994). The robustness of the NJ tree was evaluated by bootstrap analysis (Felsenstein, 1985) with 10,000 replicates. For the ML tree, the best-fit substitution model was the General time-reversible model (GTR; Tavaré, 1986) with Gamma distribution (+G; Yang, 1994), a proportion of invariable sites (+I; Shoemaker & Fitch, 1989) and 100 bootstrap replicates. For the 18S rRNA gene, NJ and ML analyses were performed using the 12 sequences obtained here and sequences obtained from GenBank for *Distaplia dubia*, two *Pycnoclavella* species (Aplousobranchia), and *Styela plicata* (Stolidobranchia) as an out-group. Analyses were also conducted without the stolidobranch as an out-group and resulted in identical tree topologies (not showed here). The best substitution model for our 18S rRNA data set was the Kimura 2-parameter (Kimura, 1980), and both NJ and ML analyses were conducted using this model and 10,000 and 1,000 bootstrap replicates, respectively.

An Automatic Barcode Gap Discovery (ABGD) analysis was performed to assign COI sequences into hypothetical species (Puillandre et al., 2012). Default settings were used for the prior intraspecific divergence (P; Pmin = 0.001, Pmax = 0.1; steps = 10), the relative gap width (X; X = 1.5) and the number of bins (Nb) for distance distribution (Nb = 20). All three available distance models (Jukes-Cantor (JC; Jukes & Cantor, 1969), Kimura 2-parameter (K2; Kimura, 1980) and p-distances (p-dist)) were used for calculating pairwise distances to eliminate potential biases presented by any one model.

2.4 | Sequencing of microbial symbionts

Eight samples representing individuals collected from the same site (Heide Trask Memorial Bridge at the Bridge Tender Marina) on the same day were selected for microbial community analysis. Half of the samples ($n = 4$; 8Mar16-4A, 8Mar16-4B, 8Mar16-5A and 8Mar 16-8) represented individuals from lineage A and possessed beige or pink tunics with brown siphon openings (colour morphs 4 and 5). The other half were from lineage B (8Mar16-1E, 8Mar16-2B, 8Mar16-3A, and 8Mar16-9) and included individuals with either dark blue tunics and yellow siphon openings or brown tunics with pink-orange siphon openings (colour morphs 1 and 2). In order to characterize the microbial communities associated with these ascidians, a ca. 300 bp fragment (V4 region) of the 16S ribosomal RNA (rRNA) gene was amplified from the tunic DNA extracts using the universal bacterial/archaeal forward primer 515f and reverse primer 806r (Caporaso et al., 2011). Each 25 µl PCR reaction consisted of 12 µl MyTaq HS Red Mix (Bioline), 1 µl of each primer and 1 uL DNA extract. Amplifications were performed in an Eppendorf Mastercycler nexus X2, with a thermocycler program consisting of an initial denaturation at 95°C for 2 min; 35 cycles of 95°C for 15 s, 50°C for 15 s and 72°C for 20 s; and a final extension at 72°C for 2 min. These initial amplifications were used to confirm the quality of the DNA extractions, and PCR viable DNA extracts were subsequently sent to Molecular Research LP for amplification, library construction and multiplexed sequencing of partial 16S rRNA gene sequences on an Illumina MiSeq platform, using the same 515f and 806r primers described above. All raw sequences were deposited in the Sequence Read Archive of NCBI (SRA accession number PRJNA640828).

2.5 | Processing of microbial sequences

Raw microbial sequences were processed using the mothur software package (v.1.39.5; Schloss et al. 2009), following a modified version of the Illumina MiSeq SOP pipeline

(Kozich et al., 2013). Raw sequences were quality-filtered and aligned against the Silva reference database (SSU Ref NR 99 128). Putative chimeric sequences were removed within mothur through self-reference searching via the UChime software package (Edgar et al., 2011). Sequences were classified and confidence-scored through a naive Bayesian classifier and bootstrap algorithm, constructed based on the improved Greengenes taxonomy (McDonald et al., 2012). Singletons and non-target sequences, including chloroplasts, mitochondria and eukarya, were removed from the data set, and all remaining high-quality sequences were assigned to operational taxonomic units (OTUs) based on 97% sequence identity and the average neighbour clustering algorithm, with the taxonomic classification of each OTU determined by majority consensus (Schloss & Westcott, 2011). To standardize sampling depth (i.e. the number of sequence reads) across samples and allow for more robust comparisons, each data set was subsampled to the lowest read count ($n = 76{,}055$), and all subsequent data analyses were performed using resulting subsampled data sets. Rarefaction curves indicated this subsampling depth was sufficient to capture the microbial community diversity of all the ascidian hosts (Figure S2).

2.6 | Microbial community diversity analysis

Alpha diversity metrics for OTU richness, evenness and diversity were calculated within mothur to compare overall microbial community diversity between the two *D. bermudensis* genetic lineages. The indices used for richness included S (observed number of OTUs) and Chao 1 (expected number of OTUs); for evenness, the Simpson evenness index ($E_{1/D}$) and the inverse Simpson index (D); and for diversity, the Shannon-Weaver diversity index (H′). Analyses of variance (ANOVA) were performed within JMP Pro 14 to statistically compare the indices between the two lineages, based on a 95% confidence interval.

2.7 | Microbial community structure analysis

To investigate potential differences in symbiotic microbial community structure between the two genetic lineages, beta diversity metrics for OTU overlap and microbial community similarity were calculated. Venn diagrams were constructed in mothur to visualize OTU overlap between *D. bermudensis* genetic lineages. Bray–Curtis similarity (BCS) matrices were constructed within PRIMER (version 7.0.13) based on square root transformed OTU relative abundances and visualized through non-metric multi-dimensional scaling plots (nMDS). A permutational multivariate analysis of variance (PERMANOVA) was performed to compare the microbial community structure of the two *D. bermudensis* lineages. A permutational multivariate analysis of dispersion (PERMDISP) was also performed to confirm that all significant PERMANOVA results were due to legitimate structural differences, rather than unequal dispersion across the different ascidian hosts. Similarity percentage (SIMPER) analyses were conducted to determine which OTUs contributed the majority (>50%) of the observed differences in microbial communities of the two genetic lineages. To correct for any potential errors that may occur with distance-based metric calculations (Warton et al., 2012), a Metastats test was performed within mothur (based on 1,000 permutations) to isolate those SIMPER OTUs exhibiting significant differences in relative abundance between the two lineages (White et al., 2009). Sequence data were further sorted into abundant (>0.1% relative abundance) and rare (≤ 0.1%) data partitions, with BCS matrices constructed for each partition and analyses of microbial community structure and diversity conducted for each partition as described above.

3 | RESULTS

3.1 | Phylogenetic positioning and species delimitation

Cytochrome c oxidase subunit I (COI) gene sequences were obtained for all 20 samples (13 sequenced here and 7 from a previous study, Table 1) and for 12 samples using the 18S rRNA gene (Table 1). Five haplotypes were identified for the COI gene (labelled a, b, c, d and e, Figure 1) and two for the 18S rRNA gene (Figure 2). For the COI gene, nucleotide variation (102 substitutions) was scattered across the entire sequence region, with nearly half of this variation yielding synonymous changes ($n = 52$; 51%) and the other half non-synonymous ($n = 50$, 49%). Only one nucleotide substitution was detected for the 18S rRNA gene fragment. NJ and ML trees were equivalent for each gene so only the tree topologies obtained with one method are shown, together with bootstrap results obtained from both methods (Figures 1 and 2). Both ML and NJ analyses based on COI sequences showed two clades for *D. bermudensis* (hereafter called Lineage A and B) with bootstrap support >70 (Figure 1). The mean distance between these two lineages was 20.5%, while mean group distance within lineage A was 0.3% and within lineage B was 0.2%. Lineage A was formed by seven sequences representing two haplotypes (d and e) from specimens ranging in colour from white to light pink (colour morphs 4 and 5, Table 1). Lineage B was formed by six sequences representing three haplotypes (a, b and c), and individuals in this clade appeared purple to dark blue, brown or dark orange (colour morphs 1–3, Table 1). All sequences except for one (GenBank access. number KY111419; Villalobos et al., 2017) belonged to

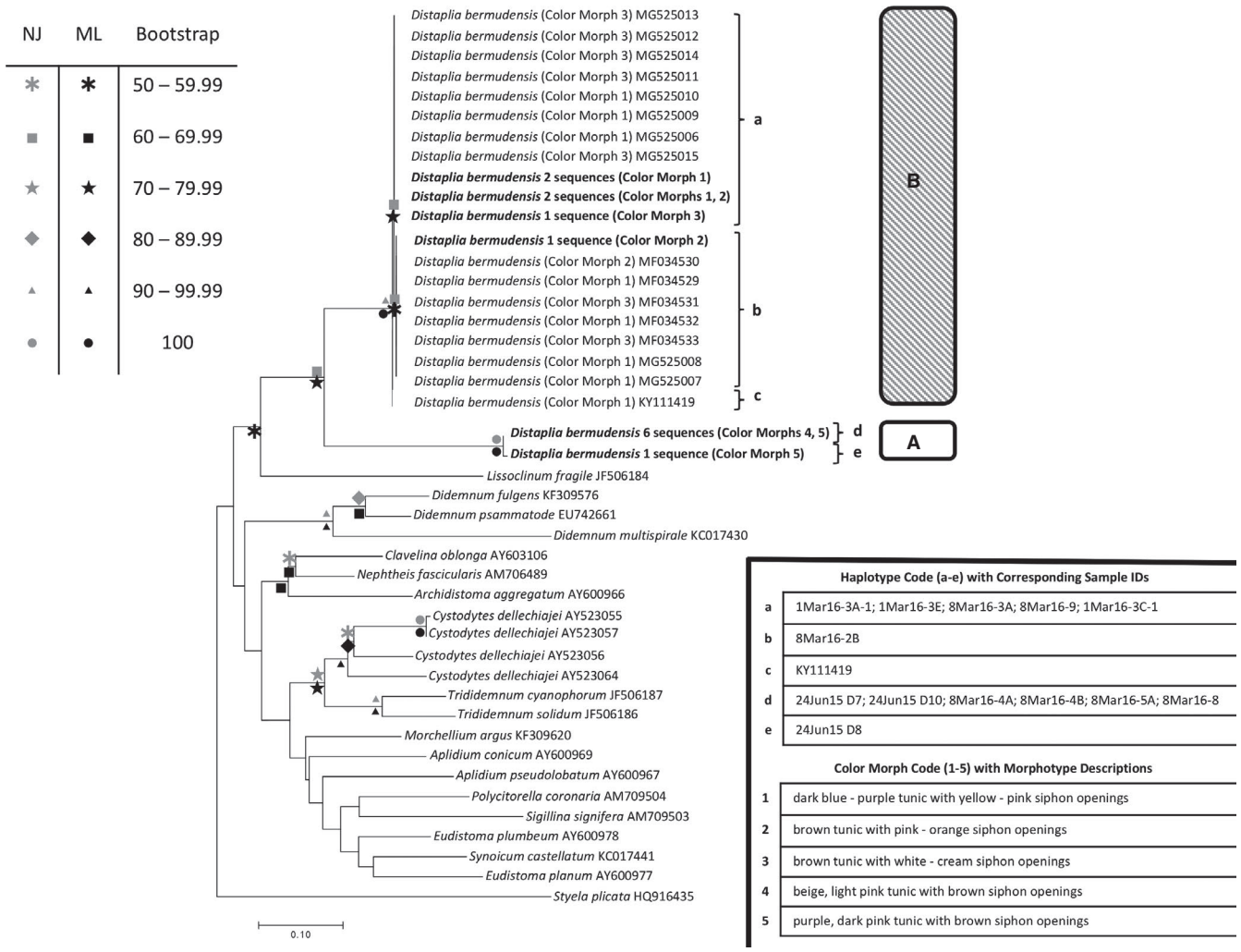

FIGURE 1 *Distaplia bermudensis* phylogeny based on partial cytochrome c oxidase subunit I (COI) sequences. Tree topology was obtained from NJ analysis. Bootstrap values (based on 10,000 replicates) greater than 50% are indicated by different shapes, where 50%–59% support is represented by asterisks, 60%–69% by squares, 70%–79% by stars, 80%–89% by diamonds, 90%–99% by triangles and 100% by circles. Bootstrap values for NJ (grey shapes) and ML (black shapes) appear above and below each node, respectively. Haplotypes obtained in this study are labelled a-e and indicated in bold, along with the number of new sequences obtained in this study ($n = 13$) corresponding to each haplotype and the corresponding colour morphology codes (1–5; in parentheses). The inset table shows sample IDs corresponding to each haplotype (a-e) and descriptions of colour morphologies indicated by codes 1–5. Sequences obtained from GenBank are identified by accession numbers. The two clades of *D. bermudensis* (A and B) are indicated by labelled bars. Scale bar represents 0.10 substitutions per site

either haplotype a or b (Figure 1). Results based on the 18S rRNA sequences showed a similar trend although with low bootstrap support (Figure 2), due to the low genetic variation (i.e. single nucleotide substitution). When these lineages were further compared with the known colour morphology of each sample, a pattern of distinct colour combinations was detected. Lineage A consisted of colonies with lighter coloured tunics and darker zooid openings, while colonies with darker tunics and lighter zooid openings made up Lineage B (Table 1 and Table S1). This pattern was unobservable after fixation.

Species delimitation analyses using ABGD to investigate COI divergence resulted in a bimodal distribution of distance values for all 3 models (Figure S3). All 3 models obtained an average COI divergence of 22.34%, 22.4% and 19.4% between Lineages A and B for JC, K2, and p-dist, respectively. Within lineage A, average COI divergence was 0.06%, 0.06% and 0.25% for JC, K2 and p-dist, respectively. Within lineage B, average COI divergence was 0.28%, 0.26% and 0.49% for JC, K2 and p-dist, respectively.

3.2 | Microbial community diversity and structure

For the microbial community analysis, 608,440 total prokaryotic sequences were obtained from eight *D. bermudensis* individuals, representing 1,302 unique microbial OTUs. From

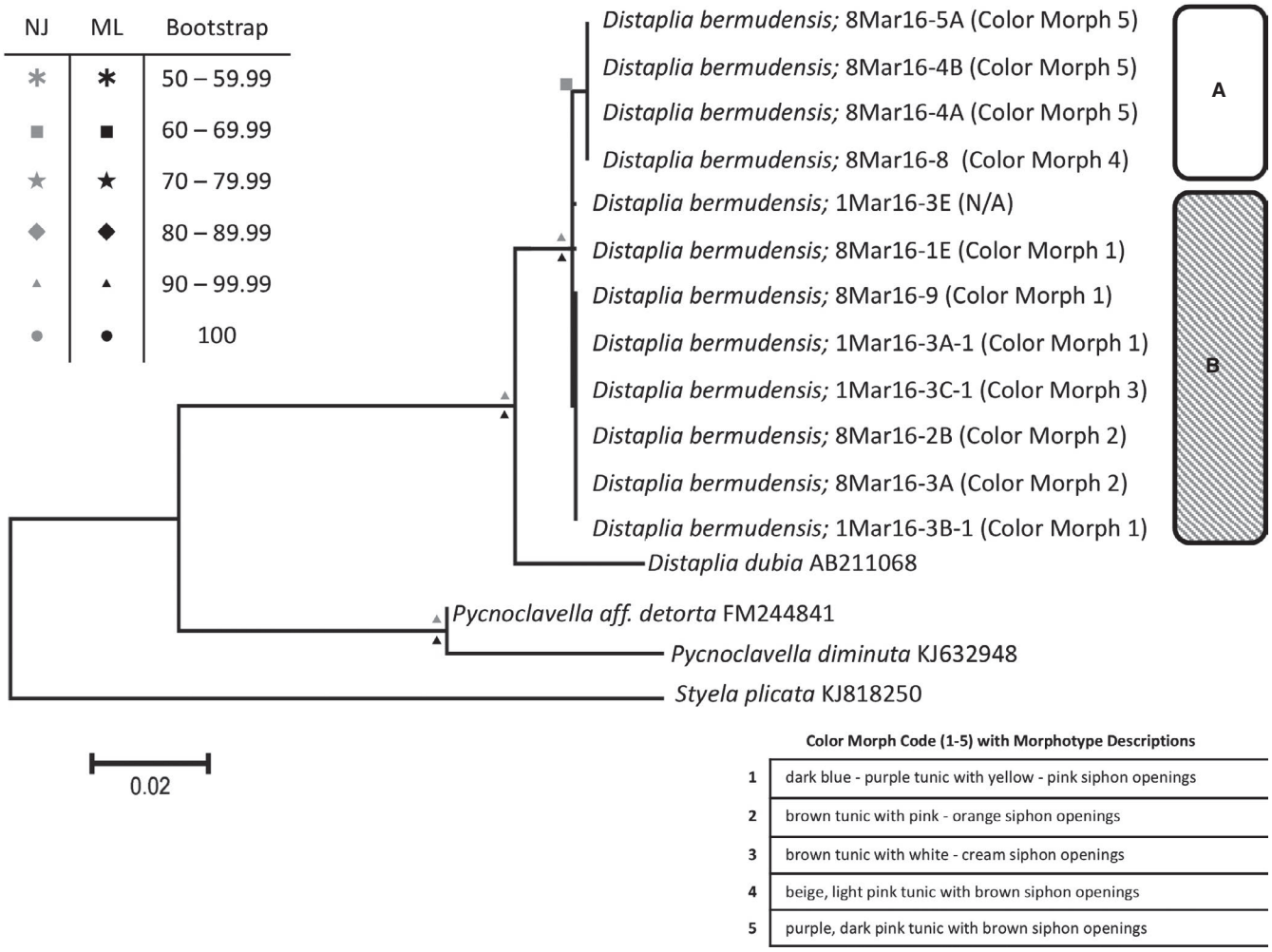

FIGURE 2 *Distaplia bermudensis* phylogeny based on partial 18S rRNA gene sequences. Tree topology was obtained from maximum likelihood (ML) analysis, with bootstrap values greater than 50% indicated by different shapes (support 50%–59% is indicated by asterisks, 60%–69% by squares, 70%–79% by stars, 80%–89% by diamonds, 90%–99% by triangles and 100% by circles). Bootstrap values for NJ (grey shapes) and ML (black shapes) appear above and below each branch, respectively. Sequences obtained from GenBank are identified by accession numbers. The two clades of *D. bermudensis* (A and B) are indicated by labelled bars. Scale bar represents 0.10 substitutions per site

these OTUs, 337 were unique to lineage A and 441 to lineage B, while the remaining 524 microbial OTUs were present in both genetic lineages (Figure S4). These OTUs spanned two archaeal (*Nanoarchaeota* and *Thaumarchaeota*) and 21 bacterial phyla. Microbial symbionts in Lineage A hosts included 16 bacterial and no archaeal phyla and were dominated by *Alphaproteobacteria* (71%), *Bacteroidetes* (12%), unclassified *Proteobacteria* (9%) and *Gammaproteobacteria* (5%; Figure 3). Microbial symbionts in Lineage B hosts spanned 18 bacterial and two archaeal phyla, and were dominated by *Alphaproteobacteria* (52%), *Gammaproteobacteria* (32%), *Bacteroidetes* (11%) and unclassified *Proteobacteria* (3%; Figure 3). Archaeal symbionts were detected only in ascidians from Lineage B and sporadically; *Nanoarchaeota* was present in just one sample (8Mar16-3A) and *Thaumarchaeota* was detected in two samples, with the majority of sequences (99%; $n = 230$) occurring within just one sample (8Mar16-9). Of the five metrics for alpha diversity and evenness calculated (S, Chao 1, $E_{1/D}$, D and H′), only the inverse Simpson index (D) indicated significant differences between microbial communities across the two *D. bermudensis* lineages, with significantly higher diversity in symbiont communities of Lineage B (ANOVA, $p < .05$; Table 2). No other indices revealed any significant differences in microbial community alpha diversity between the two lineages (ANOVA, $p > .05$; Table 2).

To further examine the observed differences in microbial community composition between the two lineages, a PERMANOVA test was conducted based on a beta diversity metric (Bray–Curtis similarity). Microbial community structure was found to differ significantly between the two genetic lineages (PERMANOVA, $p = .026$; Figure 4), with host genetic lineage alone explaining an estimated 30% of the observed variation in microbial community structure. The structure of microbial communities from ascidians within each genetic lineage was on average 50% and 49.6% similar to other specimens within the same genetic lineage, for lineages A and B,

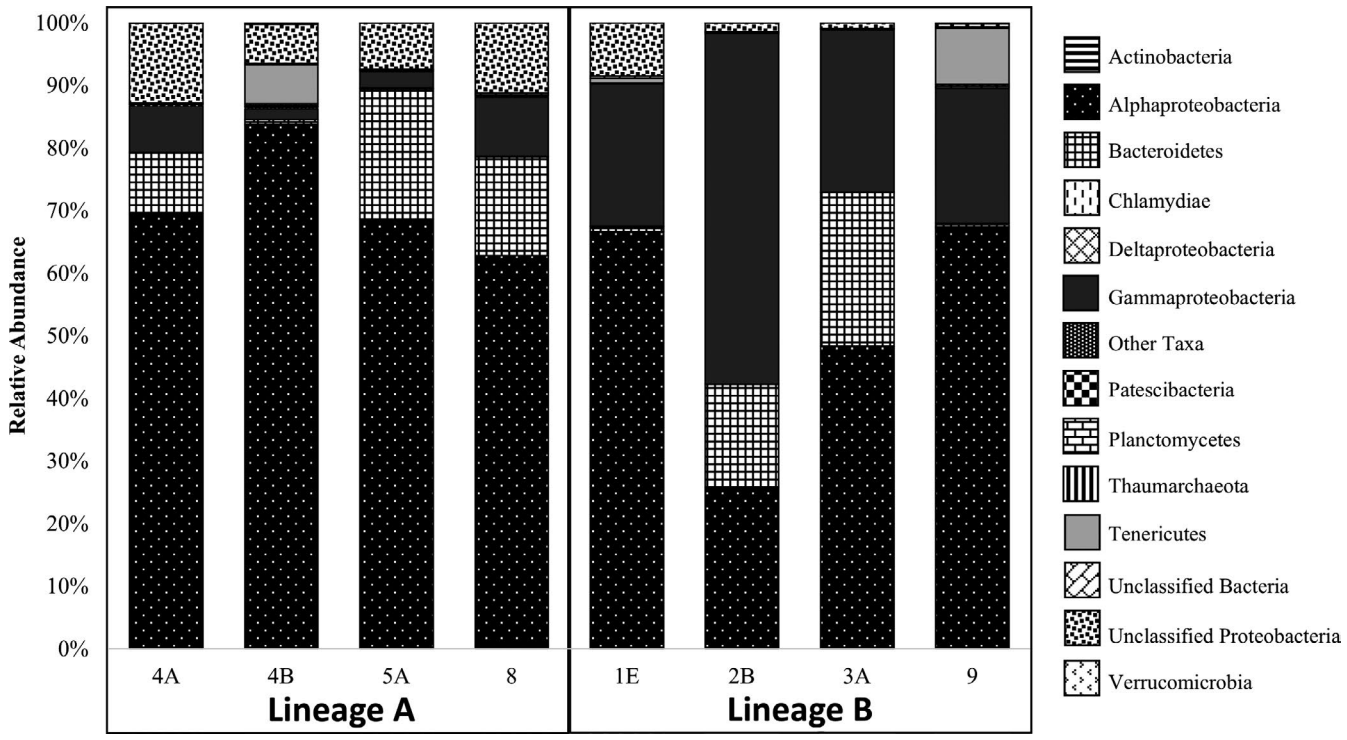

FIGURE 3 Symbiotic microbial community composition and relative abundance for each replicate sample of *D. bermudensis*. Individuals from Lineage A (samples 8Mar16-4A, 4B, 5A, 8) are shown on the left and Lineage B (samples 8Mar16-1E, 2B, 3A, 9) on the right. Phylum-level classifications are shown for all symbionts except Proteobacteria, which are divided into major classes. Here, 'other taxa' includes Acidobacteria, Chloroflexi, Cyanobacteria, Epsilonbacteraeota, Firmicutes, Fusobacteria, Latescibacteria, Lentisphaerae, Nanoarchaeota, Nitrospinae, Nitrospirae, Omnitrophicaeota and PAUC34f

TABLE 2 Diversity metrics for microbial communities associated with *D. bermudensis* Lineage A and Lineage B

Lineage	S	Chao 1	$E_{1/D}$	D	H′
A	318.3 ± 104.5^{A}	681.0 ± 66.0^{A}	0.007 ± 0.003^{A}	2.0 ± 0.408^{B}	1.2 ± 0.203^{A}
B	356.3 ± 144.7^{A}	627.7 ± 95.6^{A}	0.008 ± 0.003^{A}	2.7 ± 0.324^{A}	1.4 ± 0.134^{A}

Note: S: observed richness, Chao 1: expected richness, $E_{1/D}$: Simpson evenness, D: inverse Simpson index, and H′: Shannon-Weaver index. Average values (±1 *SD*) are shown, with different superscript letters indicating significantly different means between the two genetic lineages.

respectively. Microbial community structure between different lineages was on average 45.2% similar. No significant differences in dispersion were detected between lineages A and B (PERMDISP, $p = .886$) for the overall data partition (Table 3). For the overall data partition, SIMPER analyses revealed the identity of OTUs contributing most significantly to the observed differences between the microbial communities of the two genetic lineages of *D. bermudensis* (Table 4) and determined that a proportionally small number of OTUs ($n = 117$; 8.9% of all OTUs) were responsible for over 50% of this variation. The majority (50%) of differences between individuals within the same lineages were similarly driven by variation in relative abundance of small numbers of OTUs ($n = 2$ and 3 for lineages A and B, respectively; Table S2).

Partitioning into abundant (>0.1% relative abundance) and rare (≤0.1%) partitions resulted in 58 abundant OTUs, representing 98.6% of all microbial sequences, and 1,244 rare OTUs, representing 1.4% of all microbial sequences. Alpha diversity metrics revealed that for the abundant data partition, a significant difference in evenness exists within the two lineages (inverse Simpson test, $p < .05$), while no significant differences in diversity or evenness were detected for the rare data partition ($p > .05$). Analyses of the abundant and rare data partitions indicated significant differences in microbial community structure between the two *D. bermudensis* genetic lineages for the abundant but not the rare data partition (PERMANOVA, $p = .032$ and .297, respectively; Table 3). SIMPER analyses of the abundant data partition found that microbial communities within lineages A and B exhibited 71.2% and 67.7% average similarity among individuals within the same lineage, respectively, while microbial communities between the two lineages were on average just 62.5% similar

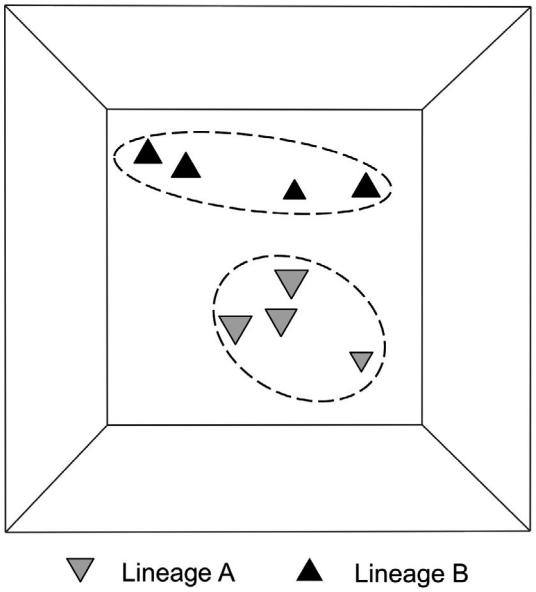

FIGURE 4 Non-metric multi-dimension scaling plot based on Bray–Curtis similarity of symbiotic microbial communities in Lineage A (grey triangles) and Lineage B (black triangles) of *D. bermudensis*. Circles encompass replicate samples within each lineage and indicate a high degree of host-specificity

TABLE 3 Statistical comparisons of microbial community structure (PERMANOVA) and dispersion (PERMDISP) between *D. bermudensis* Lineage A and B for overall, abundant and rare data partition levels

	PERMANOVA		PERMDISP	
	F	*p*	*F*	*p*
Overall	1.736	.026	0.008	.881
Abundant	3.008	.032	0.647	.515
Rare	1.163	.297	0.688	.415

for the abundant data partition. Differences between the two *D. bermudensis* genetic lineages were driven primarily (> 50%) by variation in the average relative abundance of just 5 OTUs (0.38% of all OTUs; Table S3). In contrast, for the rare data set, microbial communities of individuals within lineages A and B were on average 18.2% and 21.8% similar, respectively, with an average similarity between the two lineages of just 18.4%. This difference was driven primarily (> 50% contribution) by variation in the relative abundance of 340 OTUs (26.1% of all OTUs). No significant differences in dispersion were detected for either the abundant or rare data partitions (PERMDISP, $p > .05$; Table 3).

4 | DISCUSSION

In this study, we observed a broad range of colour morphs among *Distaplia bermudensis* individuals collected from two proximate locations. Tunics in shades of purple, orange, pink, tan, blue and white, with varying combinations of siphon opening colours, were examined. Genetic analyses using the mitochondrial COI gene and ribosomal 18S rRNA gene revealed the presence of two distinct genetic lineages within the investigated *D. bermudensis* colonies. We detected an interclade divergence of 20.5% based on the COI gene, well above or comparable to the COI divergence values reported for other cryptic colonial species (Bock et al., 2012; Bouzon et al., 2014; López-Legentil & Turon, 2006; Pérez-Portela et al., 2013; Pérez-Portela & Turon, 2008; Tarjuelo et al., 2004), and between congeneric species of Chordata (Hebert et al., 2003). When we further investigated the association between genetic identity and colour variation, a distinct pattern emerged, with lighter tunics and darker zooid openings (colour morphs 4 and 5) corresponding to Lineage A and darker tunics with lighter zooid openings (colour morphs 1, 2 and 3) corresponding to Lineage B. Although subtle, these colour differences within the living animals may represent a consistent macroscopic feature for distinguishing between these two cryptic lineages of *D. bermudensis*. Unfortunately, morphological examinations of these ascidians were only performed postfixation, at which time the above-reported pattern was unobservable due to coloration loss in the preservative. No other morphological differences were observed between either the adults or the larvae of the two lineages. However, previous studies investigating cryptic or early speciation in ascidians have similarly detected genetic differences among colour morphs and the absence of other distinguishing morphological characters between these taxa (Bouzon et al., 2014; López-Legentil & Turon, 2005, 2006; Tarjuelo et al., 2004).

To further characterize the level of divergence within *D. bermudensis*, we also investigated whether the genetic differences detected within the host ascidians would be reflected at the level of their microbial symbiont communities. By analysing 4 individuals of each lineage (A and B), we showed that each lineage harboured unique symbiont communities that differed significantly in diversity and structure. From a broader community perspective, trends across the two genetic lineages were immediately apparent, such as a greater relative abundance of gammaproteobacterial symbionts in Lineage B specimens. Thus, clear divergence in symbiont communities was observed between host lineages collected from the same site. Importantly, however, such divergence in symbiont communities was not observed between individuals from the same host lineage but different collection sites. Evans et al. (2018) investigated the ascidian-associated microbial communities in 10 specimens of *D. bermudensis* coming from two different localities and retrospectively assigned to Lineage B (5 specimens with dark blue-purple tunic and yellow-pink siphon openings from Bridge Tender Marina and 5

TABLE 4 Top 50 symbiotic microbial OTUs contributing most (>50% cumulative, SIMPER analyses) to observed dissimilarity between *D. bermudensis* Lineages A and B, based on the overall data partition

OTU ID	Relative abund. (%), Lineage A	Relative abund (%), Lineage B	Phylum	Lowest taxonomic classification	Contrib. Dissim.
2	3.28	28.73	*Proteobacteria*	(G.) *Endozoicomonas*	8.02*
3	11.25	10.27	*Bacteroidetes*	(G.) *Reichenbachiella*	4.52
4	9.38	2.77	*Proteobacteria*	(P.) *Proteobacteria*	3.62*
1	69.07	47.66	*Proteobacteria*	(O.) *Rhodospirillales*	3.39*
5	1.53	2.39	*Tenericutes*	(G.) *Candidatus_Hepatoplasma*	2.09
6	0.24	3.10	*Proteobacteria*	(F.) *Rhodobacteraceae*	1.96
7	0.30	1.63	*Proteobacteria*	(G.) *Endozoicomonas*	1.63*
11	0.27	0.01	*Proteobacteria*	(F.) *Francisellaceae*	0.78
9	0.16	0.16	*Proteobacteria*	(F.) *Vibrionaceae*	0.64
10	0.19	0.12	*Proteobacteria*	(G.) *Delftia*	0.62
13	0.14	0.03	*Proteobacteria*	(G.) *Pseudomonas*	0.58
12	0.18	0.07	*Proteobacteria*	(G.) *Pseudomonas*	0.57
14	0.13	0.02	*Proteobacteria*	(F.) *Paracaedibacteraceae*	0.5
8	1.00	0.65	*Proteobacteria*	(O.) *Rhodospirillales*	0.45*
17	0.06	0.04	*Proteobacteria*	(F.) *Francisellaceae*	0.42
18	0.07	0.003	*Proteobacteria*	(C.) *Gammaproteobacteria*	0.42
16	0.08	0.03	*Bacteroidetes*	(C.) *Bacteroidia*	0.4
15	0.07	0.08	*Tenericutes*	(G.) *Candidatus_Hepatoplasma*	0.39
19	6.57E-4	0.09	*Proteobacteria*	(G.) *Coxiella*	0.38
20	3.29E-4	0.09	*Bacteroidetes*	(G.) *Rubidimonas*	0.36
40	0.003	0.04	*Proteobacteria*	(G.) *Vibrio*	0.36*
25	0.02	0.05	Unclassified Bacteria	(K.) Bacteria	0.35
22	0.04	0.04	*Proteobacteria*	(G.) *Candidatus_Tenderia*	0.33
29	0.05	0.01	Unclassified Bacteria	(K.) Bacteria	0.31
32	0	0.06	*Proteobacteria*	(P.) *Proteobacteria*	0.30
39	3.29E-4	0.05	*Proteobacteria*	(G.) *Afipia*	0.30
26	0.04	0.02	*Proteobacteria*	(F.) *Rhodobacteraceae*	0.30
23	0.07	6.57E-4	*Proteobacteria*	(G.) *Schlegelella*	0.30
41	0.04	0	*Bacteroidetes*	(F.) *Cryomorphaceae*	0.30
30	0.01	0.05	*Chlamydiae*	(F.) *Simkaniaceae*	0.29
42	0.03	0.009	*Verrucomicrobia*	(G.) *Persicirhabdus*	0.28
31	0.05	6.57E-4	*Actinobacteria*	(G.) *Ilumatobacter*	0.28
36	0.03	0.02	*Proteobacteria*	(F.) *Vibrionaceae*	0.28
27	0	0.07	*Thaumarchaeota*	(G.) *Candidatus_Nitrosopumilus*	0.27
37	0.03	0.01	*Proteobacteria*	(G.) *Amphritea*	0.27
24	0.04	0.02	*Bacteroidetes*	(G.) *Reichenbachiella*	0.26
34	0.04	0.005	*Proteobacteria*	(G.) *Klebsiella*	0.26
51	0.003	0.03	*Proteobacteria*	(O.) *BD7-8*	0.26
33	0.05	0.007	*Planctomycetes*	(G.) *Rubripirellula*	0.26
38	0.04	0.003	*Bacteroidetes*	(F.) *Cyclobacteriaceae*	0.26
45	3.29E-4	0.04	*Proteobacteria*	(F.) *Francisellaceae*	0.25
48	0.03	0	*Proteobacteria*	(G.) *Brevundimonas*	0.25
35	0.04	0	*Planctomycetes*	(P.) *Planctomycetes*	0.25

(Continues)

TABLE 4 (Continued)

OTU ID	Relative abund. (%), Lineage A	Relative abund (%), Lineage B	Phylum	Lowest taxonomic classification	Contrib. Dissim.
47	0.03	0.006	*Proteobacteria*	(G.) *Halioglobus*	0.24
49	0.004	0.03	*Proteobacteria*	(G.) *Sulfitobacter*	0.23
53	0.02	0.01	*Fusobacteria*	(G.) *Propionigenium*	0.22
44	0.04	0	*Acidobacteria*	(G.) *Blastocatella*	0.21
88	3.29E-4	0.02	*Proteobacteria*	(F.) *Thiohalorhabdaceae*	0.21
84	0.002	0.02	*Proteobacteria*	(G.) *Stenotrophomonas*	0.21
50	0.03	6.57E-4	*Actinobacteria*	(G.) *Micrococcus*	0.21

Note: Identity (lowest taxonomic classification) of each OTU and relative abundance within each genetic lineage are indicated. Asterisks (*) denote significantly different (Metastats, $p < .05$) OTU abundances between the two genetic lineages.

Abbreviations: Contrib. Dissim, Contribution to dissimilarity (%); K, kingdon; P, phylum; C, Class; O, Order; F, family; G, genus; S, species.

additional specimens with brown tunics and white-cream siphon openings collected at the 5-Mile Ledge reef). The authors determined that *D. bermudensis* maintained a stable microbial community across the two habitats and that vertical transmission of symbionts to the offspring was essential for host functioning (Evans et al., 2018). Similarly, Casso et al. (2020) found that host genetic variation within a species, rather than geographic distance between its populations, exerted a greater influence in structuring ascidian microbial communities.

In addition to broad differences in symbiont composition, fine-scale variation at the OTU level was also detected between lineages of *D. bermudensis* and provided preliminary insight into putative functional consequences of symbiont divergence. A relatively small proportion of OTUs (9%, $n = 117$) was responsible for the majority (>50%) of the observed microbial community dissimilarity between the two host lineages. The symbiont OTU with the greatest contribution (8%) to the observed dissimilarity between the two *D. bermudensis* lineages was OTU0002, which was the second-most abundant OTU in lineage B ($n = 87{,}392$; 28.7% relative abundance) and exhibited significantly lower abundance in Lineage A ($n = 9{,}985$). This particular symbiont OTU was affiliated with the proteobacterial genus *Endozoicomonas*, which are commonly described ascidian symbionts (Cahill et al., 2016; Evans et al., 2017; Evans et al., 2018; Schreiber, Kjeldsen, Funch, et al., 2016), with some species capable of nitrate reduction (Kurahashi & Yokota, 2007; Schreiber et al., 2016). OTU0004 contributed 3% to the observed dissimilarity between the two lineages and was found in significantly greater concentration in Lineage A ($n = 28{,}537$; 9.4% relative abundance) than Lineage B ($n = 8{,}436$; 2.8% relative abundance). This symbiont OTU was affiliated with the genus *Reichenbachiella* (phylum Bacteroidetes), which represent known producers of carotenoid pigments (Nedashkovskaya et al., 2005) and have been suggested to provide UV protection in some marine invertebrates (Pichon et al., 2007). Another major contributor (0.6%) to the observed dissimilarity between the two genetic lineages of *D. bermudensis* was OTU0010. This symbiont OTU was in greater abundance in Lineage A ($n = 581$; 0.2% relative abundance) and classified to the gammaproteobacterial genus *Delftia*, which has links to organic pollutant degradation (Vásquez-Piñeros et al., 2018).

Notably, some variability in symbiont composition was observed within each lineage of *D. bermudensis*. For example, the two specimens with the greatest relative abundance of Tenericutes symbionts (8Mar16-4B and 8Mar16-9) fell within opposite lineages (A and B, respectively), and both specimens represented the largest outliers from their respective lineages. This greater relative abundance of Tenericutes was primarily due to a large overall abundance of a single OTU, OTU0005, affiliated with the genus *Candidatus* Hepatoplasma (class Mollicutes). To date, members of this genus have only been described in the gut microbiome of terrestrial isopods (Fraune & Zimmer, 2008; Wang et al., 2004). Other genera in the class Mollicutes represent known pathogens (Razin et al., 1998), suggesting *Candidatus* Hepatoplasma may also represent pathogenic symbionts. While further sampling is required to resolve the nature of this symbiont, Mollicutes-affiliated taxa were also rare in previous studies of microbial communities of *D. bermudensis* (Evans et al., 2018).

What ecological significance, if any, speciation among *D. bermudensis* colour morphs may have remains to be determined. In this context, the different microbial community compositions of the two lineages as described here may prove especially edifying. In ascidians, proposed benefits of microbial symbiosis include supplemented host nutrition from photosymbionts (Hirose & Maruyama, 2004; Maruyama et al., 2003) and improved host defence through symbiont-derived secondary metabolites (Schmidt et al., 2005). Diverse microbial communities may facilitate the successful introduction of some ascidian species to new

habitats by enhancing host fitness and tolerance to a wide range of environmental parameters (Dror et al., 2019; Evans et al., 2017). Thus, if symbiont capabilities such as those described above (e.g. nitrate reduction, UV protection and organic pollutant processing) improve the host ascidian's overall fitness, differences between genotypes in their symbiotic microbial communities may ultimately favour the long-term survival of one lineage over the other. All in all, the high degree of host-specificity exhibited by ascidian microbiomes (Erwin et al., 2014; Tianero et al., 2015), the stability of *D. bermudensis* microbial associations across different habitats (Evans et al., 2018), and the differences in both host genetics and microbial symbiont composition between Lineages A and B detected herein, indicate that two cryptic species of *D. bermudensis* coexist in North Carolina.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest as defined by Zoologica Scripta.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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