Symbiosis
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Mutualistic dinoflagellates with big disparities in ribosomal DNA variation may confound estimates of symbiont diversity and ecology in the jellyfish *Cotylorhiza tuberculata*

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Abstract

The precise identification of mutualistic dinoflagellates is critical for understanding the physiology, ecology and evolution of their mutualisms with animals. *Cotylorhiza tuberculata* (Macri 1778) is a common scyphozoan endemic to the Mediterranean Sea and relies in part on endosymbiotic dinoflagellates (zooxanthellae) for survival and growth. To further study the diversity of symbionts associated with these animals, we analyzed specimens of *C. tuberculata* collected across the western Mediterranean Sea and from public aquaria, using a combination of next generation sequencing (NGS) of ITS2 rDNA and direct Sanger sequencing of partial 28 S rRNA and mitochondrial cob genes. Two diagnostic ITS2 profiles were characterized during our analysis of NGS data. Combined with information from additional genetic markers, each profile corresponds to a single species of symbiont, not diverse community assemblages as are sometimes inferred. *Breviolum psygmophilum* was common in all specimens, while *Philozoon medusarum* occurred at lower abundances in many individuals. The ribosomal array of *B. psygmophilum* was highly heterogeneous and contained ~ 15 co-occurring sequence variants found in the same relative proportions across all samples obtained in this study, while the ribosomal array in the genomes of *P. medusarum* was relatively homogeneous represented mostly by one abundant sequence variant. This precise interpretation of rDNA data improves understanding of the ecology and evolution of these mutualisms. *Cotylorhiza tuberculata*'s association with dinoflagellate symbionts from different genera is consistent with previous findings and suggests that evolutionary divergent symbionts with dissimilar niches are better able to coexist *in hospite*.

Keywords *Breviolum* · Coexistence · Eukaryotic rDNA · Mediterranean · *Philozoon* · Scyphozoa · Symbiodiniaceae

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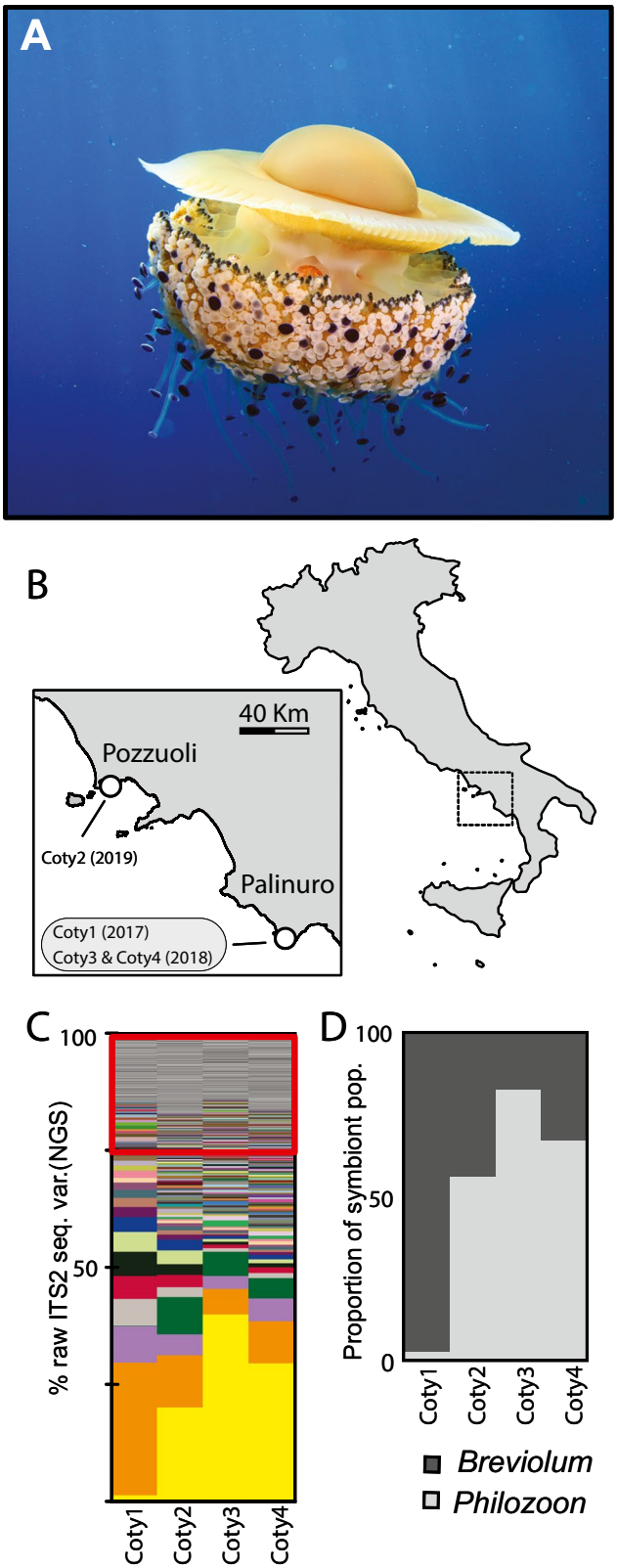

Fig. 1 Symbiosis ecology of *Cotylorhiza tuberculata*. **A** Medusa ▶ stage of *Cotylorhiza tuberculata* in the water column. **B** Collection locations in 3 different years of four *Cotylorhiza tuberculata* specimens from coastal waters in southwestern Italy. **C** Deep sequencing (> 20,000 reads per sample) and sorting of rDNA sequence variants found in each sample by relative abundances using the SymPortal analysis program. The red box signifies rare sequence variants and pseudogenes, as well as the large numbers of technical artifacts created by the sequencing platform present in raw datasets. **D** Determination of symbiont composition based on 'defining intragenomic variants' estimated by SymPortal. (Photo credits: M. Cannavacciuolo)

1 Introduction

Currently, a non-standardized system of complex and confusing nomenclature is predominantly used to report results in studies of dinoflagellate diversity in host animals. This provisional taxonomy first emerged when evolutionarily divergent genetic lineages, or clades, in the dinoflagellate family Symbiodiniaceae were initially assigned letter designations (i.e. A, B, C, etc…; Rowan and Powers 1991); and later numbers were ascribed to dominant ITS2 rDNA sequence variants that defined ecologically/functionally distinct entities within each clade (e.g. LaJeunesse 2002, Sampayo et al. 2009). Now the increasing use of NGS has added large quantities of sequence variants to the database, which have amplified confusion about symbiont identity and species diversity, and blurred assessments of ecological patterns and processes fundamental to these mutualisms (LaJeunesse and Thornhill 2011; Hume et al. 2019).

The inconsistent ecological narratives found in the current literature stem primarily from an over interpretation of extensive sequence diversity (As cautioned by: Thornhill et al. 2007, Sampayo et al. 2009, LaJeunesse and Thornhill 2011, Hume et al. 2019). The numerous ITS2 sequence variants often characterized via NGS are too often interpreted in ways similar to how microbial diversity is assessed using 16 S sequencing (e.g. Apprill and Gates 2007). The presentation of sequence diversity and abundances in bar graphs gives the impression that hosts contain multiple and complex combinations, or communities, of symbionts in their tissues (e.g. Ong et al. 2022). But this interpretation dismisses the fact that eukaryotic genomes have numerous rDNA copies (Prokopowich et al. 2003), and that they possess numerous sequence variants (Thornhill et al. 2007). However, these conflicting interpretations can be reconciled with additional genetic markers and careful examination of the sequence abundance profiles created by NGS.

Mediterranean collections of the "fried egg jellyfish", *Cotylorhiza tuberculata* (Macri 1778), were used here to demonstrate how combined genetic analysis resolves the identity of symbiotic dinoflagellates (Fig. 1A). Among scyphozoans, approximately 20% (most belonging to the order Rhizostomeae) have mutualistic symbionts that provide them with metabolites derived from photosynthesis

(Djeghri et al. 2019; Davy et al. 2012). *Cotylorhiza tuberculata* is the only pelagic scyphomedusa native to the Mediterranean Sea reliant on dinoflagellate symbionts (Fig. 1; Kramp 1961). Past studies on the biology and ecology of *C. tuberculata* focused mainly on its population dynamics and life cycle (Kikinger 1992, Ruíz et al. 2012; Astorga et al. 2012), while efforts to characterize the identity and distribution of its symbionts remain limited (Visram et al. 2006; Dall'Olio 2016; LaJeunesse et al. 2021). The medusa 'jellyfish' stage is seasonally present in oligotrophic and eutrophic waters of the Mediterranean Sea (Boero 2013). Populations of this animal commonly reach high abundances during the summer season in shallow semi-enclosed marine areas, such as Vlyho Bay in Greece (Kikinger 1992) and the Mar Menor coastal lagoon in Spain (Pérez-Ruzafa et al. 2002; Ruiz et al. 2012). The jellyfish, which grows to 40 cm in diameter (Palomares and Pauly 2022), is known to provide nursery habitats to juvenile fish including the economically important Atlantic horse mackerel (*Trachurus trachurus*), as well as harboring many marine invertebrates (D'Ambra and Malej 2015).

Dall'Olio et al. (2022) provided the first comprehensive analysis of symbiont diversity in *Cotylorhiza tuberculata* from different localities around the Mediterranean Sea, including the Algerian Basin, southern Tyrrhenian, northern Adriatic, and Ionian Seas; and identified one of two dinoflagellate species, either *Philozoon medusarum* or *Brevolium* spp in each of their specimens. Moreover, from samples collected along different years in the northern Adriatic, they inferred the relative prevalence of *P. medusarum* and *Brevolium* spp may shift from year to year. However, LaJeunesse et al. 2021 found that individual *C. tuberculata* medusae from the southern Tyrrhenian Sea hosted simultaneously two species of symbiont, *Breviolum psygmophilum* LaJeunesse, Parkinson & Coffroth and *Philozoon medusarum* Geddes (LaJeunesse et al. 2021). Further biogeographic sampling of *C. tuberculata* would provide additional insight concerning the distribution and prevalence of these and possibly other symbionts.

For the resolution of symbiont diversity, we used a combination of genetic approaches to characterize the dinoflagellates in specimens of *C. tuberculata* collected across the western Mediterranean and from captive animals maintained for years in aquaria. Next generation Illumina sequencing was also used to profile the resident symbiont population in each animal, as well as to characterize the intragenomic diversity of ITS2 rDNA diagnostic of each symbiont (Arif et al. 2014; Hume et al. 2019), a process formerly performed by denaturing gradient gel electrophoresis (Thornhill et al. 2007; LaJeunesse and Pinzon 2007; Sampayo et al. 2009). Data from high throughput sequencing was augmented with direct sequencing of the 28 S (D1-D3 domain) rRNA as well as the mitochondrial cob genes to confirm whether interpretations of ITS2 sequence variation are accurate and verify the identities of resident symbiont species.

2 Materials and methods

2.1 Collections of *Cotylorhiza tuberculata*

From the southern Tyrrhenian Sea, three specimens of *C. tuberculata* scyphomedusae were collected in Palinuro (Campania, Italy) during August 2017 (Coty1) and August 2018 (Coty3 and Coty4; Fig. 1B). Another specimen (Coty2) was then collected in the waters off the town of Pozzuoli (Campania, Italy) in October 2019 (Supplemental Table S1). Animals collected in Palinuro (Coty1, Coty3 and Coty4) were immediately frozen at -20 °C until further processing at the Stazione Zoologica Anton Dohrn (SZN, Naples, Italy), while the scyphomedusa collected in Pozzuoli (Coty2) was brought alive to the laboratory. Whole animals of *Cotylorhiza tuberculata* were collected in 2020 near the coastline at locations across the western Mediterranean Sea (Supplemental Table S1). Portions of the swimming bell and oral arms were chemically preserved using DMSO preservation buffer (20% DMSO, 0.25 M EDTA, in super-saturated NaCl) or 96% ethanol (Supplemental Table S1).

Three specimens of *C. tuberculata* were also obtained from public aquaria: two from the Honriman Museum London England, and one from the Oceanogràfic (Valencia, Spain). Specimens from both aquaria are presumed to have originated from the Bay of Vlyho (Greece, Ionian Sea, Eastern Mediterranean).

2.1.1 Symbiont isolation, DNA extraction

Tissue samples from the oral arms were placed in a 1.5 µl microtube and homogenized using 0.5 mm glass bead in a bead beater for 2 min. DNA extractions followed the protocol described by LaJeunesse et al. (2003). Freshly collected cells from a live animal collected at site 6 were pelleted in a 1.5 µl microtube and then re-suspended in lysis buffer (Tissue and Cell lysis Solution by MasterPure DNA and RNA purification Kit, Epicenter, Madison, WI, USA) and stored at -20 °C. DNA extraction was then performed following the protocol specified in the MasterPure DNA and RNA purification Kit (Epicenter).

2.2 PCR amplifications and DNA sequencing

The ITS2 of the symbionts in samples from Italy was amplified using Symbiodiniaceae-specific primers SYM_VAR_5.8 S and SYM_VAR_REV (see Hume et al. 2013, Hume et al. 2015). All PCR mixtures (25 µL final volume) were composed of: 0.5 ng (2.5 µL) of extracted DNA, 0.5

µM of each primer, 3% of DMSO (dimethyl sulfoxide), 200 µM of dNTPs, 5x of High-Fidelity Phusion Reaction Buffer and 0.02 u/µL of Phusion DNA polymerase (Finnzymes, Thermo Fisher Scientific, Waltham, MA, USA). Amplification was achieved using with an initial denaturation step at 98 °C for 30s, followed by 38 cycles including 10 s at 98 °C, 30 s of annealing at 57 °C, 30 s of elongation at 72 °C, and a final elongation step of 10 min at 72 °C. PCR products of amplified ITS2 rDNA were Illumina sequenced (BMR Genomics™, Padova, Italy). Paired-end ITS2 reads (2 × 300 bp) were then assembled using mothur (v.1.33.0) (v.1.33.0; Schloss et al. 2009) according to developers' instructions (http://www.mothur.org/wiki/MiSeq_SOP). Contigs pairs were assembled and differences in base calls in the overlapping region were solved using ΔQ parameter (Kozich et al. 2013). Primers were trimmed (pdiffs = 3), and ambiguities removed. Reads shorter than 200 bp, longer than 400 bp and with homopolymers longer than 10 bp were filtered. Remaining reads were de-replicated and inspected for chimaerae with UCHIME using *de novo* mode (Edgar et al. 2011). Two different ITS2 alignments were needed due to the large sequence divergences that existed between them.

ITS2 rDNA (280–320 bp) from samples obtained from across the western Mediterranean were amplified using ITS2intfor2 and ITS2rev as described by LaJeunesse and Trench (2000). Successful amplifications were verified via gel electrophoresis and duplicate reactions were pooled together for a volume of 40 µl per sample to be used for library preparation. Pooled samples were purified using calibrated Ampure XP beads and then used to construct the Illumina DNA library. Sequencing was performed at MR DNA (Shallowwater, TX, USA) on a MiSeq following the manufacturer's guidelines. Sequence data was joined with sequences < 150 bp or with ambiguous base calls removed. Sequences were quality filtered using a maximum expected error threshold of 1.0 and de-replicated.

Additional gene markers including the D1/D2 domain of the large ribosomal subunit (LSU rDNA) and mitochondrial *cob* genes were amplified and directly sequenced from a subset of samples according to Zardoya et al. (1995) and Zhang et al. (2008), respectively.

2.2.1 SymPortal analyses of ITS2 sequence variants

ITS2 sequences from each round of MiSeq were submitted to the SymPortal analytical framework (SymPortal.org) for quality control and analyses. SymPortal algorithm assesses the presense of ITS2 sequence variants that consistently occur in specific combinations and abundances. Those variants whose co-occurrence is non-random are deemed intragenomic sequence variants and used to delineate different ITS2-type profiles. These sequences were also compared against a growing database, generated by earlier studies, to match sequence variants with previously characterized sequences and to assign alpha-numeric designators to new variants. thus allowing continual expansion and comparison of genotype representative ITS2 profiles between analyses. For more details on the SymPortal framework, refer to Hume et al. (2019).

2.3 Phylogenetic analyses

All phylogenetic analyses were conducted using PAUP Version 4.4a147 (Swofford 2014) to construct Maximum Parsimony phylogenies (with any insertion-deletions assigned to a 5th character state) with a total of 1000 bootstrap replicates to assess statistical significance of internal branching. The numerically common sequence variants (> 3–4% of total) obtained from the SymPortal analysies output were aligned and their similarity measured as described above.

2.4 Observations of animal larvae

The specimen collected in Pozzuoli (Coty2) was dissected at the SZN upon arrival. Larvae obtained from this animal were used to observe the presence or absence of endosymbionts.

3 Results

3.1 High through-put sequence analyses of ITS2 rDNA

The results from an initial round of Illumina MiSeq sequencing on four samples from Italy collected in three different years, Coty1, Coty2, Coty3, and Coty4, produced 56,698 reads (4453 distinct variants); 39,407 reads (2429 distinct variants); 64,172 reads (4698 distinct variants) and 67,824 reads (4437 distinct variants), respectively (Fig. 1B). After quality control and removal of shorter sequences (> 200 bp), SymPortal analysis of variants in each sample, which filters out most of the numerous PCR and sequence artifacts generated by NGS, identified ITS2 sequences corresponding to the genus *Breviolum* and *Philozoon* (Fig. 1C). Each genus was found in different proportions in different samples (Fig. 1D). Calculation of symbiont proportions in each sample assumed that each symbiont has a similar rRNA gene copy numbers. Samples Coty3 and Coty4 contained more *Philozoon* (58.2% and 75.3% of total reads, respectively), while Coty1 and Coty2 contained mostly *Breviolum* (53.0% and 98.1% of reads, respectively).

Illumina MiSeq sequencing applied to samples from across the western Mediterranean produced an average of 29,307 reads (10,923 distinct variants) were obtained per sample, ranging from 14,321 to 48,441 reads (6881–17,013

distinct variants) across the six locations (Fig. 2A). SymPortal analysis reduced this variation to 3 ITS2 rDNA profiles. One profile corresponded to *Philozoon* was recovered from a subset of samples where they comprised 0.5 to 20% of the total sequence composition; and were most abundant in samples from sites 5 and 6 (Fig. 2A) (Fig. 2B). All samples from these collections comprised mostly *Breviolum* sequences. These profiles, comprising ~ 15 ITS2 variants corresponded to *Breviolum* contained most of the same variants in similar non-random relative abundances (Fig. 2C), however some differed by the presence or absence of specific variants (e.g. B289, B075, and B2w; Fig. 2D and E). The two profiles with and without these minor variants occurred randomly across the study region (Fig. 2C). By contrast, all *Philozoon* sequence profiles were dominated (50–90%) by one sequence variant (P1bo; Fig. 2D inset). These profiles also contained a second divergent variant always present at considerably lower abundances (P1fb). The variants corresponding to *Breviolum* were similar in sequence and produced a star phylogeny radiating from the variants B2 and B19K, the most common variants found in each sample (Fig. 2E).

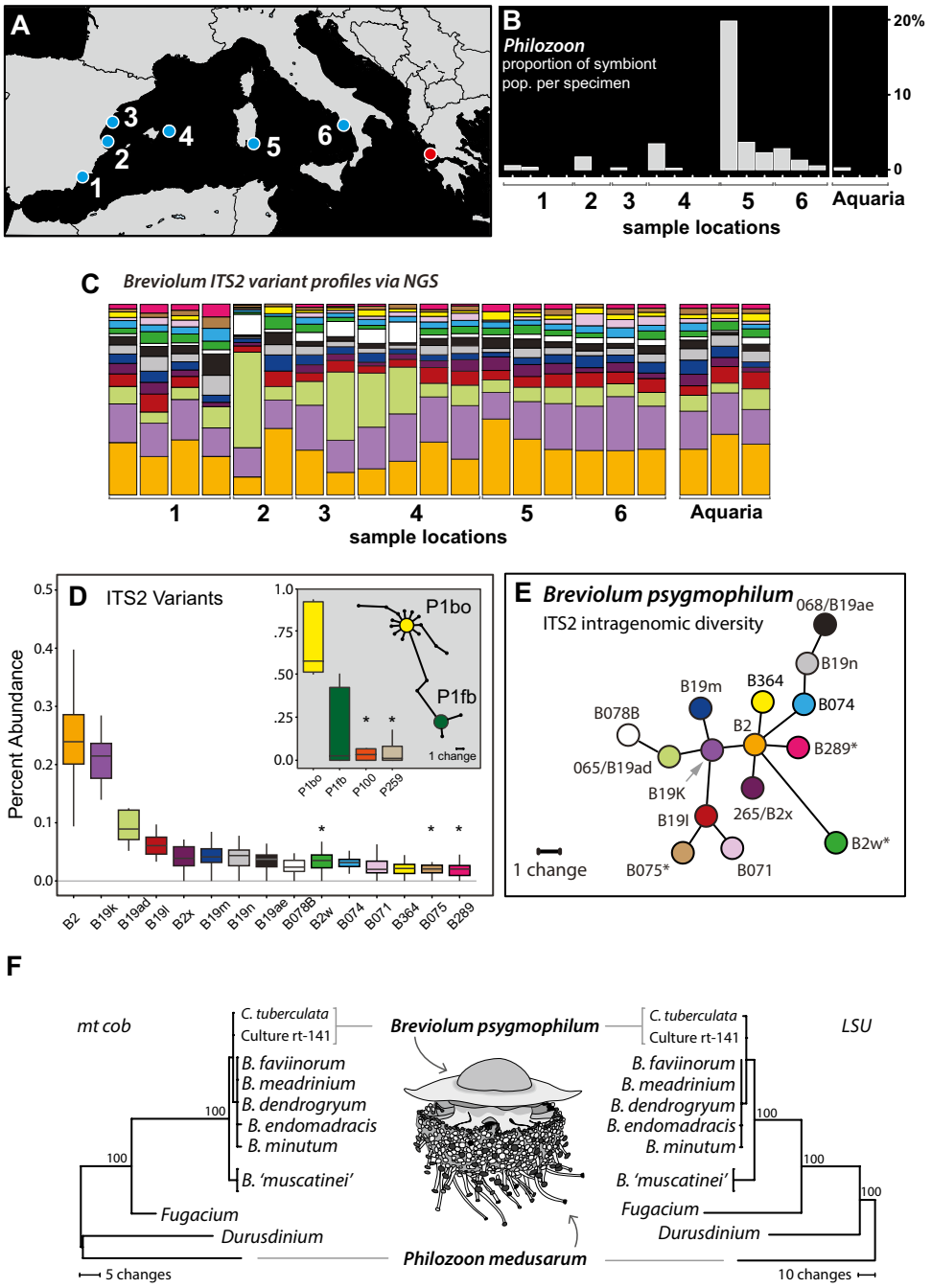

Fig. 2 The genetic analysis of dinoflagellate symbionts in *C. tuberculata* across a 1500 Km expanse of the western Mediterranean. **A** Sampling locations including the putative source of aquarium specimens (red circle; see Supplemental Table S1). **B** Proportion of each sample dominated by *Philozoon* based on relative sequence abundances, which assumes similar rDNA copy numbers in the genomes of each symbiont taxon. **C** The complex yet consistent ITS2 profiles involving up to 15 sequence variants arranged by mean abundances diagnostic of *Breviolum*. **D** Graphical representation showing the proportions of individual sequence variants corresponding to *Breviolum* found in a sample. Sequence variant 'B2' is the most common across the dataset followed by 'B19K' and so on. Each variant is color coded. Asterisks correspond to sequence variants not always detected in a sample. Inset shows relative abundances of four commonest sequence variants and their phylogenetic relationships corresponding to *Philozoon*. **E** An unrooted phylogeny of the 15 most common variants showing their similar sequence relatedness. **F** Mitochondrial cytochrome b (cob) gene and LSU gene phylogenies relating the *B. psygmophilum* found in *Cotylorhiza* to other members in the genus *Breviolum* and this species' evolutionary divergence from *Philozoon medusarum*. Bootstrap values, based on 1000 replicates, are shown

All numerically common diagnostic sequences as generated by Illumina sequencing and after processing through the SymPortal QC pipeline, are deposited on Dryad submission doi.org/10.5061/dryad.stqjq2c6p.

3.1.1 LSU and mitochondrial gene sequence phylogenies

Direct sequencing of the LSU often produced chromatograms which contained consistent secondary peaks indicative of PCR product with multiple sequence variants produced by intragenomic variation and similar to the variation observed for ITS2. The consensus of these sequences corresponded closest to *Breviolum psygmophilum* LaJeunesse, Parkinson and Coffroth (Fig. 2F). LSU sequences corresponding to *Philozoon medusarum* matched with sequences obtained previously from *C. tuberculata* collected from the Gulf of Trieste (Slovenia), Mjiet Lake (Croatia) and Ustica (Sicily, Italy) in the central and eastern Mediterranean (unpublished Genbank data). Sequences of mitochondrial *cob* also matched with *B. psygmophilum* and *P. medusarum*, respectively (Fig. 2F).

3.1.2 Light microscopy observations

The planulae obtained in specimen 'Coty2' from Pozzuoli, did not contain symbionts (Suppl. Figure 1).

4 Discussion

Late 19th century studies from the Mediterranean Sea were the first to discover single-celled algae in the tissues of some common invertebrates (Krueger 2017). Indeed, analyses of *Cotylorhiza tuberculata* contributed to the earliest paper that proposed strange yellow cells inside animals were algal symbionts important to the animal's health and ecological success (Geddes 1882). While they were eventually recognized as dinoflagellates (Hovasse 1922), learning of the exact identities and the evolutionary relationships of these symbionts in animals and protozoa would have to wait for the application of molecular genetic analyses starting in the 1990s (Rowan and Powers 1991; Gast and Caron 1996; Siano et al. 2010; Probert et al. 2014; LaJeunesse et al. 2021). The use of genetic data for description of these symbiotic microbes is critical for investigations into their physiology and ecology, yet issues remain regarding data interpretation, especially regarding the widely used ITS2 sequences when characterizing symbiont diversity (Davies et al. 2022). Despite recent systematic revisions erecting numerous genera from the original genus *Symbiodinium sensu lato* (LaJeunesse et al. 2018; Nitschke et al. 2020; Pochon and LaJeunesse 2021), species taxonomy, and how to consistently identify species once they are formally established, continues to languish.

Based on the combined genetic evidence from samples analyzed in this study, *Cotylorhiza tuberculata* appears to exhibit fidelity for two evolutionarily divergent species of symbiodiniacean dinoflagellate, *Brevolium psygmophilum* and *Philozoon medusarum*, across a broad geographic range, including the locality where Geddes had obtained specimens during his original research (site 6, Tyrrhenian Sea); and supports recent findings by Dall'Olio et al. (2022). The known geographic distribution of *Breviolum psygmophilum* reaches from sub-tropical and temperate waters of the Mediterranean Sea to the western Atlantic, where it is also mutualistic with the temperate corals in the genera *Astrangia* and *Oculina* (Grupstra et al. 2017; Visram et al. 2006; Casado-Amezúa et al. 2016). Until the systematic revision of the genus *Philozoon,* these symbionts were referred by many names, including "Mediterranean A" by Hunter et al. (2007), "Phylotype A" by Barbrook et al. (2006), "AI" by Hansen and Daugbjerg (2009) or A1_Med & NAt1 by Grajales et al. (2016). *Philozoon* currently has eight species displaying high host specificity (LaJeunesse et al. 2021), many of which occur in the Mediterranean. Only one of these, *P. medusarum*, is known to associate with *C. tuberculata*. Therefore, this animal's symbiont flexibility appears limited to just two species.

These conclusions are mostly consistent with the recent findings of Dall'Olio et al. (2022), which found that individual specimens of *C. tuberculata* collected at sampling sites in the Algerian Basin (westernmost western Mediterranean), southern Tyrrhenian, northern Adriatic, and Ionian Seas hosted one of two possible symbionts, corresponding to *P. medusarum* and *Breviolum spp.* (Type B2 and related sequence variants; see the phylogeny presented in their Fig. 2). Their findings differed from the present study in that they did not observe mixtures in the specimens they analysed, as well as finding many more specimens with only *Philozoon* detected. Moreover, their recovery of numerous *Breviolum* LSU and ITS2 sequences could be interpreted as representative of distinct entities within the genus (Dall'Olio et al. 2022). However, differences between Dall'Olio et al. (2022) and the present study are reconcilable when the techniques used for symbiont identification are compared.

The symbiont analyses by Dall'Olio et al. (2022) relied on the sequencing of PCR amplified rDNA using bacterial cloning and therefore may have missed the detection of the other symbiont present at low abundance background levels. Cloning from a diverse pool of PCR amplicons can be highly selective, where, for example, smaller fragments are preferentially ligated into plasmids (Thornhill et al. 2007). The application of high throughput NGS avoids this artefact by sequencing most or all the constituents represented in a PCR reaction while also producing data on their relative numerical proportions in each sample (e.g. Figure 2D). Given the limited coverage provide by cloning and sequencing, this

approach was unable to recognize that the numerous rDNA sequences arbitrarily recovered from the cloning process corresponding to *Breviolum*, are likely intragenomic variants (Thornhill et al. 2007; LaJeunesse and Thornhill 2011; Sampayo et al. 2009). An alternate interpretation, based on the data presented here, is that the high similarity in ITS2 sequence compositions from sample to sample obtained across the western Mediterranean Sea (Fig. 2D) represents co-occurring intragenomic variants stemming from a single species; and not, as is sometimes assumed, assemblages of multiple closely related symbionts co-occurring in the same relative proportions in each animal (e.g. Quigley et al. 2018, Howe-Kerr et al. 2020, Ong et al. 2022, Huang et al. 2020). Unless reconciled, these conflicting interpretations create confusion about the ecology and evolution of animal-dinoflagellate mutualisms (Thornhill et al. 2007).

The large difference in sequence homogeneity and intragenomic rDNA sequence variation evident in *Philozoon medusarum* (1–2 common variants) and *Breviolum psygmophilum* (~ 15) appears to be a property of each species' genome (e.g. Miranda et al. 2012). High rDNA homogeneity or heterogeneity is ultimately dependent on the rate of concerted evolution in a population. Concerted evolution acts to homogenize the gene copies of the ribosomal array, however, its effectiveness differs among species and is influenced by the number of gene copies present in the genome as well as the frequency of sexual recombination (Dover 1982; Nei and Rooney 2005). Members of the genus *Breviolum*, similar to *Cladocopium*, haver greater numbers of ITS2 copies in their genomes relative to other Symbiodiniaceae (Saad et al. 2020; unpulished data). With many more gene copies, there is a greater probability for the existence of multiple intragenomic sequence variants. Thus rDNA data from symbionts in *Cotylorhiza tuberculata* presents a case study how to appropriately interpret the composition of sequence variants recovered from each sample via NGS.

The highly repeatable sequence 'profiles' recovered by NGS corresponding to *Breviolum* and presented in Fig. 2C, appear diagnostic of a single entity. Some variation in the relative abundances among these sequence variants is likely a feature of genome differences among individual strains that dominate each host, as well as artifacts generated from independent PCR reactions. The additional evidence provided by direct sequencing of the LSU and sequences of the low copy mitochondrial cob gene supports this one species interpretation (Fig. 2F; see also LaJeunesse and Thornhill 2011). Moreover, the conclusion of this host associating with only two symbiont species, is consistent with theoretical expectations about the conditions necessary for maintaining stability in a mutualism (Douglas 1998), as well as general principles of ecology (Harper et al. 1961). The expectations being that the number of co-occurring symbionts are minimized to avoid competition and cheating. The predominance of evidence in the form of low-, or single-copy genetic markers applied to spatial and temporal samplings independently support the concept that most zooxanthellate cnidarians host monotypic symbiont populations, or mixtures involving species from separate genera (Thornhill et al. 2009; LaJeunesse and Thornhill 2011; Pettay et al. 2011; Baums et al. 2014; Lee et al. 2016; Wham et al. 2017). Presently, there is no plausible ecological mechanism proposed that could explain the maintenance of highly diverse communities of endosymbiont in a host, nor is there independent genetic evidence to support this alternate interpretation.

4.1 Ecology of a mutualism involving two symbionts

The results from this study and that of Dall'Olio et al. (2022) raise several questions regarding factors influencing the ecological dominance of each symbiont in *Cotylorhiza tuberculata* populations over space and time. And whether these differences are important to the ecology of the animal and its population growth. Persistent differences in light and temperature related to water depth and latitude influence host-symbiont pairings over local and regional spatial areas (Rowan and Knowlton 1995; Sampayo et al. 2007; Bongaerts et al. 2010; Finney et al. 2010; LaJeunesse et al. 2010, 2014; Silverstein et al. 2012; Baker et al. 2013; D'Angelo et al. 2015; Hoadley et al. 2019). Prevailing water temperatures in a given year, or region, may explain why different *C. terbuculata* populations were dominated by one symbiont or the other (Dall'Olio et al. 2022). The Mediterranean Sea experiences strong seasonal changes in temperature and light among its different basins (Casado-Amezúa et al. 2016; Coll et al. 2010). Shift in ecological dominance would most likely occur during the start of a new generation. Planula larva collected from the wild, and aposymbiotic polyps developed from them, lacked symbionts (Suppl. Fig. 1; D'Ambra et al. 2021), supporting previous conclusions that *C. tuberculata* rely on horizontal symbiont transmission to achieve symbiosis (Kikinger 1992; Astorga et al. 2012). While both *P. medusarum* and *B. psygmophilum* are adapted to endure environmental conditions characteristic of shallow temperate waters (Thornhill et al. 2008; LaJeunesse et al. 2021), subtle differences in their ability to utilize light under a range of temperatures could shift their competitive advantage over the other prior to summer blooms of these jellyfish.

Possibly, differential sampling from the bell or the proximal or distal regions of the oral arms could produce an artifact of variability in symbiont dominance. One symbiont may dominate different anatomical regions of the medusa. For some samples, the abundance of *P. medusarum* differed between sub-samples obtained from different parts of the

same animal (Fig. 1D vs. Fig. 2B). Thus, the complex medusoid morphology may partially explain the frequent coexistence of two symbionts in specimens (Fig. 2B).

The continued study of these and other temperate animal-dinoflagellate mutualisms is likely to provide valuable information about the ecological dynamics of animal-dinoflagellate mutualisms in response to large seasonal oscillations and environmental gradients. Further comprehensive spatial and temporal sampling of *C. tuberculata*, including the polyp stage, throughout the Mediterranean would provide additional biogeographic and ecological insight. Moreover, the quantification of mixed symbiont populations would benefit from using low or single-copy genetic markers. As mentioned above, species differences in rDNA copy number will effect calculations of the relative abundances of co-occurring symbionts. Without use of a correction factor, our analysis likely over estimated, albeit consistently, the dominance of *Breviolum* relative to *Philozoon*.

5 Use of rDNA sequence variants for symbiont characterization and identification

Characteristic of eukaryote genomes, numerically dominant ribosome gene sequence variants can provide useful proxies for species diagnoses (Sampayo et al. 2009). These, abundant sequence variants are surprisingly stable in the genomes of species distributed over large geographic scales (Fig. 3C; e.g. LaJeunesse et al. 2014, Turnham et al. 2021). Once linked to formal taxonomic descriptions, specific combinations of ITS2 sequence variants are potentially valuable for the rapid diagnosis of distinct species (e.g. Saad et al. 2021). The presence or absence of rarer variants may differentiate different genotypes within a species (Fig. 2C). In summary, next generation sequencing of ITS2 rDNA provides a reliable high-resolution assessment of intragenomic rDNA variation that improves upon previous characterizations of rDNA using DGGE (Sampayo et al. 2009; LaJeunesse 2002). When paired with independent genetic, and available ecological, biogeographic and morphological evidence, these data are highly useful in characterizing and assessing 'zooxanthellae' species diversity (Smith et al. 2017; Wham et al. 2017).

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