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REPORT

# The diversity, distribution, and temporal stability of coral 'zooxanthellae' on a pacific reef: from the scale of individual colonies to across the host community

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**Abstract** The ecological and evolutionary consequences of partner fidelity and flexibility among coral–dinoflagellate mutualisms are widely debated. Resident symbionts can modulate the resilience of their hosts to environmental stressors, which explains, in part, why host–symbiont combinations differ over broad geographic ranges and across physical–environmental gradients in light and temperature. Therefore, flexibility in these mutualisms may influence the longevity of coral populations and communities subjected to ocean warming. However, despite decades of research, basic knowledge about these mutualisms remains incomplete, hindering the development of predictive ecological theory. In particular, few studies have investigated the long-term composition of symbiont populations within individual colonies. To further examine the extent to which coral colonies have stable relationships with specific symbionts over multiple years, diverse coral taxa (Scleractinia) from a West Indo-Pacific fore reef (Palau) were tagged and sampled at various intervals—ranging from six months to several years—over nine years' time. Symbiont identity was examined using multiple genetic markers that resolved symbiont diversity to species and individual genotypes (i.e., clonal strains). Members of the genus *Cladocopium* (formerly *Symbiodinium* Clade C) were prevalent across the host community. Generally, corals with open modes of symbiont acquisition harbored a host–generalist symbiont, while corals with vertical symbiont transmission were associated with co-evolved host-specific symbionts. Consistent with previous colony monitoring studies, symbiont populations in a majority of colonies were dominated by one species and one strain (based on multilocus genotyping) over multiple years. Thus, the distribution of symbiont diversity at the genus, species and clone level, comprising specific and stable partner combinations, scale predictably to reef habitat, host taxon, and individual colony. Recognizing these fundamental ecological patterns establishes a more comprehensive understanding of the population and community structure of these mutualisms.

**Keywords** Biodiversity · Coral reef ecosystems · Ecological generalists · Homeostasis · Scleractinia · Symbiosis

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## Introduction

Mutualisms between reef corals and endosymbiotic dinoflagellates in the family Symbiodiniaceae are abundant in warm coastal waters. Fortified with the ability to photosynthesize,

large numbers of these animals construct and sustain biologically diverse ecosystems of significant importance to Earth's biosphere. Since their origin in the Mesozoic Era, scleractinian–dinoflagellate mutualisms have endured large and sometimes geologically abrupt shifts in the planet's environment. Paradoxically, shifts to a warmer climate in recent decades now pose a threat to the long-term viability of these diverse and productive systems. Biological responses from both animal and dinoflagellate partners range from rapid physiological processes of acclimatization to long-term evolutionary adaptation through generational turnover and natural selection. Additional ecological processes may involve the emergence and spread of certain host–symbiont partnerships better adapted to endure the changed environment.

The physiological attributes of both the animal and symbiont play a consequential role in the response of coral colonies to episodic or chronic warming and cooling events (Baird et al. 2009; Hoadley et al. 2019). Rising trends in ocean warming and greater environmental variability have a destabilizing effect on host–symbiont combinations sensitive to thermal stress (Berkelmans and van Oppen 2006, LaJeunesse et al. 2009a). The displacement of a heat-sensitive symbiont by a heat-tolerant lineage within a colony would constitute a 'rapid' ecological response to near-term shifts in sea surface temperatures (e.g., Berkelmans and van Oppen 2006; LaJeunesse et al. 2009). Thus, the flexibility and/or stability exhibited by these partnerships may partially determine the persistence of reef coral communities in the coming decades.

Knowing the temporal and spatial stability of symbiotic interactions provides greater insight and understanding of their basic ecology. However, fundamental knowledge of the extent of host–symbiont fidelity at different biological, spatial, and temporal scales remains fragmented. This limitation, along with poor taxonomic resolution of the symbiont partner, has restricted our ability to accurately forecast the ecological response of coral symbioses to climate change. Recent improvements in symbiont systematics are reliant on the use of multiple genetic markers to resolve a natural hierarchy of genera, species, and individual genotypes, thus helping substantially reduce scientific confusion and allowing investigators to study the ecology of these mutualisms more precisely.

There are several studies documenting symbiont change in coral colonies. Most shifts in symbiont identities have been observed among corals subjected to severe physiological stress by abruptly moving colonies to environmentally different habitats (Rowan et al. 1997; Toller et al. 2001; Baker 2001; Grottoli et al. 2014; Bay et al. 2016), or exposing them to acute thermal stress in experimental tank systems and following the identity of the symbiont through the recovery phase (Berkelmans and van Oppen 2006; Cunning et al. 2015; Howe-Kerr et al. 2020). Other studies have monitored tagged individuals (Stat et al. 2009; Kemp et al. 2014; Palacio-Castro et al. 2023) or, less precisely by randomly sampling different colonies from set locations before and after natural episodes of thermal stress (Baker et al. 2004; Jones et al. 2008; LaJeunesse et al. 2009). There are fewer long-term observational studies assessing whether change in the dominant symbiont, or 'switching,' occurs in naturally growing colonies under normal environmental conditions and seasonal cycles (Thornhill et al. 2006; Lee et al. 2016). Moreover, rarely has the symbiont identity been monitored across a broad and diverse assemblage of reef coral species in any location.

To further deduce ecological processes important to coral–dinoflagellate mutualisms, numerous colonies living on a barrier reef in Palau were individually tagged and their dinoflagellate symbionts were examined for up to nine years. Host diversity represented 14 genera approximating 6 scleractinian families and included animals whose larva must acquire symbionts from the environment at each new generation (horizontal transmission) and those that acquire symbionts transmitted directly to the egg during oogenesis (vertical transmission). Since 2010, the reef corals of Palau have experienced minimal thermal stress and thus constitute mutualisms thriving under relatively stable environmental conditions. Multiple genetic analyses were applied to each sample to identify the dominant symbiont species and characterize individual symbiont genotypes (i.e., individual clones or strains) comprising the symbiont population in each colony. To test the possibility that changes in symbiont species or genotype over time might be explained by sampling location on a colony, we also conducted randomized spatial sampling to estimate the number of colonies likely to have associations comprising more than one clone, or identify possible cohabitation of an additional species. Combined with previous research, these findings provide a generalized high-resolution view of the temporal stability and within-colony homogeneity of symbionts among coral colonies from a representative Indo-Pacific barrier reef.

## Materials and methods

### Study location and transects

Permanent transects were established in August of 2013 with the objective of tracking symbiotic associations over time. Three twenty-five meter transects were positioned along a western north-facing barrier reef containing diverse and healthy coral assemblages typical of the equatorial West Pacific Ocean, Rebotel reef (7.2497°N, 134.2288°E). The transects were spaced over an area spanning approximately 100 m of reef at a depth ranging approximately between 8 and 12 m (at high-tide). To mark the endpoints of each

transect, steel rebar stakes were driven into the carbonate substrate using a sledgehammer.

### Colony tagging and temporal sampling of transect colonies

Transect tape was strung between rebar stakes to guide colony selection and sampling efforts (Fig. 1A). To ensure tagged corals were easily identifiable at later time points, selected colonies were within three meters from the central transect line. Numbered thermoplastic polyurethane tags were attached to corals using a hammer and steel nails (mounding and encrusting coral species; Fig. 1B) or by UV-stabilized nylon cable ties (branching species; Fig. 1B). Individual colonies were photographed to verify coral species. A fragment of skeleton and tissue (approximately ~ 2 $cm^2$) was removed for genetic analysis using a small chisel and hammer and placed in numbered plastic bags with seawater. Coral fragments were transported by boat to the Palau International Coral Reef Center (PICRC) where they were preserved in 20% Dimethyl sulfoxide salt (DMSO-EDTA-NaCl) buffer and stored at -20 °C (Seutin et al. 1991).

A variety of taxonomically diverse corals were selected for long-term monitoring. Coral host taxa were identified visually from photographs. Transect collections included 162 individual coral colonies representing 10 families and 21 genera: *Acropora* and *Isopora* (Acroporidae), *Stylophora, Seriatopora,* and *Pocillopora* (Pocilloporidae), *Lobophyllia* (Lobophylliidae), *Diploastrea* (Diploastreidae), *Podabacia* (Fungiidae), *Goniastrea, Favites, Platygyra,* and *Leptoria* (Merulinidae) (Budd et al. 2012, Huang et al. 2014a, Huang et al. 2014b), as well as three additional genera, *Turbinaria* (Dendrophylliidae), *Psammocora* (Psammocoridae), *Leptastrea* (Leptastreidae), and *Pachyseris* (Pachyseridae) (WoRMS 2024 https://www.marinespecies.org; Table S1).

Return visits to resample colonies referenced waterproof site maps showing the relative position of each colony, along with colony photos, and tag number verified their identity. The position of the sample along the surface of the colony was randomized with each sampling. Sampling times were dependent on travel and varied, initially occurring at approximately six month to one-year intervals. Sampling was conducted at nine independent time points, spanning a total observation period of nine years for several colonies.

### Spatial sampling of transect colonies

Intra-colony diversity was investigated by spatial collections. In July 2016, two (paired) samples were collected from each tagged colony. The first sample was obtained from the left side of the colony when facing the colony with the reef slope behind it. The location of the second sample was randomized along the top axis of the colony using a percent distance method. When viewed from above, the colony was visually segmented into ten equal-sized partitions, each representing 10% of the colony's transverse axis. Next, a random number was generated to assign the numbered partitions between paired samples ($^{\#}1$–$^{\#}10$). Using this method, the distance between paired samples ranged from samples taken next to each other to samples collected at the opposite end of the colony (illustrated in Fig. 1C). Skeletal fragments were individually placed into separate pre-labeled collection bags and preserved following the methods described above.

### DNA extraction and analysis of diversity using conventional genetic markers

DNA extractions were performed on 5 × 5 mm skeletal fragments containing animal tissue with associated symbionts using a modified Promega Wizard genomic DNA extraction protocol described by LaJeunesse et al. (2003).

Samples were analyzed by denaturing gradient gel electrophoresis (DGGE) profiling of the ribosomal internal transcribed spacer 2 (ITS2). Prominent and discrete bands in DGGE of diagnostic profile fingerprints were excised in a subset of samples and directly sequenced as described by LaJeunesse (2002). Thus, by targeting only the prominent bands through direct sequencing, we isolated the numerically dominant ITS2 rDNA sequence in the nuclear genomes of resident symbionts. DGGE-fingerprinting was also used to identify samples containing a mixed population of two or more symbionts when present at greater than 10% of the total population (Thornhill et al. 2006). For additional verification of symbiont species identity, the nuclear large-subunit ribosomal DNA (*LSU*) was amplified following conditions specified by Zardoya et al. (1995). All sequencing was conducted on an Applied Biosciences sequencer (Applied Biosciences, Foster City, CA) at the Pennsylvania State University Genomics Core facility.

### High-resolution genotyping of the symbiont population

Microsatellite loci were used to resolve multilocus genotypes of clone-dominated symbiont populations (i.e., synonymous with resolving individual strains, or clones). A total of 10 microsatellite loci developed for *Cladocopium* spp. were used including 9 loci (Sgr 34, SgrSpl 13, SgrSpl 22, SgrSpl 24, SgrSpl 25, SgrSpl 26, SgrSpl 78, Spl 1, and Spl 16) described by Wham et al. (2014) and 1 locus (C1.05) described by Bay et al. (2009). Genotyping was applied to those colonies harboring *Cladocopium madreporum*, *Cladocopium patulum*, *Cladocopium* C21 and *Cladocopium* $C^{Stylophora}$. These loci are unlinked and using 10 provides sufficient variability to unambiguously identify distinct clonal cell lines. Each locus was amplified in reaction volumes of 10 µL each comprised of 1 µL of 10 mM dNTPs, 1

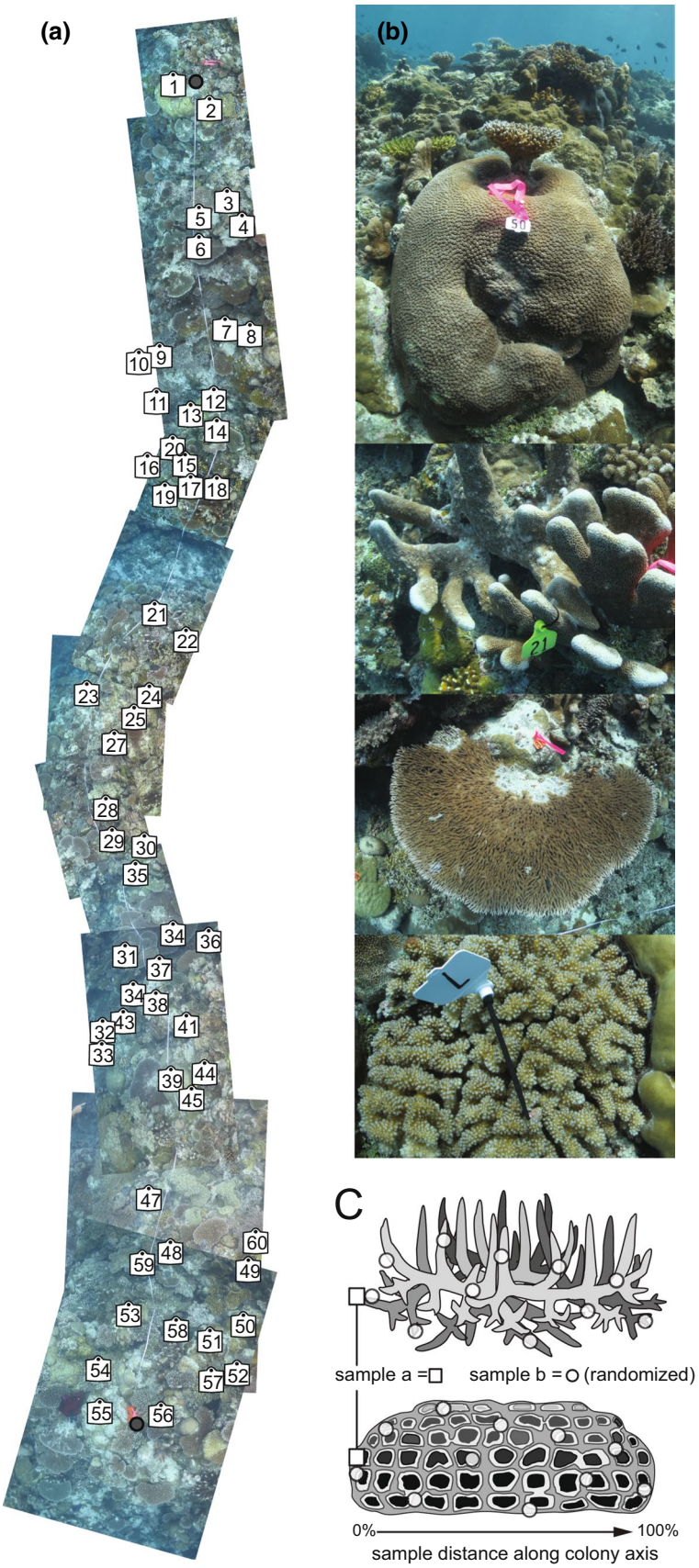



**Fig. 1** Establishment of permanent transects, colony tagging, and sampling protocol. **A**, Photomosaic of transect 1 (white). **B**, Example of colonies tagged using cattle ear tags, zip ties, and steel nails. **C**, Random spatial sampling methodology. After a single edge sample is removed, a second sample was taken based on randomized distances from the first sample along an axis passing through the center of the colony

µL 25 mM MgCl2, 0.2 U Taq DNA Polymerase, and 1 µL of standard Taq Buffer (New England Biolabs, Ipswich, MA), 1 µL of forward and reverse primers at 10 µM and 1 µL of 100–50 ng DNA template). PCR amplifications were performed according to the specifications given by Wham et al. (2014) and Bay et al. (2009). Microsatellites were analyzed on an ABI 3730 Genetic Analyzer (Applied Biosystems) using a 500-bp standard (LIZ-labeled) at the Penn State University Nucleic Acid Facility. Fragments sizes were scored visually using Geneious (Geneious version 6.1.8 created by Biomatters, Newark, NJ, USA).

The number of alleles amplified at each locus identified samples homogeneous for only one genotype as opposed to a mixed population. Two alleles at each locus, expected for *Cladocopium*, represent a single genotype (Wham et al. 2014; Wham and LaJeunesse 2016), while three or more alleles may be indicative of a combination of multiple co-occurring genotypes or non-target (i.e., non-specific) PCR amplifications. Distinctive symbiont genotypes characterized for each symbiont species (i.e., unique multilocus genotypes, MLGs) were assigned a number designation (Table S2). Matching symbiont clones were identified when alleles were identical across all microsatellite loci.

While genotypes of mixed populations in a colony could not be characterized for some colonies, there were instances when both mixed and pure single genotypes were observed at different time points for a particular colony. Thus, over the course of temporal and spatial sampling, in instances when a sample was homogeneous for one, the MLG for each was deduced.

### Use of high-resolution *psbA*$^{ncr}$ sequences to verify clone identity

The non-coding region of the *psbA* minicircle (*psbA*$^{ncr}$) was amplified and sequenced according to the conditions described by Moore et al. (2003). Data from these hypervariable nucleotide sequences were used to verify symbiont species identity, further assess intra-species genotype identity (i.e., clone), and display this clone diversity on a phylogeny (LaJeunesse and Thornhill 2011). Sequences were aligned using the Internet version of ClustalOmega followed by manual editing.

### Phylogenetic analysis

PAUP v4.0a169 was used to create an unrooted maximum parsimony phylogeny based on a heuristic search (Swofford 2014). Because each phylogeny was congruent, they were concatenated, and a phylogenetic tree created with gaps (and insertions) treated as a 5th character and scored as one change. Bootstrap values based on 1,000 iterations were evaluated to statistically assess branch support.

### Sea surface temperature data

Averaged monthly surface seawater temperature was obtained from HOBO temperature loggers deployed at Ulong Rock near the offshore reef site (7.29042° N; 134.24105° E) provided by the Coral Reef Research Foundation. Daily 5-km Satellite Coral Bleaching Thermal Stress Monitoring Product Suite data were obtained from the National Oceanic and Atmospheric Administration's Coral Reef Watch website. Daily surface seawater temperatures (SST) for Palau were determined by calculating the average of the daily low (SST_MIN) and high (SST_MAX) SST between July 2013 and July 2022 (NOAA Coral Reef Watch). Both surface seawater measurements were plotted for the period of observation to evaluate seasonality and identify potential anomalous temperature events (Fig. 4b).

## Results

### DNA sequence evidence of symbiont identity and homogeneity among transect colonies

Initial analysis using DGGE-fingerprinting of ITS2 rDNA detected one symbiont 'type' in the majority of samples (Supplemental Figure S1). The characterization of highly repeatable banding profiles by excising and sequencing the brightest bands established the existence of many definable symbiont taxa (Fig. 2). Analysis of fainter bands in each fingerprint determined that they corresponded to the low abundance intragenomic variants present in each symbiont's genome. It was determined by additional sequence analyses that some of these profiles were diagnostic of described species (detailed below).

### Phylogenetic relationships of symbiont species diversity from transect colonies

Symbionts in the genus *Cladocopium* dominated the coral community. Phylogenetic analysis based on the combined mitochondrial *cob,* ribosomal ITS2 (diagnostic sequence variant) and partial LSU rDNA gene sequences resolved 12 *Cladocopium* lineages, some of which were recently described formal species (Fig. 2). The most prevalent of these was the widespread host generalist *C. madreporum* (formerly 'type' C40), which occurred only in coral taxa reliant on horizontal transmission. *Cladocopium* C21 was especially common to multiple species in the genus *Acropora*. Corals with vertical symbiont transmission, including members of *Porites*, *Montipora*, *Pocillopora*, *Stylophora*, and *Seriatopora*, respectively, possessed only host-specific symbionts. Two species in the genus *Durusdinium* occurred rarely. The host-specialist *D. glynnii* was found in several

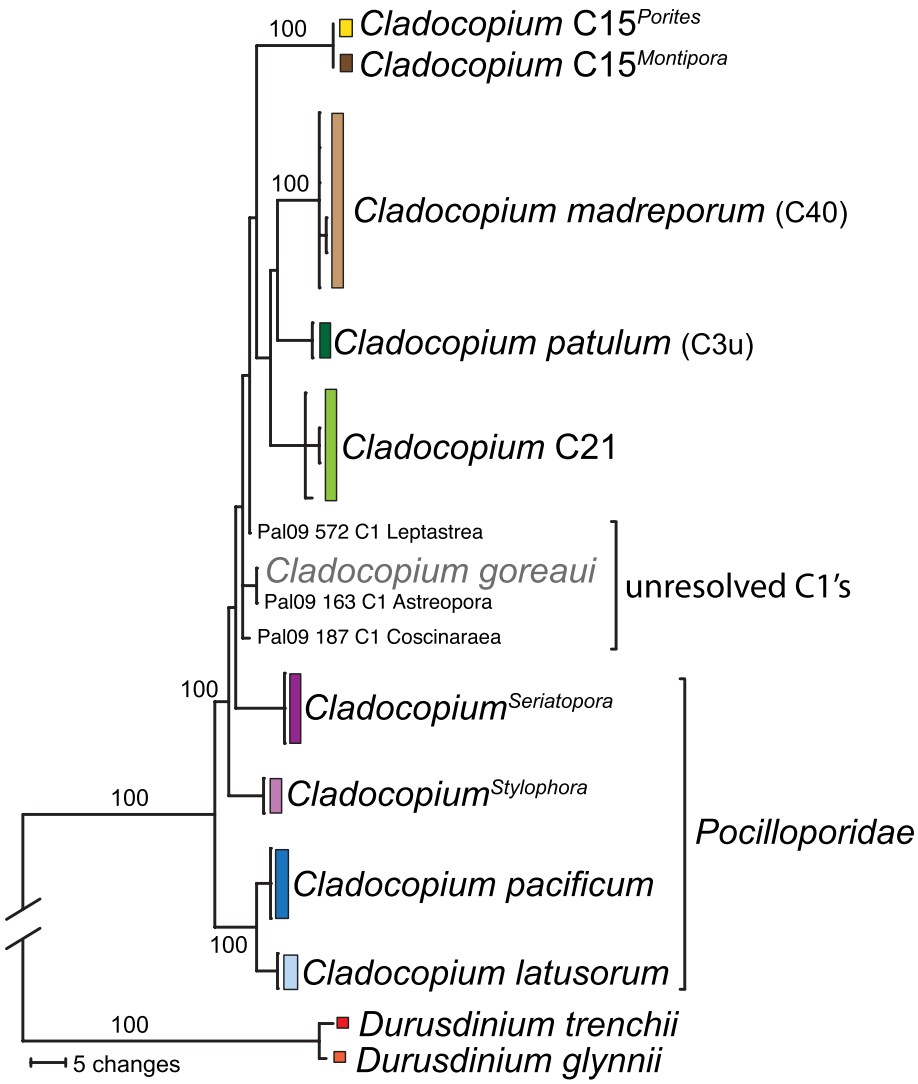



**Fig. 2** Maximum parsimony phylogeny reconstruction showing the Symbiodiniaceae diversity found in colonies representing 21 genera and ~ 50 species from all three transects. The phylogenetic reconstruction was based on of the mitochondrial *cob*, ITS2, and partial LSU gene sequences (combined alignments representing ~ 1959 bases). Certain branches correspond to formally described species while others represent species lineages that are presently undescribed. Sequence data for *C. goreaui* (light gray label) were included for comparison purposes. Bootstrap values based on 1000 iterations using the software PAUP (Swofford 2014)

colonies of *Pocillopora verrucosa,* while host-generalist *D. trenchii* was detected at high abundances in some colonies of *Pachyseris rugosa* and in colonies of the massive reef builder, *Diploastrea heliopora* (Fig. 3).

### Diversity and similarity of symbiont microsatellite genotypes from transect colonies

Population genetic microsatellite markers applied to a subset of colonies harboring *C. madreporum* (C40), *C. patulum* (C3u), *Cladocopium* C21, and *Cladocopium* sp. from *Stylophora* ($C^{Stylophora}$) identified unique MLGs in each species. Overall, *Cladocopium* genotypes were highly diverse. Some clone genotypes were observed in more than one colony, including clones of *C. madreporum* and *C. patulum*. In particular, colonies of *Isopora palifera* distributed over ~ 100 m of reef shared the same clone of *C. madreporum* (Supplemental Fig. S2). All clone genotypes of *Cladocopium* C21 and $C^{Stylophora}$ genotypes were unique. Thus, most tagged colonies hosted a unique symbiont genotype.

### The composition of symbiont populations in colonies over spatial and temporal scales

Missing tags and death from storm damage meant that colonies were 'lost' between sampling time points. Thus, the duration of symbiont monitoring varied by colony and ranged from as little as a year to 9 years. In addition, some colonies were not located during some time points creating gaps in the temporal data (Fig. 4).

The frequency of mixed genotypes sampled over time was comparable to the frequency of two genotypes observed through spatial sampling at one time point (Fig. 4; Supplemental Table S2). Over the course of repeated sampling, additional symbiont genotypes were found in ~ 18% of colonies. Only one new genotype was found for the majority of these colonies, except for a colony of *Goniastrea* (*Favia*)

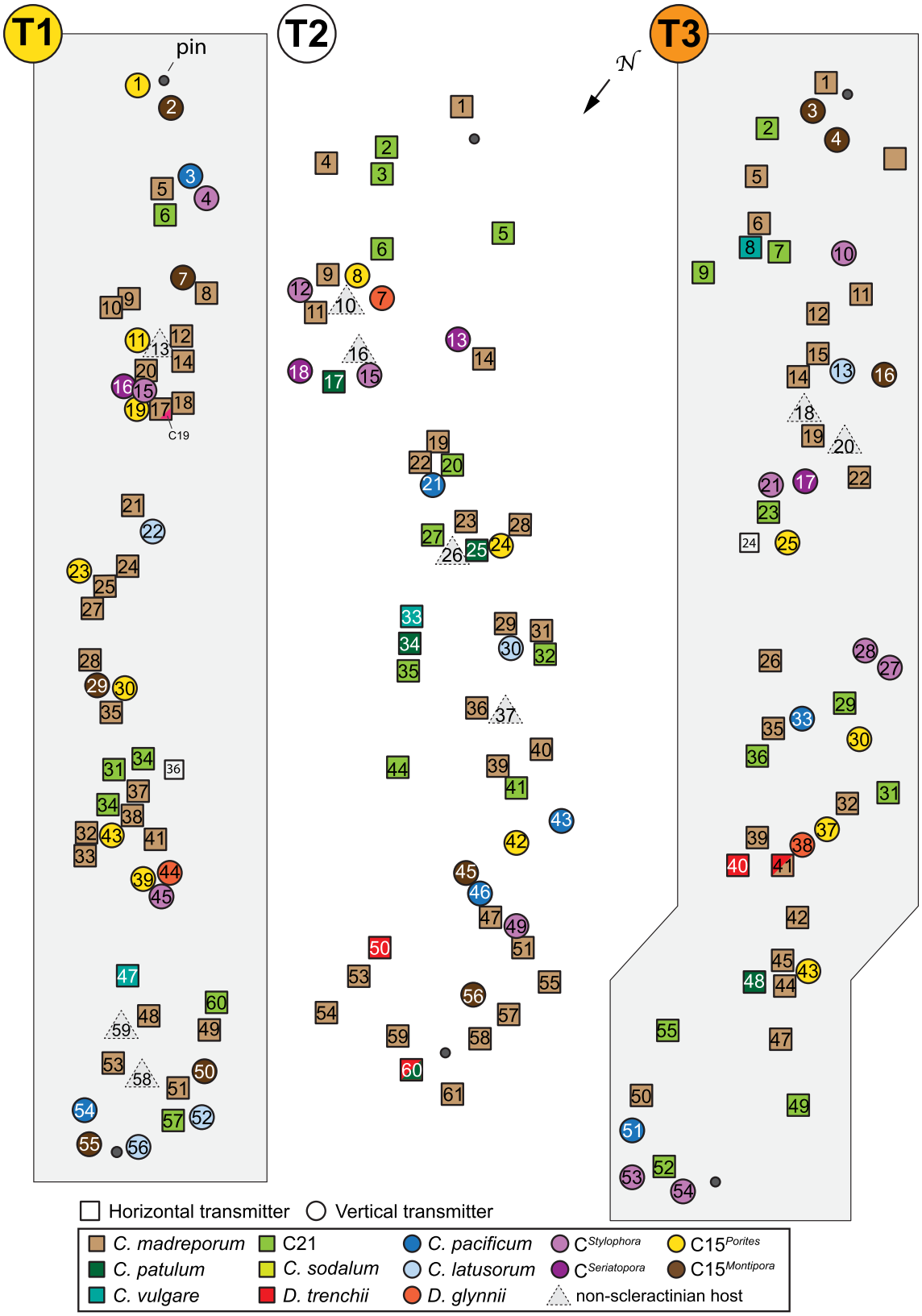



**Fig. 3** Transect schematics showing the location of individual colonies, their mode of symbiont acquisition (symbol shape: circle or square) and the distribution of symbiont species diversity (color) across the host landscape. Split color-coding corresponds to colonies co-dominated by two symbiont species during sampling in August 2013

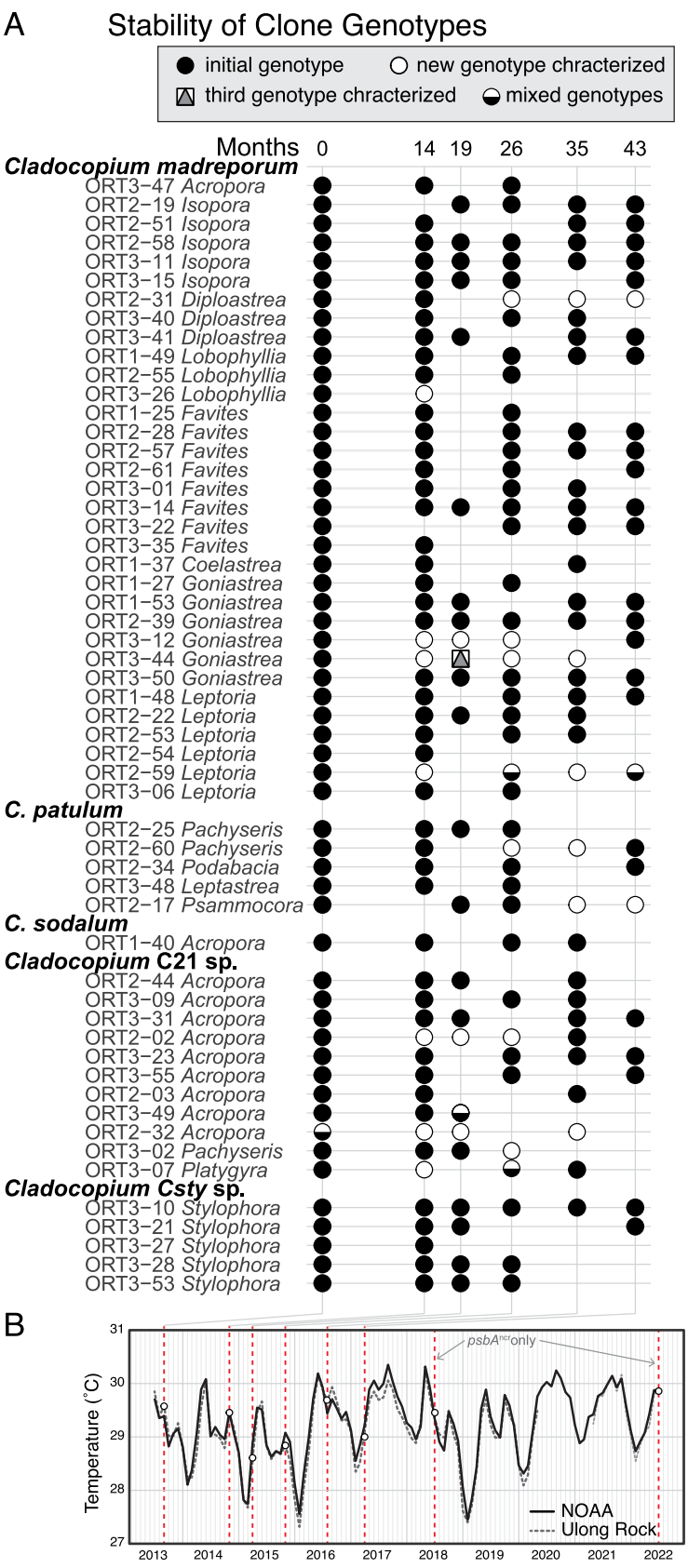



**Fig. 4** Genotype diversity and temporal stability and variation in symbiont genotypes among tagged coral colonies over multiple years. **A**, Genotype monitoring. Individual colonies (n = 55) are represented by each row. Colonies are ordered by their family and species. The initial symbiont species found at the start of monitoring is listed along the left side of each row. Circles represented along the vertical axis correspond to an analyzed sample beginning at month 0 (first sampling in August 2013) for a respective coral colony. A solid circle indicates a single dominant symbiont genotype corresponding to the species listed. A split circle (white top/black bottom) indicates the presence of two co-occurring genotypes. For each colony, the detection of a novel symbiont genotype (not previously observed within the colony) is indicated by a white circle. A triangle inside a square was used in the one case where a third unique genotype was characterized. Samples from the last two collections were analyzed via sequencing of the *psbA*$^{ncr}$ only **B**, The average monthly sea surface temperatures from July 2013 to July 2022 (NOAA Coral Reef Watch (black line) and Ulong Rock by the Coral Reef Research Foundation (dotted gray line)). Dashed vertical red lines and open circles indicate sampling times (see Table 1)

**Table 1** Diversity of colonies analyzed using phylogenetic and population genetic markers (microsatellites) over approximately 4 years beginning in August 2013 and then subsequently sampled when possible in October 2014, March 2015, July 2016, March 2017, and October 2017. Additionally, in July 2018 (year 5) and June 2022 (year 9) remaining colonies were sampled for analysis of LSU rDNA and *psbA*$^{ncr}$ of LSU rDNA *psbA*$^{ncr}$ to verify the species and clone genotype, respectively. These dates correspond to the timeline and temperature graph in Fig. 4

| Host Family | Genus | August 2013 (month 0) | October 2014 (month 14) | March 2015 (month 19) | July 2016 (month 26) | March 2017 (month 35) | October 2017 (month 43) |
|---|---|---|---|---|---|---|---|
| Acroporidae | *Acropora* | 10 | 10 | 4 | 5 | 8 | 3 |
| | *Isopora* | 5 | 4 | 3 | 5 | 1 | 5 |
| Diploastraeidae | *Diploastrea* | 3 | 3 | 1 | 2 | 3 | 2 |
| Leptastreidae | *Leptastrea* | 1 | 1 | – | 1 | – | – |
| Lobophylliidae | *Lobophyllia* | 3 | 3 | – | 2 | 1 | 1 |
| Merulinidae | *Coelastrea* | 1 | 1 | – | – | 1 | – |
| | *Favites* | 8 | 7 | 2 | 6 | 5 | 5 |
| | *Goniastrea* | 6 | 6 | 3 | 6 | 4 | 4 |
| | *Leptoria* | 6 | 6 | 1 | 5 | 4 | 2 |
| | *Platygyra* | 1 | 1 | – | 1 | – | 1 |
| Pachyseridae | *Pachyseris* | 3 | 3 | 2 | 3 | 1 | 1 |
| Pocilloporidae | *Stylophora* | 5 | 5 | 4 | 3 | – | 2 |
| Psammocoridae | *Psammocora* | 1 | – | 1 | 1 | 1 | 1 |

*stelligera*, where three different genotypes of *C. madreporum* were characterized at different sampling times (Fig. 4; ORT3-44). Analysis of two samples collected from different locations on a colony in July 2016 found that ~ 17% of colonies contained two genotypes (Fig. 1C). Each of these dual genotypes were the same as those independently characterized from the sampling a tagged colony over time.

### Relationship between *psbA*$^{ncr}$ haplotypes and microsatellite genotypes

Sequence haplotypes of the *psbA*$^{ncr}$ obtained from a subset of sampled colonies corresponded to MLGs characterized with microsatellite alleles. Most *psbA*$^{ncr}$ haplotypes, even those with sequences that differed by a few nucleotides, were matched with a unique MLG (Fig. 5A–C). Thus *psbA*$^{ncr}$ haplotyping was used as a proxy to assess the stability of the mutualism in samples from later time points (years 5–9).

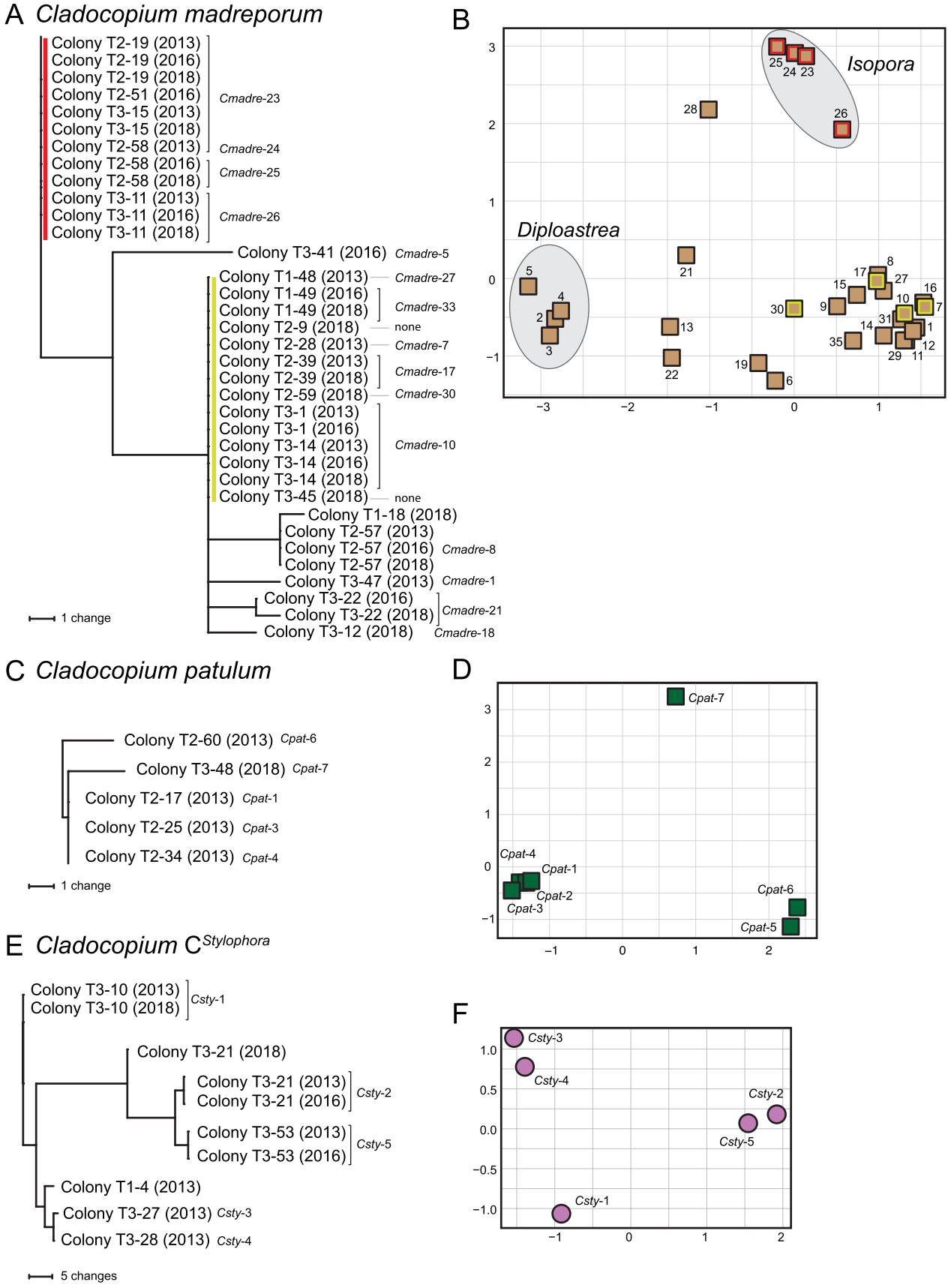
Principal component analysis (PCoA) applied to microsatellite data revealed correspondence between the genetic relatedness of symbiont genotypes and the phylogenetic similarity of *psbA*$^{ncr}$ haplotypes (Fig. 5A–F). PCoA also showed that some *C. madreporum* genotypes (i.e., genets) obtained from the same host genus were more genetically similar than other *C. madreporum* genets from different host taxa (Fig. 5B). This clustering was observed among samples from *Isopora* and *Diploastrea* (Fig. 5B).

### Sea surface temperatures from July 2013 to July 2022

Averaged monthly sea surface temperatures obtained from the National Oceanic and Atmospheric Administration Coral Reef Watch database and recorded at Ulong Rock showed that SSTs for the duration of the transect experienced some seasonal differences across sampling points, but rarely rose above 30˚C (Fig. 4). While extreme and anomalous thermal events occurred in the western Pacific in 2016, non-anomalous elevated temperature events were generally not observed in Palau.

## Discussion

The use of complementary genetic analyses of Symbiodiniaceae in the same colonies at multiple time points revealed a distribution of symbiont 'diversity,' characterized to the genus, species and clone levels, organized into stable, specific, non-random, and highly repeatable partner combinations. These taxonomic ranks corresponded to the offshore reef habitat (*Cladocopium* dominant), host taxon (host-specific species within), and individual host colonies (clones of distinct *Cladocopium* spp.). Ascertaining fundamental ecological patterns at various biological scales (discussed below) provides a more comprehensive understanding of the population and community structure of these mutualisms.


A Cladocopium madreporum
Colony T2-19 (2013)
Colony T2-19 (2016)
Colony T2-19 (2018)
Colony T2-51 (2016)
Colony T3-15 (2013)
Cmadre-23
Colony T3-15 (2018)
Colony T2-58 (2013)
Cmadre-24
Colony T2-58 (2016)
Colony T2-58 (2018)
Cmadre-25
Colony T3-11 (2013)
Colony T3-11 (2016)
Colony T3-11 (2018)
Cmadre-26
Colony T3-41 (2016) Cmadre-5
Colony T1-48 (2013) Cmadre-27
Colony T1-49 (2016)
Colony T1-49 (2018)
Cmadre-33
Colony T2-9 (2018) none
Colony T2-28 (2013) Cmadre-7
Colony T2-39 (2013)
Colony T2-39 (2018)
Cmadre-17
Colony T2-59 (2018) Cmadre-30
Colony T3-1 (2013)
Colony T3-1 (2016)
Colony T3-14 (2013)
Colony T3-14 (2016)
Colony T3-14 (2018)
Cmadre-10
Colony T3-45 (2018) none
Colony T1-18 (2018)
Colony T2-57 (2013)
Colony T2-57 (2016) Cmadre-8
Colony T2-57 (2018)
Colony T3-47 (2013) Cmadre-1
Colony T3-22 (2016)
Colony T3-22 (2018)
Cmadre-21
Colony T3-12 (2018) Cmadre-18
1 change
B
Isopora
Diploastrea
C Cladocopium patulum
Colony T2-60 (2013) Cpat-6
Colony T3-48 (2018) Cpat-7
Colony T2-17 (2013) Cpat-1
Colony T2-25 (2013) Cpat-3
Colony T2-34 (2013) Cpat-4
1 change
D
Cpat-7
Cpat-4
Cpat-1
Cpat-2
Cpat-3
Cpat-6
Cpat-5
E Cladocopium C Stylophora
Colony T3-10 (2013)
Colony T3-10 (2018)
Csty-1
Colony T3-21 (2018)
Colony T3-21 (2013)
Colony T3-21 (2016)
Csty-2
Colony T3-53 (2013)
Colony T3-53 (2016)
Csty-5
Colony T1-4 (2013)
Colony T3-27 (2013) Csty-3
Colony T3-28 (2013) Csty-4
5 changes
F
Csty-3
Csty-4
Csty-2
Csty-5
Csty-1

◂**Fig. 5** Phylogenetic relationships of *psbA*$^{ncr}$ haplotypes and two-dimensional visualization of principal component analyses (PCoA) of multilocus genotypes (based on 10 microsatellite loci) characterized from each of three symbiont species. These analyses are presented together to assess the correspondence of *psbA*$^{ncr}$ sequence similarity to allele similarity among clone genotypes. **A**, *Cladocopium madreporum psbA*$^{ncr}$ unrooted haplotype maximum parsimony phylogeny and **B**, the PCoA for multilocus genotypes characterized. The first two PCs account for 27.5% and 18.2% of total genetic variance, respectively. **C**, *Cladocopium patulum psbA*$^{ncr}$ unrooted haplotype maximum parsimony phylogeny and **D**, the PCoA for the seven multilocus genotypes characterized. The first two PCs account for 49.8% and 33.5% of total genetic variance, respectively. **E**, *Cladocopium*$^{Stylophora}$ unrooted haplotype maximum parsimony phylogeny and **F**, the PCoA for the five multilocus genotypes characterized. The first two PCs account for 69.5% and 20.3% of total genetic variance, respectively. Numbers 1–n were arbitrarily assigned to distinct genotypes corresponding to each symbiont species (e.g., *Cmadre*-1 is genotype 1 of *Cladocopium madreporum*)

## Zooxanthellae species diversity from a West Indo-Pacific barrier reef

A combination of described and undescribed Symbiodiniaceae species was identified from a broad diversity of reef-building scleractinian corals (Fig. 2). Their host specificities ranged substantially depending on the host's mode of symbiont acquisition. Few of them associated with many evolutionarily divergent coral taxa reliant on horizontal symbiont acquisition (i.e., host generalists; Butler et al. 2023), while most others formed mutualisms with animals representing a single genus or species and often exhibiting vertical symbiont transmission as part of their life histories, including colonies of *Montipora*, *Pocillopora*, *Stylophora*, *Seriatopora*, and *Porites*, which transmit symbionts from parent to offspring through egg development (Hirose and Hidaka 2006; Turnham et al. 2021). The prevalence of few host-generalist symbionts mutualistic with horizontal transmitters, which includes most coral taxa from the Indo-Pacific (Richmond and Hunter 1990), is why symbiont diversity comprised mostly host-specific species (i.e., ecological specialists; Figs. 2, 3).

Many other, mostly undescribed, symbiont 'species' were noted in animals other than scleractinians that were sampled along each transect and from surrounding habitats (unpubl. data), including various octocorals (e.g., soft and leather corals), hydrozoans (fire corals), scyphozoans (jellies), and molluscs (i.e., giant clams; Lee et al. 2015, 2020). None of these symbionts occurred in scleractinian corals, and moreover, none of these 'other' host taxa possessed scleractinian symbionts. As the vast majority of papers documenting symbiont diversity are limited to samples from reef-building corals, a large portion of symbiodiniacean diversity remains poorly described.

## Ecological dominance of host–generalist symbionts

Prevailing environmental conditions appear to dictate the relative prevalence and dominance of host generalists (LaJeunesse et al. 2010a; Butler et al. 2023), and why highly diverse Indo-Pacific corals from a given habitat, or region, associate with one or a few generalist symbiont taxa (LaJeunesse et al. 2010a). Extrapolating from tagged colonies sampled along transects, the most prevalent and abundant symbiont associated with the Palauan barrier reef coral community was *C. madreporum* (Fig. 3).

Upon settlement and metamorphosis, the larvae of most Indo-Pacific corals must obtain symbionts from environmental sources. While this early phase in establishing the mutualism may involve multiple compatible symbionts (and clone genotypes) occurring in the environment, homogenization for one symbiont species in the developing colony, and often one clone representative of that species, likely proceeds via different symbiont cell division rates (Jones and Yellowlees 1997). The competitive sorting, or 'winnowing,' between compatible symbionts is context dependent (Coffroth et al. 2001; Poland et al. 2013) and is strongly influenced by prevailing environmental conditions as well as attributes unique to compatibility of each host and symbiont pairing (e.g., biological factors influencing the growth dynamics of competing symbiont species). Mean temperatures and light availability characteristic of Palauan barrier reefs, together with its broad host compatibility (Butler et al. 2023), may explain why *C. madreporum* is ecologically dominant over other host-generalist taxa occurring in the region (e.g., *C. patulum* and *C. vulgare*).

While *C. madreporum* dominated most corals reliant on horizontal symbiont transmission, numerous colonies of *Acropora* harbored *Cladocopium* C21 (Fig. 3). Given the ecological dominance of these branching corals, the standing biomass of *Cladocopium* C21 on Palauan reefs must also be considerable, and therefore, like *C. madreporum*, is of immense importance to the primary productivity of the host community and the larger ecosystem.

## Clone diversity and intra-host clonality within zooxanthellae species

The long-term monitoring of symbiont diversity using multiple genetic markers revealed that most coral colonies, representing numerous host taxa, maintained genetically homogeneous zooxanthellae populations over multiple years (see below). Moreover, instances of intra-colony variation in symbiont genotype were attributed to coexisting clonal cell lines and not novel symbiont acquisition or change.

As organisms that rely on clonal growth inside a host, the differential success of certain clone genotypes over others at larger special scales may be important to the ecology and

evolution of Symbiodiniaceae species. Moreover, physiological variation among different clone genotypes may influence the degree to which a host copes with thermal stress (Parkinson et al. 2015), and why individual genotypes of both the host *and* symbiont possibly explain patterns of differential bleaching and mortality (Baird et al. 2009; LaJeunesse et al. 2010b).

Most clone genotypes occurred in only one colony (Supplemental Table S2, Fig. S2), and individual clones distributed in multiple host colonies was rare (see also, Hoadley et al. 2019). This pattern differs for many host populations in the Western Atlantic, where clone genotypes are often found in neighboring colonies (Santos et al. 2003; Baums et al. 2014; Pettay et al. 2015). As most sampled colonies had a unique symbiont clone, the diversity of clone genotypes comprising each symbiont species must be exceedingly high when extrapolated to the many thousands of colonies in the area. If these patterns are indicative of other reefs, symbiont populations on western Pacific reefs comprise exceptional clone diversity, even at small spatial scales.

For the host–generalist symbiont *C. madreporum*, evidence of population subdivision corresponding to specific host taxa was sparse (Fig. 5a). While the distribution of some genetically similar clones appeared to exhibit specificity for the same host taxon, there was little overall correspondence among closely related genotypes for a particular host taxon (Fig. 5a). This was similar to previous findings of *D. trenchii* genotypes randomly distributed across a diverse host population from inshore habitats (Hoadley et al. 2019). This lack of population subdivision indicates that present selection pressures appear to have maintained the genetic cohesion of the host generalist populations in the region.

While the genotype diversity of *C. madreporum* appeared randomly distributed (i.e., homogenized) across the host community, a notable exception was discovered for *Isopora palifera* colonies, which hosted similar symbiont genotypes across an area spanning all three transects (Figs. 5a, S2). Several similar genotypes with the same *psbA*$^{ncr}$ haplotype occurred exclusively in colonies of this animal (Suppl. Fig S2). *Isopora* is one of the few Pacific Scleractinia known to brood their larvae without vertical transmission (Harii et al. 2009), and this fundamental difference in life history is one reason this genus was erected to distinguish it from *Acropora* (Wallace et al. 2007). The proximity between parent *Isopora* and developing offspring could explain this unusual pattern of association with particular genotypes of *C. madreporum* at spatial scales of 10 to 100 m. However, this pattern does not persist across larger reef areas (*psbA*$^{ncr}$ data in Butler et al. 2023). Ultimately, the aposymbiotic larvae of *Isopora* are open to symbiont infection a few days after release and thus must rely on horizontal transmission (Harii et al. 2009), and why brooding in this case does not have the same effect on symbiont diversification observed in animals that transfer symbionts to the egg during oogenesis.

Characterizing clone genotypes with microsatellites is relatively costly and laborious. Comparing these genotypes with their corresponding *psbA*$^{ncr}$ haplotypes indicated that slight differences in the chloroplast marker approximated clone identity (Fig. 5a–c). Because different haplotypes of the *psbA*$^{ncr}$ corresponded with distinct microsatellite genotypes, sole use of this marker can act to rapidly assess clone diversity and track certain clones across a host community (Fig. S2), and why samples from 2018 and 2022 were analyzed only with the *psbA*$^{ncr}$ marker. Ultimately, the combined use of independent high-resolution genetic markers provided a robust assessment of the genetic diversity of clones within individual colonies and from the community of corals in the area.

### Symbiont population homogeneity and temporal stability at the colony level

Understanding symbiont population spatial and temporal dynamics inside individual corals helps to deduce processes critical to the ecology and evolution of these mutualisms. These findings support an existing body of evidence that symbiont populations within most coral colonies are highly stable and often homogeneous (Thornhill et al. 2006, 2017; Stat et al. 2009; Pettay et al. 2011; Baums et al. 2014; Lewis et al. 2022). Independent genetic analysis reliant on DGGE-ITS2 profiling, microsatellite genotyping, and sequencing of the *psbA*$^{ncr}$ indicated that one species of symbiont persisted in each colony (see discussion of background symbionts below). The identity of the resident symbiont was determined primarily by the identity of the host and its mode of symbiont acquisition (Fig. 3). Symbiont species unique to only one host taxon occurred in animals reliant on vertical symbiont transmission, while host–generalist species were exclusive to animals with horizontal transmission. In rare cases where two symbiont species co-dominated a colony, the second was *D. trenchii*, a host-generalist from a different genus and prevalent among host populations from inshore and lagoon habitats across the region (Fabricius et al. 2004; Hoadley et al. 2019; Kemp et al. 2023). When combined with previous studies, a general pattern emerges that most colonies are dominated indefinitely by a single symbiont species under the prevailing environmental conditions characteristic of the reef habitat and geographic location.

While in most host colonies, the symbiont population comprised a single clone genotype (Fig. 4), re-sampling colonies over time revealed the existence of an additional genotype in ~ 18% of the colonies. This same percentage (~ 17%) was observed when colonies were sampled twice at the same time (Fig. 1C, Supplemental Table S2). Hence, genotype 'switching' appears to be when a colony is

dominated by multiple, usually two strains (clones), which persist over time in different parts of the colony. Thus, the supposed 'changes' in symbiont's clone genotype observed during temporal sampling appear to be an artifact of different parts of the colony sampled at different times and may also explain why seasonal changes in symbiont dominance remain unverified (Chen et al. 2005; Suwa et al. 2008; Hsu et al. 2012; Lee et al. 2016). Thus, if different genotypes inhabit distinct portions of the host colony or occur as mixed assemblages in overlapping regions, the perception of a 'change' may simply be the case when a different part of the colony was dominated by a different clone genotype or symbiont species was sampled, and probably not indicative of symbiont 'switching' or 'shuffling' per se.

Interpreting this appraisal of symbiont 'change' is further supported by other studies where multiple samples from a single colony also found additional symbiont clones, or species, abundant in other parts of the colony (Pettay et al. 2011; Baums et al. 2014). Extreme cases of coexisting symbiont species includes shallow colonies (between two and 10 m depth) of the Caribbean reef-building *Orbicella faveolata* (Rowan and Knowlton 1995; Kemp et al. 2015) and among certain colonies of *Acropora 'tenuis'* and *A. valida* from the central GBR (Ulstrup and Van Oppen 2003), where irradiance strongly determines symbiont distributions within colonies from certain habitats. It appears that, for colonies along the Palau transects, multiple symbiont strains in a colony, and co-occurring species in a few cases (i.e., *D. trenchii*), remained stable over time. Interestingly, the distribution to two clone genotypes across a colony seemed random and did not appear influenced by light intensity. Thus, reasons why some colonies exhibit stable symbiont populations comprising more than one clone genotype, and the possible physiological and ecological implications of this coexistence require further study (see discussion below; Berkelmans and van Oppen 2006; LaJeunesse et al. 2009; Grottoli et al. 2014).

When monitored over time, symbiont identity for most host taxa remains stable under normal environmental conditions (e.g., Goulet and Coffroth 2003; Thornhill et al. 2006; Stat et al. 2009; Pettay 2011; Baums et al. 2014). Most reported instances of symbiont displacement or replacement (i.e., 'switching') occur in colonies that are physically moved to new environments (Grottoli et al. 2014; Baker 2001; Bay et al. 2016) or during episodes of extreme thermal stress in natural (Jones et al. 2008; LaJeunesse et al. 2009) or experimental tank settings (Berkelmans and van Oppen 2006; Cunning et al. 2015; Howe-Kerr et al. 2020). In most cases of episodic stress, symbiont 'change' appears to be temporary, often involving rare, opportunistic, taxa, but, with sufficient time, the symbiont population reverts to the original native symbiont (Thornhill et al. 2006; LaJeunesse et al. 2009) and further emphasizes that while these mutualisms are susceptible to disruption, their natural inclination is partner fidelity and stability.

### The presence of low abundance background symbionts

This study did not attempt to assess the coexistence of other symbiont species present at low abundances, arbitrarily defined here as comprising < 5% of the total symbiont population. While certain Symbiodiniaceae taxa may persist in some colonies at low or trace (< 0.5) proportions of the total population (e.g., Boulotte et al. 2016), they probably contribute little to the productivity of the mutualism; and in most cases have minimal functional or ecological significance, especially under normal environmental conditions (Lee et al. 2016). When tracked over time, many background 'symbionts' are transient appearing and disappearing randomly over time in temporally sampled colonies. Indeed, many may not be host-compatible or even mutualistic (Lee et al. 2016; LaJeunesse et al. 2010b). A notable exception, however, is *D. trenchii* (and perhaps other species of the genus), which may displace or replace the original resident symbiont under thermally stressful conditions (Berkelmans and van Oppen 2006; LaJeunesse et al. 2009; Grottoli et al. 2014; Cunning et al. 2015).

Thermally tolerant coral communities from the Rock Island lagoon habitats of Palau are dominated by *Durusdinium trenchii* (Fabricius et al. 2004; Hoadley et al. 2019; Kemp et al. 2023). However, *D. trenchii* dominated few offshore colonies (Fig. 3). Because this species exists in high abundances in nearby habitats and is compatible with numerous coral taxa, perhaps it is prevalent at low, undetected, abundances in offshore colonies. DGGE-fingerprinting of ITS2 detects co-occurring symbionts at proportions ranging from 1–20%. The sensitivity of DGGE-fingerprinting as well as other PCR-based protocol targeting rDNA, including next-generation sequencing of ITS2-PCR amplicons (Arif et al. 2014; Hume et al. 2019; LaJeunesse et al. 2022), depends upon the rDNA copy number in the genomes of the species under detection (e.g., LaJeunesse et al. 2008; Saad et al. 2020). Because of its relative low copy rDNA number, detection of *Durusdinium* spp. in background is diminished considerably when co-occurring in populations dominated by *Cladocopium* spp. (LaJeunesse et al. 2008). Therefore, more sensitive methods of symbiont detection, such as primer specific quantitative PCR (qPCR) assays (Mieog et al. 2009; Correa et al. 2009), could be employed to evaluate the prevalence of low abundance background populations among offshore colonies. Given the potential ecological significance of background *Durusdinium trenchii* during and following thermal stress events, further analyses and detection of persistent background populations are warranted (Turnham et al. in review).

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