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FEATURE ARTICLE

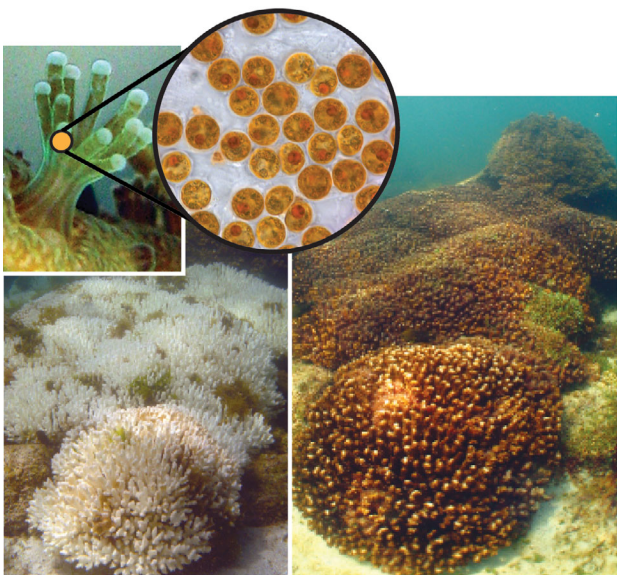
Symbiodinium spp. in colonies of eastern Pacific *Pocillopora* spp. are highly stable despite the prevalence of low-abundance background populations

Michael P. McGinley[1,*], Matthew D. Aschaffenburg[1], Daniel T. Pettay[1], Robin T. Smith[2], Todd C. LaJeunesse[3], Mark E. Warner[1]

[1]College of Earth, Ocean, and Environment, University of Delaware, Lewes, Delaware 19958, USA
[2]Department of Biology, Florida International University, Miami, Florida 33199, USA
[3]Department of Biology, The Pennsylvania State University, University Park, Pennsylvania 16802, USA

ABSTRACT: A shift in the dominant *Symbiodinium* species within a coral colony may allow rapid acclimatization to environmental stress, provided that the new symbiont is better suited to prevailing conditions. In this study, the *Symbiodinium* diversity in *Pocillopora* corals was examined following a cold-water bleaching event in the Gulf of California. Individual colonies were differentially impacted by this event based upon their association with either the *Symbiodinium* ITS-2 type C1b-c (sensitive) or ITS-2 type D1 (tolerant). Real-time PCR indicated a high prevalence of an alternate and compatible *Symbiodinium* sp. (i.e. C1b-c or D1) residing at low-abundance background levels within many colonies both during and after a 1 yr recovery interval (46 to 52%). However, despite the potential for 'switching,' the dominant resident symbiont remained at high abundance during the recovery, with only 2 of 67 colonies (3%) undergoing a change to the other *Symbiodinium* type. *Pocillopora* residing in the Gulf of California therefore maintain long-term associations dominated by a specific *Symbiodinium* sp., where potential competition by a second symbiont type is suppressed despite the temporary change in environmental conditions that would favor a shift in symbiosis toward a more stress-tolerant species.

KEY WORDS: Coral bleaching · Real-time PCR · *Symbiodinium* · Symbiosis · *Pocillopora* · Eastern Pacific



Resident *Symbiodinium* spp. remained stable within *Pocillopora* corals following a cold-water stress event off Baja California, Mexico, in 2008.

Photos: T. C. LaJeunesse, M. D. Aschaffenburg, D. T. Pettay

INTRODUCTION

The survival of a coral colony is dependent on the physiological limitations of both the cnidarian host and its endosymbiotic dinoflagellate (*Symbiodinium* spp.) under prevailing environmental conditions (Iglesias-Prieto & Trench 1997). The physiological contribution of each partner determines the level of sensitivity or resiliency of a coral to environmental

*Email: mpm312@gmail.com

stress (Brown et al. 2002, Rowan 2004, Berkelmans & van Oppen 2006, Baird et al. 2009, Wicks et al. 2012). When grown under identical conditions, genetically distinct isolates of *Symbiodinium* exhibit considerable differences in their ability to acclimatize to both light (Iglesias-Prieto & Trench 1997, Reynolds et al. 2008, Hennige et al. 2009) and temperature stress (Berkelmans & van Oppen 2006, Robison & Warner 2006), both of which are important factors responsible for large-scale coral bleaching events (Fitt et al. 2001). Therefore, the physiological attributes of a coral's symbiont are important factors determining the persistence of coral communities subjected to acute and long-term changes in their resident environment (Sampayo et al. 2008, LaJeunesse et al. 2010a).

If corals continue to survive under the current rate of climate change (IPCC 2007), one mechanism for rapid physiological change could involve a shift in coral symbioses to *Symbiodinium* spp. better adapted to more stressful environmental conditions (e.g. higher temperatures; Baker 2001). Therefore, severe physiological stress and disruption of the normal symbiosis (i.e. bleaching) may present an opportunity for rapid acclimatization by allowing for the proliferation of a tolerant background symbiont population, a condition known as symbiont 'shuffling' (Rowan et al. 1997, Berkelmans & van Oppen 2006, Jones et al. 2008, LaJeunesse et al. 2009). A coral species capable of associating with several symbiotic partners could possess an ecological advantage in its ability to adjust to different stressors imposed by global climate change.

Although most corals appear to develop highly specific and stable associations with particular algal partners (Goulet 2006), the recent use of increasingly sensitive molecular techniques (i.e. real-time PCR) have detected low-level, background symbiont populations that belong to a clade group different from the typical dominant symbiont (Loram et al. 2007, Mieog et al. 2007, Correa et al. 2009, Silverstein et al. 2012). These findings were used to infer that the potential of symbiont shuffling is higher than previously thought. However, in only a few cases has it been shown that background symbiont populations may be ecologically important during episodes of increasing stress (e.g. Berkelmans & van Oppen 2006, LaJeunesse et al. 2009). Understanding the flexibility of different coral-algal symbioses, as well as the ecological role of background symbionts, is crucial to fully comprehending the biological impact that climate change will have on coral communities.

In 2008, an anomalous cold-water bleaching event differentially impacted colonies of *Pocillopora* residing in the southern Gulf of California, in which corals harboring the sensitive symbiont, *Symbiodinium* ITS-2 type C1b-c, were severely bleached, while the colonies associated with *Symbiodinium* ITS-2 type D1 were largely unaffected (LaJeunesse et al. 2010a). The present study compared the temporal stability of these *Symbiodinium* communities within *Pocillopora* spp. at the end of the bleaching event (May 2008) and following a 1 yr recovery interval (June 2009) using real-time PCR. This sensitive detection technique confirmed that despite the potential for populations of D1 symbionts to proliferate and outcompete type C1b-c symbionts, the dominant symbiont remained remarkably stable during recovery for the majority of colonies.

MATERIALS AND METHODS

All experimental coral fragments were obtained from Punta Galeras Reef, Gulf of California (24° 21′ 15″ N, 110° 17′ 05″ W), near La Paz, Mexico. In May 2008, fragments (~4 to 5 cm in size) from 77 distinct parent colonies of *Pocillopora* spp. were sampled following a cold-water bleaching event, caused by an unusually strong La Niña cycle in this region (see LaJeunesse et al. 2010a), based on the visual characterization of bleached (completely white colony; n = 43) or non-bleached (brown colony; n = 34). This visual characterization of bleaching was confirmed by symbiont isolation and quantification, such that bleached colonies contained significantly fewer symbionts relative to coral fragments sampled 1 yr prior to the bleaching event at this same location in 2007, while there was no significant change in symbiont number for the same comparison in non-bleached fragments (LaJeunesse et al. 2010a). Initially, a fragment from each colony was analyzed for its original *Symbiodinium* composition (see below), mounted with marine epoxy to a labeled 1.5 inch PVC pipe coupler, and transplanted back to the reef site for a 1 yr recovery interval. In June 2009, all previously transplanted colony fragments were collected and again sampled for the genetic analysis of their *Symbiodinium*. Ten of the colonies (2 C1b-c and 8 D1 corals) were excluded from our analysis due to mortality or problems with extracting quality DNA (n = 67 remaining colonies).

Small fragments were removed from each coral using bone cutters and preserved in a high salt 20% DMSO DNA preservation buffer prior to transport back to the USA (Seutin et al. 1991). A Wizard DNA extraction protocol (Promega) was applied using

1.0 mm glass beads to homogenize the tissue from each fragment. The genetic identity of the *Symbiodinium* sp. was initially examined using PCR-denaturing gradient gel electrophoresis (DGGE) of the ITS2 rDNA to characterize the dominant *Symbiodinium* sp. as either C1b-c or D1 (LaJeunesse et al. 2008). All samples were amplified using the 'ITS2 Clamp' and 'ITSintfor2' using a touchdown PCR protocol according to LaJeunesse (2002). The resulting PCR products were electrophoresed on an 8% polyacrylamide denaturing gradient gel (45 to 80% denaturant) for 16 h at 115 V (CBScientific System).

The samples were additionally analyzed using previously developed Clade B, C, and D specific real-time PCR primers designed to target differences in the rDNA (Correa et al. 2009). This assay provides a greater level of sensitivity than PCR-DGGE for detecting the presence/absence of any low-level, background populations of *Symbiodinium* (Mieog et al. 2007). The DNA concentration for each sample was diluted to 10 ng μl^{-1} with autoclaved H_2O. Reactions were performed on an ABI Prism 7500 Sequence Detection System (Applied Biosystems) using 2 technical replicates for each sample. Each plate contained a no-template control and a known reference sample (cultured *Symbiodinium* DNA from Clade B, C, or D), to account for differences between subsequent runs. Each PCR reaction consisted of 2 µl H_2O, 5 µl SensiMix SYBR mastermix (Bioline), 1 µl each of forward and reverse primers (10 µM Clade C and 1 µM Clade D), and 1 µl DNA (10 ng μl^{-1}) in 10 µl total volume reactions. The subsequent thermal cycler conditions followed Correa et al. (2009). Cycle threshold (C_T) values were established using a common fluorescence threshold value of 0.01 and an automatic baseline.

The upper limit of a 'positive' amplification was conservatively set at 31.6, 30.3, and 32.3 C_T for Clade B, C, and D *Symbiodinium*, respectively. These cutoff values were chosen as our limit of detection following the analysis of a series of seven 10-fold serial dilutions (10.0 to 0.00001 ng μl^{-1}) of B1 (Culture #13), C1b-c, or D1 DNA in the presence of a constant DNA concentration (10 ng μl^{-1}) of the opposing symbiont. The DNA samples used to construct each dilution were extracted from pelleted *Symbiodinium* cells (isolated from the host or a culture [B1] via centrifugation) and screened by real-time PCR to ensure the absence of any background symbionts. The conservative cutoff point represents the highest C_T values at which the dilution series remained linear (C_T versus log scale concentration), with a standard deviation of <0.25 among the technical replicates.

The resulting C_T values for the C and D primer sets were used to designate each sample into one of the following categories: C1b-c or D1 only (a single clade detected), C1b-c/D1 mixture (<3 C_T difference between clades), C1b-c dominated with a D1 in background (C1b-c $\gg$ D1; Clade D >3 C_T values higher than Clade C), and D1 dominated with a C1b-c in background (D1 $\gg$ C1b-c; Clade C >3 C_T values higher than Clade D). The frequencies of each *Symbiodinium* type characterized using real-time PCR during May 2008 and June 2009 were analyzed by an exact Pearson's chi-squared test for independence (StatXact 4; Cytel Software) against the null hypothesis that the symbiont distribution was homogeneous between the 2 sampling points.

RESULTS AND DISCUSSION

As previously reported (LaJeunesse et al. 2010a), DGGE analysis of the ITS2 PCR amplifications revealed that the majority of the bleached *Pocillopora* colonies were dominated by *Symbiodinium* C1b-c (n = 36), with only a few colonies harboring either a C1b-c/D1 mixture (n = 3) or solely D1 (n = 2; Table 1). Conversely, most of the non-bleached colonies associated with *Symbiodinium* D1 (n = 24), while C1b-c was the dominant symbiont in only 2 of these colonies (for an extensive survey, see LaJeunesse et al. 2010a). Our subsequent application of real-time PCR identified a considerably higher proportion of low-abundance, background populations of Clade C or D residing within these corals than was detected by ITS2-DGGE (Fig. 1). Interestingly, Clade B *Symbiodinium* was not detected in any

Table 1. *Symbiodinium* spp. associated with *Pocillopora*. ITS2-DGGE identification during (May 2008) and after (June 2009) recovery from the bleaching event

	Bleached			Non-bleached			Total		
	C1b-c	D1	C/D mix	C1b-c	D1	C/D mix	C1b-c	D1	C/D mix
May 2008	36	2	3	2	24	–	38	26	3
June 2009	38	–	3	2	24	–	40	24	3

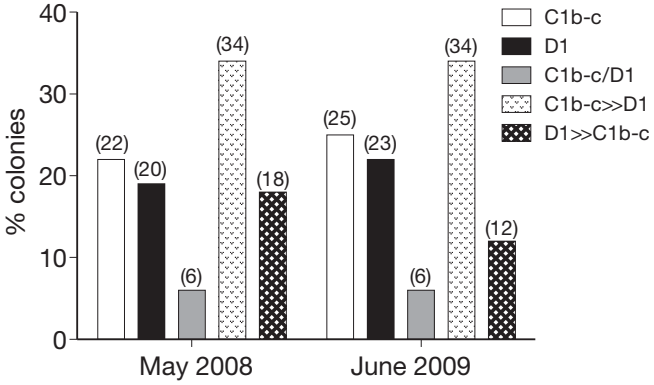


Fig. 1. *Symbiodinium* community structure in *Pocillopora* colonies. Real-time PCR characterization (n = 67) immediately after a cold-water bleaching event (May 2008) and following a 1 yr recovery interval (June 2009). All corals were classified into 1 of 5 categories: C1b-c only, D1 only, C1b-c/D1 mixture, dominant C1b-c with background D1 (C1b-c ≫ D1), and dominant D1 with background C1b-c (D1 ≫ C1b-c). Numbers above each bar represent the percentage of corals corresponding to a particular category

of the corals from either time point, although *Symbiodinium* B1 was identified in a small number of bleached colonies sampled in May 2008 in areas outside of our recovery plot (LaJeunesse et al. 2010a). In May 2008, ~2 mo following the cold-water bleaching event when affected colonies were bleached but recovering, 52% of all *Pocillopora* colonies contained a single dominant *Symbiodinium* type (C1b-c or D1) along with a detectable background population of the alternate symbiont (34% C1b-c ≫ D1, 18% D1 ≫ C1b-c; Fig. 1). Following the 1 yr recovery (June 2009), the *Symbiodinium* community structure was remarkably stable despite the high percentage of background symbionts (Fig. 1; exact Pearson's chi-squared test, p = 0.891).

Recent applications of real-time PCR have detected the prevalence of low-abundance *Symbiodinium* types associated with corals that were previously thought to harbor only a single or small subset of different symbionts (Loram et al. 2007, Mieog et al. 2007, Correa et al. 2009, Silverstein et al. 2012). These studies suggested that the prevalence of mixed symbiont populations and/or potential for symbiont shuffling within many corals is greater than previously reported (Goulet 2006). However, the ecological significance and overall contribution of such background symbionts to the holobiont fitness remains enigmatic, as many of the corals examined are rarely (if ever) dominated by one of the additional *Symbiodinium* types detected (Thornhill et al. 2006b, Goulet 2007, Stat et al. 2009, LaJeunesse et al. 2010b).

If symbiont shuffling was frequent among eastern Pacific *Pocillopora* colonies, one may expect a greater proliferation of *Symbiodinium* D1 given the increased tolerance during bleaching conditions (both warm and cold water) imparted by harboring this particular symbiont (Glynn et al. 2001, LaJeunesse et al. 2010a). However, a comparison of fragments within the individual colonies during and after the recovery period revealed that a large majority of colonies remained dominated by the same *Symbiodinium* type (67%) or showed minor differences in the appearance or disappearance of the background species (30%; Fig. 2). Overall, only 3% of *Pocillopora* colonies (n = 2) experienced a switch in their dominant symbiont population (i.e. a shift from D1 to C1b-c [n = 1] or a shift from D1 to a C1b-c/D1 mixture [n = 1]), suggesting that the occurrence of a shuffling event is exceptionally rare.

The dynamics of the background symbiont populations may suggest limited influence of preferential growth or demise of a particular *Symbiodinium* sp. in response to the bleaching event (Fig. 2b,c). For example, the appearance of a background D1 population was detected in 6 coral colonies after the recovery (i.e. absent in May 2008 and found in June 2009), while this same symbiont was no longer detected as a background population in 7 other colonies following the same interval (Fig. 2b,c). The haphazard pattern of background symbiont dynamics is possibly a sampling artifact due to the removal of coral tissue from different locations between the respective years. The relative abundance of each *Symbiodinium* sp., as well as distinct genotypes within a single symbiont species, can vary at different locations within some individual colonies (Pettay et al. 2011).

The monitoring of background symbionts over time allows us to examine in detail the dynamics of *Symbiodinium* diversity in colonies following environmental stress. The present study confirms the presence of additional low-abundance *Symbiodinium* spp. using a technique with greater detection resolution (real-time PCR; Loram et al. 2007, Mieog et al. 2007, Correa et al. 2009, Silverstein et al. 2012) and examines their dynamics during bleaching recovery (LaJeunesse et al. 2009). In contrast to certain expectations (Baker 2001, Silverstein et al. 2012), remarkable stability in resident *Symbiodinium* populations suggest that processes are at work that suppress the proliferation of a compatible symbiont present in background over the prevailing dominant symbiont population. The fidelity in partner combination during and after a bleaching event, in the presence of background populations of a second compatible symbiont, supports the idea that currently unknown molecular or cellular processes govern the dynamics

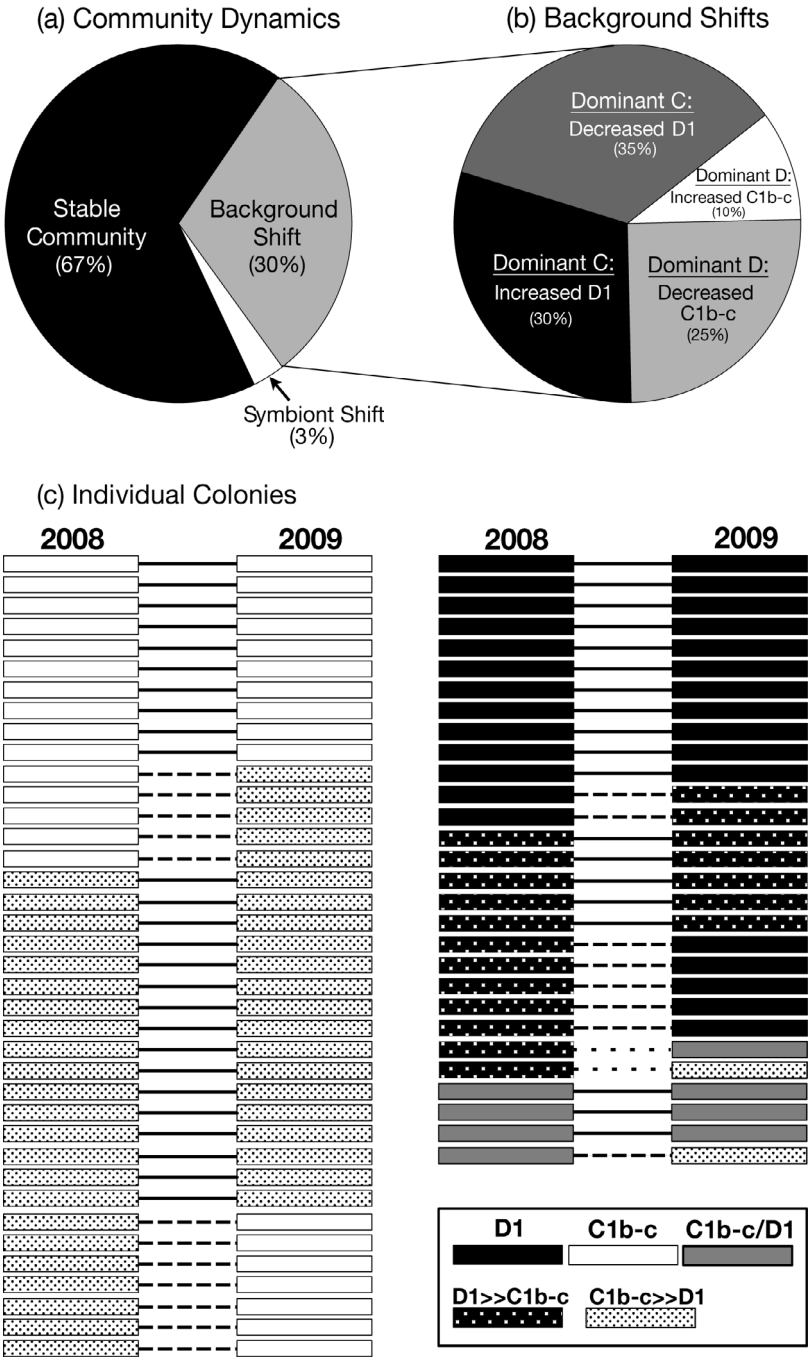


Fig. 2. *Symbiodinium* community dynamics within the *Pocillopora* colonies over the recovery interval. (a) Comparison of the *Symbiodinium* community within a coral colony, labeled as stable, background shift (stable dominant symbiont with the disappearance/appearance of a background population), or symbiont shift (change in the dominant symbiont). (b) Corals experiencing a background shift, further categorized based on their dominant symbiont type as well as the appearance ('Increased') or disappearance ('Decreased') of a background population after the 1 yr recovery interval. (c) Real-time PCR characterization of the *Symbiodinium* within individual *Pocillopora* colonies immediately after the bleaching event (2008) and following a 1 yr recovery (2009). Symbiont dynamics between sampling points: —— = stable community; – – – = background shift; ····· = symbiont shift

between these host-symbiont associations (Weis et al. 2001, Rodriguez-Lanetty et al. 2006, Wood-Charlson et al. 2006, Dunn & Weis 2009) and suggests that the selection process may be imprinted on these corals during early development (Nyholm & McFall-Ngai 2004). However, environmentally mediated forces and/or physiological characteristics of each symbiont type could be equally or more important in preventing the proliferation of a background symbiont (i.e. environmental patterns did not favor a symbiont change). For example, a rapid recovery in the remaining C1b-c symbionts may have been facilitated by more efficient nutrient acquisition and faster growth in this alga in the initial recovery interval. More research is required to evaluate factors influencing the stability of these symbioses and how specific stressors (e.g. exposure to anomalously warm vs. cold-water temperature) may differentially influence these associations.

The stable symbiosis of these *Pocillopora* colonies differs significantly from similar investigations with different types of *Symbiodinium* C and D, such as *Acropora millepora*, which exhibited a higher proportion of Clade D dominated corals following a bleaching event (Berkelmans & van Oppen 2006, Jones et al. 2008). This different outcome is not surprising given that the fidelity of a coral-algal symbiosis can vary depending on both the geographic location and the combination of each symbiotic partner (Thornhill et al. 2006a, LaJeunesse et al. 2009). Furthermore, different strategies of symbiont acquisition could also be important between these 2 coral species. *Pocillopora* in the Gulf of California are broadcast spawners but vertically transmit their symbionts to their progeny during egg development (Glynn et al. 1991), whereas *A. millepora* horizontally acquires its symbiont from the surrounding environment (van Oppen 2004). The symbioses of corals with vertical modes of symbiont transmission usually remain stable during and after exposure to physiological stressors (Thornhill et al. 2006b, Sampayo et al. 2008, Stat et al. 2009). The

coupling of host and symbiont life histories in eastern Pacific *Pocillopora* colonies may favor greater integration and stability that is less responsive to external environmental conditions and limits or prevents competition from other symbionts.

Silverstein et al. (2012) proposed that a gradient of specificity exists based on the frequency at which a coral contains background populations of an additional *Symbiodinium* clade. What is the ecological significance of background *Symbiodinium* sp. detected among various colonies? This answer can only be gained through the long-term examination of colonies in natural and experimental settings exposed to environmental change. Indeed, the existence of multiple symbionts within the colonies of certain host species may undergo rapid change when exposed to different environmental conditions (e.g. *Montastraea* complex; Rowan et al. 1997, Baker 2001, Toller et al. 2001, Chen et al. 2005). For other coral species, environmental stress may lead to the temporary dominance (for months to years) of an uncommon opportunistic symbiont (Thornhill et al. 2006a, Jones et al. 2008, LaJeunesse et al. 2009). Our data indicate that some coral-dinoflagellate associations are stable over time despite the presence of background populations of a different yet homologous (i.e. compatible) symbiont species. Therefore, although real-time PCR can routinely detect a high frequency of low-abundance *Symbiodinium* representing different clades in various host species, the stability and relative abundance of these populations must be examined over time to assess the ecological significance of the background symbionts.

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