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NOTE

A microsampling method for genotyping coral symbionts

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Abstract Genotypic characterization of *Symbiodinium* symbionts in hard corals has routinely involved coring, or the removal of branches or a piece of the coral colony. These methods can potentially underestimate the complexity of the *Symbiodinium* community structure and may produce lesions. This study demonstrates that microscale sampling of individual coral polyps provided sufficient DNA for identifying zooxanthellae clades by RFLP analyses, and subclades through the use of PCR amplification of the ITS-2 region of rDNA and denaturing-gradient gel electrophoresis. Using this technique it was possible to detect distinct ITS-2 types of *Symbiodinium* from two or three adjacent coral polyps. These methods can be used to intensely sample coral-symbiont population/communities while causing minimal damage. The effectiveness and fine scale capabilities of these methods were demonstrated by sampling and identifying phylotypes of *Symbiodinium* clades A, B, and C that co-reside within a single *Montastraea faveolata* colony.

Keywords *Symbiodinium* · Coral sampling · Zooxanthellae · Microhabitat · Symbiosis · Genotyping

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Introduction

The presence of photosynthetic endosymbionts commonly referred to as zooxanthellae contribute substantially to the productivity, survival, and success of coral reefs (Muscatine and Porter 1977). In the last 25 years, significant advances have been made in understanding the genetic diversity of these algae. Zooxanthellae are primarily represented by the genus *Symbiodinium* whose cladal and subcladal members exhibit differential host-specificity, ecological niche specialization, and temperature stress sensitivity (Rowan and Powers 1991; Iglesias-Prieto et al. 1992, 2004; Rowan and Knowlton 1995; Rowan et al. 1997; Warner et al. 1999, 2006; LaJeunesse and Trench 2000; LaJeunesse 2002; Rowan 2004; Tchernov et al. 2004; Goulet et al. 2005). With projected sea temperature increase, genetically diverse zooxanthellae may play an important ecological role in the survival of corals (Little et al. 2004; Berkelmans and van Oppen 2006; Ulstrup et al. 2006).

To date, genotypic surveys of coral symbionts have employed removal of branches, use of hammers and chisels, or coring in order to acquire coral tissue for DNA analyses. These methods provide abundant material for extractions of *Symbiodinium* DNA, but can inflict considerable damage to the coral colony that may increase susceptibility to diseases or initiate other necrotic processes (Mascarelli and Bunkley-Williams 1999; Henry and Hart 2005; Aeby and Santavy 2006).

This study describes a coral-zooxanthellae sampling protocol that enables zooxanthellae genotype identification from minimal amounts of coral tissue, and demonstrates the fine scale capabilities of these techniques on a common Caribbean coral *Montastraea faveolata*, which is known to simultaneously associate with diverse assemblages of *Symbiodinium* (Rowan and Knowlton 1995).

Materials and methods

Coral tissue (containing zooxanthellae) was collected from *M. faveolata* using a pre-labeled 3 cc syringe that was tipped with a 16 gauge needle which was blunted (for safety reasons) with a file or grinding wheel. Typically, the needle was inserted and rotated to thoroughly penetrate a coral polyp and samples were withdrawn by gentle suction (Fig. 1). One to three separate coral polyps (depending on species) were extracted from the intact colony (Fig. 2) resulting in approximately, 1.5–2.0 mg ash free dry weight (AFDW) of coral-zooxanthellae tissue. These amounts of polyp mass can be highly variable due to seasonal variations in coral tissue and zooxanthellae densities (Fitt et al. 2000). During polyp extraction, surrounding seawater and coral mucus was inadvertently collected, thereby making it possible that some free-living *Symbiodinium* and microbial symbionts may have been included in the samples, as is the case with all bulk sampling methods.

Fig. 1 Application of the syringe technique on a *Montastraea faveolata* colony. Typically, two to three polyps were sampled. The rope was marked every 10 cm and used as a transect for sampling

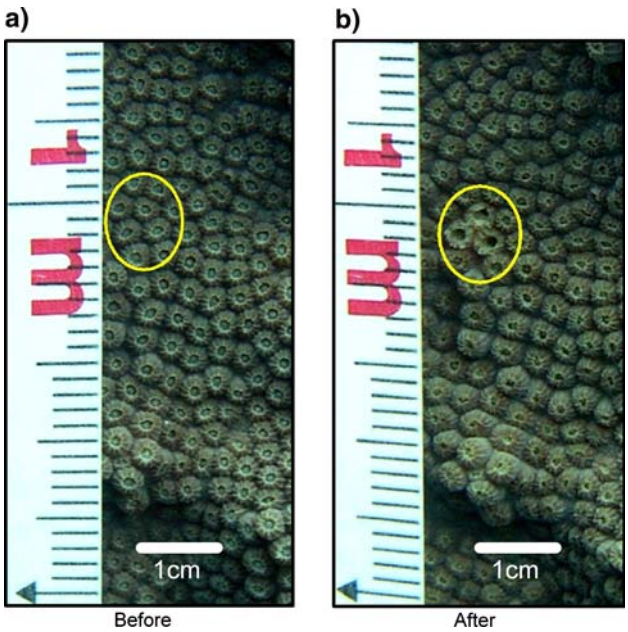

Fig. 2 **a** *M. faveolata* colony before using the syringe technique. **b** Same area showing minimal damage after sampling. Highlighted area is where three polyps were sampled

Damage to the colony was difficult to see macroscopically since the calcified skeleton was not exposed. Moreover, healing usually occurred rapidly (weeks to months) by somatic proliferation from adjacent host tissues.

Syringes containing the samples were placed in coolers containing seawater, to maintain the ambient temperature, and then transferred to the laboratory to be processed further. Each sample was expelled from the syringe to pre-labeled 1.5 or 2.0 ml microcentrifuge tubes, centrifuged (~5,000*g*) for 1–2 min to pellet zooxanthellae released from the coincidentally disrupted coral tissue. The supernatant was decanted and either 80% ethanol alcohol or DMSO buffer (20% dimethylsulfoxide, 0.25 M EDTA in saturated aqueous sodium chloride; Seutin et al. 1991) was added for DNA preservation. Symbiont DNA was extracted subsequently with the protocols prescribed for the Wizard DNA preparation kit (Promega) and refined by LaJeunesse et al. (2003). The extracted DNA was dried, resuspended in 100 µl ddH_2O, and quantified using a Nano Drop ND-1000 spectrophotometer.

PCR-based fingerprinting techniques were used to identify *Symbiodinium* from the collected samples. Restriction fragment length polymorphisms (RFLPs) analysis was conducted by amplifying the small subunit (18S) rDNA with dinoflagellate biased primers "ss5" and "ss3z" (Rowan and Powers 1991), digested with Taq I restriction endonuclease, then separated in a 2% 1X TAE agarose gel to generate the RFLPs. RFLPs were compared to cultured standards for cladal identification. Denaturing gradient gel electrophoresis (DGGE) of the internal transcribed spacer 2 region (ITS-2) of the nuclear ribosomal RNA was done using the primers "ITSintfor2" and GC-rich clamp "ITS2Clamp" following protocols of LaJeunesse et al. (2003).

Results and discussion

The microsampling technique allowed the collection and analysis of numerous coral-zooxanthellae samples from precise positions on a single coral colony, while causing minimal damage to the colony itself (Fig. 2). In this study, the yield of DNA varied between 45.0 and 91.0 ng μl^{-1}. DNA concentrations could be highly variable due to nucleic acid extraction efficiency between samples and/or unequal sample amount of host-symbionts that vary

because of symbiont density and/or polyp size and vigor. The sampling protocol used here employed sampling two to three coral polyps, used the same sized needle and syringe, and removed ~1.5–2.0 mg of AFDW of coral tissue per sample. Because of the significant biological and mechanical variation, quantitative analyses, although possible would need to be used with caution.

All the DGGE banding profiles patterns were compared directly to previously sequenced standards for symbiont identification (Fig. 3). In every case, RFLP cladal identifications coincided directly with DGGE genotypes (data not shown). Because of the greater specificity which DGGE allows, only ITS-2 genotypes are reported (Fig. 3). There are no apparent reasons why this microsampling technique could not be used with alternative *Symbiodinium* identification applications such as microsatellite analyses (e.g., Santos et al. 2003), single-stranded conformation polymorphisms (SSCP) (e.g., Little et al. 2004), or quantitative real time PCR (qPCR) (e.g., Ulstrup and van Oppen 2003).

Symbiodinium discrimination by molecular genetics has revealed distinct host-symbiont populations and community structure (LaJeunesse 2002; Baker 2003; Thornhill et al. 2006). Niche partitioning between distinct *Symbiodinium* phylotypes has also been documented to a limited extent, revealing environmental specialists, particularly with respect to high and low light habitats (Rowan and Knowlton 1995; Toller et al. 2001; LaJeunesse 2002; Ulstrup and van Oppen 2003; Garren et al. 2006; Sampayo et al. 2007). Additionally, there have been numerous ecological surveys focusing on *Symbiodinium* biogeography (Loh et al. 2001; LaJeunesse 2002; LaJeunesse et al. 2003; Santos et al. 2003; van Oppen et al. 2005). While much insight has been gained on the degree of coral-zooxanthellae specificity and/or flexibility that occurs, this is still a topic of debate (see Goulet 2006, 2007; Baker and Romanski 2007). A major deficiency, however, in many of these studies has been reliance on bulk sampling methods that do not spatially resolve coral polyp heterogeneity with respect to their most prevalent symbiont. Such crude methodologies may account for much of the confusion, for certain host species, as to whether some symbiont phylotypes are dominant while minor symbionts prevail or co-inhabit cryptic niches. Alternative conclusions from bulk coral sampling might equally be interpreted as competitive/differential proliferation stemming from symbiont segregation among adjacent polyps as a result microhabitat specialization. As illustrated in Fig. 3, some coral colonies have heterogeneous populations of *Symbiodinium* that are spatially distributed on a micro-scale (as determined by PCR-DGGE of ITS-2 rDNA from syringe samples of adjacent coral polyps). Fine scale distribution and coral-symbiont complexity would not be resolved by analysis of large coral pieces.

To date, the technique demonstrated in this study has also been used successfully to identify *Symbiodinium* from other Caribbean hard corals (*Montastraea annularis, Montastraea franski, Montastraea cavernosa*, *Siderastrea siderea, Porites asteroides and Stephanocoenia intersepta*).

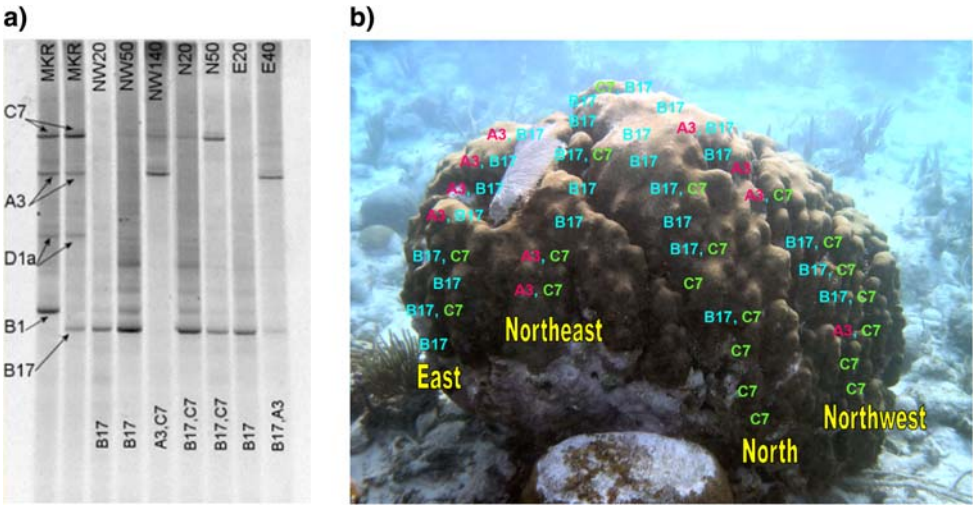

Fig. 3 The distribution of *Symbiodinium* genotypes (ITS2 region) from *M. faveolata* from Carrie Bow Cay, Belize. **a** PCR-DGGE fingerprint profiles of the ITS2 region showing the genotypes involved. The gel profile is presented as a reverse image. The first two gel lanes are markers with common ITS2 PCR-DGGE profiles of *Symbiodinium* found in the Caribbean. Non-labeled, minor shadow bands in lanes NW50, N20, NW20 N50 and E20 are presumably heteroduplexes or intragenomic variants and, due to their consistent co-occurrence, provide an additional fingerprint for phylotype B17. The top of each lane indicates compass direction and the distance (cm) from the top of the colony where syringe samples of 2–3 neighboring polyps were taken every 10 cm. Below the gel subclade designated symbiont phylotypes are assigned; capital letters indicate clades, and numbers represent ITS2 type (LaJeunesse 2002). **b** Microhabitat distribution of *Symbiodinium* genotypes (ITS2 region) in *M. faveolata* (2 m depth)

The only alterations to the methodologies have involved variations of the needle gauge size. Smaller needles (18–26 gauge) tended to work better for smaller polyped corals, but clogged more frequently, it is better, therefore, to use as large gauge needles as possible. The method can also potentially be extended to molecular certification of host identities and their possible genetic variation, along with the identification of additional microbial symbionts and pathogens that are also found to associate with corals (Knowlton and Rohwer 2003).

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