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FAST-TRACK ARTICLE

Recognizing diversity in coral symbiotic dinoflagellate communities

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Abstract

A detailed understanding of how diversity in endosymbiotic dinoflagellate communities maps onto the physiological range of coral hosts is critical to predicting how coral reef ecosystems will respond to climate change. Species-level taxonomy of the dinoflagellate genus *Symbiodinium* has been predominantly examined using the internal transcribed spacer (ITS) region of the nuclear ribosomal array (rDNA ITS2) and downstream screening for dominant types using denaturing gradient gel electrophoresis (DGGE). Here, ITS2 diversity in the communities of *Symbiodinium* harboured by two Hawaiian coral species was explored using direct sequencing of clone libraries. We resolved sixfold to eightfold greater diversity per coral species than previously reported, the majority of which corresponds to a novel and distinct phylogenetic lineage. We evaluated how these sequences migrate in DGGE and demonstrate that this method does not effectively resolve this diversity. We conclude that the *Porites* spp. examined here harbour diverse assemblages of novel *Symbiodinium* types and that cloning and sequencing is an effective methodological approach for resolving the complexity of endosymbiotic dinoflagellate communities harboured by reef corals.

Keywords: coral, DGGE, *Porites*, rDNA ITS2, *Symbiodinium*, symbiosis

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Introduction

The high rates of calcification and production that characterize typically oligotrophic coral reef ecosystems are largely credited to the mutualistic symbiosis between the scleractinian corals and photosynthetic dinoflagellates belonging to the genus *Symbiodinium*. The alarming frequency, intensity, and geographical extent of coral bleaching and mortality associated with shifting climatic conditions (Huppert & Stone 1998) has focused research on understanding how coral–symbiont associations respond to these disturbances, and if particular types of symbiotic dinoflagellates are more physiologically or ecologically robust or vulnerable to the changing environmental conditions. Central to this endeavour is an accurate and comprehensive methodology for describing symbiont diversity.

Early *Symbiodinium* classifications were based on the morphology of the nonmotile, coccoid symbiotic cells (reviewed by Blank & Trench 1986). Ultrastructural, physiological and behavioural differences in cultured isolates expanded the defined diversity within the group (Schoenberg & Trench 1980a, b, c), but our understanding was radically improved by the application of molecular systematics to the group in the early 1990s. Studies by Rowan & Powers (1991a, b) using the small subunit ribosomal RNA (18S) uncovered three major clades within the genus, each containing five to six subtypes based on restriction fragment length polymorphisms (RFLP) and direct sequencing. Building upon this work, investigators have since resolved eight major groupings in the genus by exploring *Symbiodinium* diversity using the large subunit region (28S) (Rowan 1998; Baker 1999), chloroplast large subunit (23S) (Santos *et al*. 2002), mitochondrial protein-coding gene [cytochrome oxidase subunit I (COX1); Takabayashi *et al*. 2004] and the more rapidly evolving, noncoding internal transcribed spacer regions, ITS1 and ITS2 (Hunter *et al*. 1997; Baillie *et al*. 2000; LaJeunesse 2001; van Oppen *et al*. 2001). The latter two markers have provided insight into subclade or 'ecological species' level classification, and are suggested to be the most

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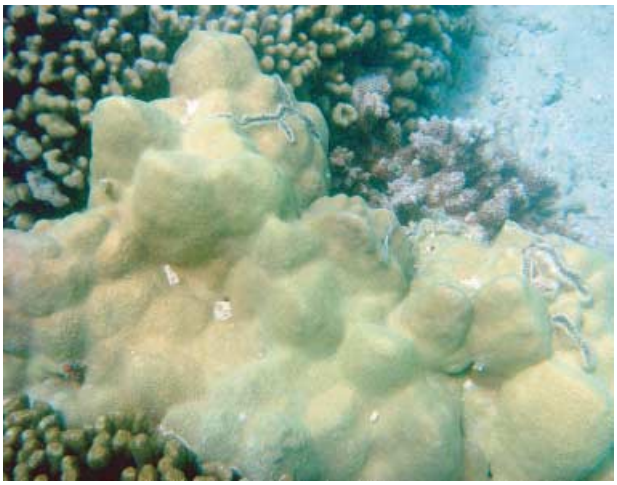
broadly applicable and appropriate markers for examining and describing *Symbiodinium* diversity (LaJeunesse 2001). Indeed, over the past 5 years, the symbionts found in numerous cnidarian hosts have been characterized using the ITS2 and the identities of dominant types in mixed communities assigned using migration characteristics and band intensities in denaturing gradient gel electrophoresis (DGGE), and sequencing of excised bands (LaJeunesse 2002; Rotjan *et al*. 2006; Thornhill *et al*. 2006; Warner *et al*. 2006; among others).

As a result of this effort, data obtained using the ITS2/DGGE methodology now ground much of our understanding of coral-symbiont relationships. For example, these data have revealed that many coral species engage in highly specific interactions with their symbionts, harbouring only one or two distinct ITS2 types (LaJeunesse 2002; LaJeunesse *et al*. 2004a; Dove *et al*. 2006). They have also demonstrated that corals and their endosymbiotic dinoflagellates share deep evolutionary histories (LaJeunesse 2005) and are stable across both geographical boundaries (LaJeunesse *et al*. 2004b) and in the face of environmental variability related to seasons and disturbance events (LaJeunesse *et al*. 2003; Thornhill *et al*. 2006). Further and more directly relevant to the function, differences in the photo-physiological performance of coral-symbionts have been shown to correspond to differences in ITS2 symbiont types (Iglesias-Prieto *et al*. 2004; Warner *et al*. 2006).

Clearly, this body of literature provides compelling evidence that the ITS2/DGGE methodology has significant merit in addressing important questions in coral symbioses. However, in the few studies where polymerase chain reaction (PCR) amplified ITS1 and ITS2 regions have been cloned and sequenced, all have noted higher symbiont diversity than demonstrated in any DGGE study (Diekmann *et al*. 2003; Rodriguez-Lanetty & Hoegh-Guldberg 2003) and van Oppen *et al*. (2005) showed that much of this variation is also visible on single-strand conformation polymorphism (SSCP) gels. Cloning and sequencing are standard methods that are used alone or in combination with genetic fingerprinting techniques to identify individual species in mixed environmental samples. This approach has been used extensively to explore diversity in eukaryotic plankton communities (D'ez *et al*. 2001) and fungi (Buchan *et al*. 2002), and characterize the bacteria associated with soils (Borneman *et al*. 1996) and corals (Rohwer *et al*. 2002). While these methods are broadly applied in other fields, they have been criticized as suboptimal for evaluating *Symbiodinium* diversity because of the potential introduction of sequence errors during cloning (Baker 2003; LaJeunesse *et al*. 2004a). As such, the utility of cloning and sequencing as a downstream method for analysing PCR-amplified ITS2 sequences has not been comprehensively assessed for the endosymbiotic communities in corals.

Here, we compare the identity and diversity of symbiont types obtained using cloning and sequencing of ITS2 with that obtained using the more commonly applied downstream analytical techniques of DGGE. We focus on the widely distributed massive corals *Porites lobata* and *Porites lutea* (synonymous with *Porites evermanni*, Fenner 2005) (Fig. 1) from Kaneohe Bay, Hawaii. Both species have previously been analysed for symbiont diversity using the ITS2/DGGE methodology and are described as containing a single dominant ITS2 type, C15 (GenBank Accession no. AY239369) (LaJeunesse *et al*. 2004a).

(a)



(b)

Fig. 1 Representative photos of the similarly morphed corals *Porites lobata* (a) (bounded by *Porites compressa* and *Montipora capitata*) and *Porites lutea* (b).

Materials and methods

Coral collection and dinoflagellate isolation

Cores of *Porites lobata* and *Porites lutea* (6 mm diameter; $n = 4$ and 3, respectively) were collected from reefs in

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Kaneohe Bay, Oahu, Hawaii in January 2004. The coral tissues containing the symbiotic dinoflagellate cells were removed from the coral skeleton using a dental Waterpik and 0.2 µm filtered seawater. The resulting blastate was blended and repeatedly centrifuged to disrupt the coral tissues and pellet the dinoflagellates. Characteristic morphological features in the coral skeletons were examined for taxonomic identity of the coral hosts, and coral ITS2 sequences obtained during the cloning and sequencing process confirmed the identity of each sample (as per Forsman *et al*. 2006).

ITS2 analyses — cloning and sequencing

Genomic DNA was extracted from the washed algal pellets using the DNeasy Plant MiniKit (QIAGEN). A portion of the 5.8S, entire internal transcribed spacer 2 region (ITS2) and portion of the 28S rDNA were amplified using the primers 'ITSintfor2' (LaJeunesse 2002) and 'ITS2rev' (5'-GGGATCCATATGCTTAAGTTCAGCGGGT-3') (lacks the GC clamp, otherwise identical to 'ITS2CLAMP'; LaJeunesse 2002), and a touchdown PCR protocol with annealing conditions of 62–52 °C and decreasing 0.5 °C every cycle, with 12 cycles held at the final annealing temperature of 52 °C. Amplified products were cloned using the TOPO TA Cloning kit (Invitrogen) with the Top10F' *Escherichia coli* strain and pCRII-TOPO plasmid vector. Cells were grown in LB broth with ampicillin, prepared for sequencing using the Fast Plasmid Mini Kit (Eppendorf) and sequenced using an ABI 3100 Genetic Analyser (Applied Biosystems). ITS2 sequences were first identified as coral or *Symbiodinium* using BLAST (GenBank), and then aligned using BIOEDIT and CLUSTAL W software. Sequences were deposited in GenBank (Accession nos DQ182634–DQ182656). A phylogenetic analysis was conducted on aligned sequences compared to ITS2 sequences available in GenBank. The analysis was constructed using PAUP version 4.0b10 (Swofford 1999), with analysis based on parsimony under the heuristic search mode using the delayed transformation character state optimization (Deltran) and with gaps and insertions considered a fifth character state.

To independently validate the presence of a common variable region in many of the ITS2 types we encountered, including the most abundant type (named KB1 throughout), we developed a primer to target a region of KB1 that differs in sequence from the previously defined subgroup, C15. The primer 'KB1begF' (5'-ACCAATGGCGAAGGTGTG-3') was paired with 'ITS2rev' to amplify ITS2 fragments from genomic DNA (original templates used to generate the clone libraries), and two plasmids representing KB1 and C15, respectively. PCR consisted of 30 cycles annealing at 59 °C. The PCR products were visualized and size confirmed on a 3% Nu-Sieve low-melting temperature gel that ran for 60 min at 70 V.

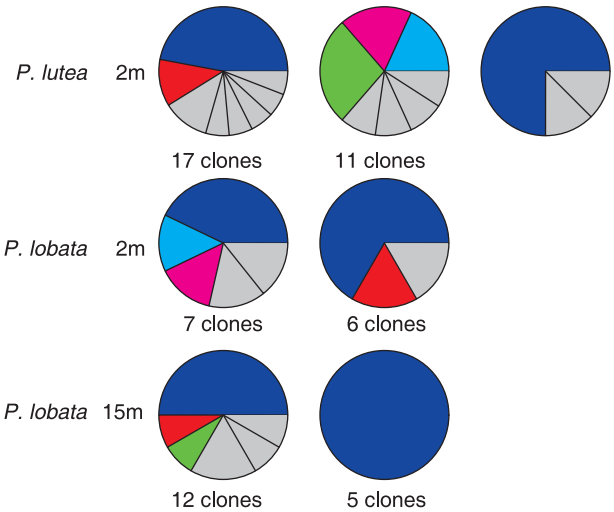

Fig. 2 Diversity of rDNA ITS2 symbiont types isolated from colonies of *Porites lobata* and *P. lutea*. Each pie chart represents one coral colony and sectors correspond to individual ITS2 sequence types. The size of the sector indicates within colony abundance relative to the total number of clones examined, which is given below each chart. Blue, red, cyan, pink, and green sectors correspond to KB1, KB2, KB3, KB4, and C15, respectively, ITS2 sequence types present in multiple hosts. Grey sectors represent types unique to a single host.

ITS2 analyses — DGGE

For denaturing gradient gel electrophoresis, the genomic DNA of a selected *P. lobata* colony (colony 135) and ITS2 clones generated from that DNA were amplified using 'ITSintfor2' paired with the reverse-adapted primer 'ITS2CLAMP' as per LaJeunesse (2002). The PCR products were loaded onto an 8% polyacrylamide denaturing gel containing a gradient of 25–55% where the 100% denaturant contained 7 M urea and 40% formamide. The gel ran for 5 h at 130 V and 60 °C. The PCR amplification and DGGE analysis were repeated to confirm results.

Results and discussion

Sequence analysis of PCR-amplified ITS2 clone libraries identified a total of 11 ITS2 types in *Porites lobata* and 17 in *Porites lutea* with individual colonies hosting from one to six and three to eight ITS2 types for *P. lobata* and *P. lutea*, respectively (Fig. 2). Of the clones examined, 93% of the *P. lobata* and 83% of the *P. lutea* sequences are not listed in GenBank. The novel ITS2 types KB1, KB2, KB3, and KB4 (blue, red, cyan, and pink in Fig. 2) were found in one or more colonies of both coral species, while other novel sequences were only found in a single colony of one species (individual grey sectors presented in Fig. 2). KB1 was the most frequently occurring ITS2 type, being present in all *P. lobata* colonies examined from 2 m and 15 m depth,

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and two of three *P. lutea* from 2 m depth. Only 7% of the *P. lobata* and 17% of the *P. lutea* symbiont clones sequenced represented ITS2 type C15, and this sequence type was encountered in only two of the seven clone libraries examined (green in Fig. 2).

To examine the relationship between the sequences obtained here and related types published in GenBank, we constructed a phylogeny based on parsimony (Fig. 3). The most dominant types in our clone libraries, KB1, KB2, KB3 and KB4 are closely related to one another and group together in a separate monophyletic group that is distinct from the subclade group that contains C15, C60, C55, C56, and several other types. Four of our newly recovered sequences, KB119_16, KB179A_18, KB129_4, and KB179B_24, nest within this previously defined group (note: individual symbiont types are classified by the colony number followed by the clone number, and those that are found in multiple colonies are assigned a KB identity which represents the site, Kaneohe Bay, from which the host corals originated). These analyses reveal a high level of phylogenetically unique ITS2 diversity in *P. lobata* and *P. lutea* and suggest that the repeatedly recovered KB symbiont types represent potentially important components of the symbiont communities that are harboured by these corals. Additionally, several of these corals harbour types that have been previously described as subclade C15 in corals from diverse locations, or other types that are phylogenetically similar to the C15 lineage (LaJeunesse *et al*. 2003, 2004a).

To explore how *Symbiodinium* ITS2 sequences perform in DGGE analysis, we compared the migration patterns of ITS2 fragments amplified from a genomic DNA with profiles obtained for cloned and sequenced plasmids representing a range of ITS2 types originating from the same genomic DNA. The heterogeneous genomic DNA appeared primarily as a single band that migrated to a similar position on the gel as clones representing a variety of ITS2 sequence types (Fig. 4). For example, types KB1 (135_2 and 135_16) and C15 (135_36) differ by 15 base pairs, but comigrate as the primary band visualized from the genomic DNA (Fig. 4). In contrast, KB1 and 135_33, vary by only a single base pair but exhibit very different migration characteristics (Fig. 4). This observation is unexpected based on theoretical migrations, but may occur if the genomic DNA contain nonhomologous sequences that have similar migration characteristics and 'stack' at a single position on the gel, a problem similar to that reported for DGGE analyses of closely related bacterial types (Sekiguchi *et al*. 2001). The fact that a clean read of a single sequence can be obtained from a cut, re-amplified and sequenced DGGE band (LaJeunesse 2002) may seem to discount this possibility. However, successful sequence reads from 'stacked' bands may reflect template abundance and/or bias in the re-amplification of excised bands using the modified primer necessary for sequencing (lacking the GC repeat; Weisburg *et al*. 1991; Farrelly *et al*. 1995; Fabricius *et al*. 2004). Such biases may also explain why some excised bands do not re-amplify at all and are dismissed as heteroduplexes, codominant sequences or artefacts (LaJeunesse *et al*. 2003, 2004a).

Other recent efforts have tested the sensitivity and robustness of PCR and DGGE detection techniques for *Symbiodinium* typing. Thornhill *et al*. (2006) characterized the sensitivity of PCR/DGGE for detecting different ratios of clades B and C symbionts using cultures and found a higher specificity for detection of C symbionts. It is likely that this result stems from PCR-based biases that reflect greater primer affinity for sequences from clade C symbionts (Weisberg *et al*. 1991), sensitivity to template concentration (Chandler *et al*. 1997), and/or differential amplification due to multiple RNA copies (Farrelly *et al*. 1995). In fact, the presence of intragenomic variation driven by the multicopy nature of rDNA arrays has been shown to be extensive in other dinoflagellates (Galluzzi *et al*. 2004), and there is no reason to believe that such sequences are not embedded in our dataset for the nonfunctional coding ITS2 region. However, it is also important to note that none of the data published using the suite of markers on the ribosomal array have been examined in the context of intragenomic variation (van Oppen & Gates 2006). Defining the levels of ITS2 diversity in single *Symbiodinium* cells will rapidly resolve this important issue and is clearly an important future research endeavour.

As mentioned earlier, sequencing of clone libraries has previously been criticized as a suboptimal downstream method for evaluating *Symbiodinium* diversity because of the potential introduction of sequence errors during cloning (Baker 2003; LaJeunesse *et al*. 2004a). The percent of error seen in a clone library has been evaluated as quite high, with at least 14% of closely related sequences containing aberrations of polymerase errors, mutational hot spots, heteroduplexes and chimeras (Speksnijder *et al*. 2001). However, the majority of these errors occur during PCR amplification and not as a result of cloning (Speksnijder *et al*. 2001) and as such, all PCR based methods are subject to the same sources of error. In this context, several studies have now demonstrated that the number of cycles of PCR in any method is important and that fewer PCR cycles reduce the opportunity for error or bias in the reaction (Suzuki & Giovannoni 1996; Qiu *et al*. 2001; Speksnijder *et al*. 2001). The protocols used in generating clone libraries employ fewer cycles of PCR than DGGE and thus the resulting sequences should contain fewer errors. Single nucleotide PCR-based errors have been shown to appear in previous studies (Kobayashi *et al*. 1999; Speksnijder *et al*. 2001) and a portion of the 22 novel symbiont sequences reported here contain single nucleotide differences that may represent artefacts. However, many of our sequences

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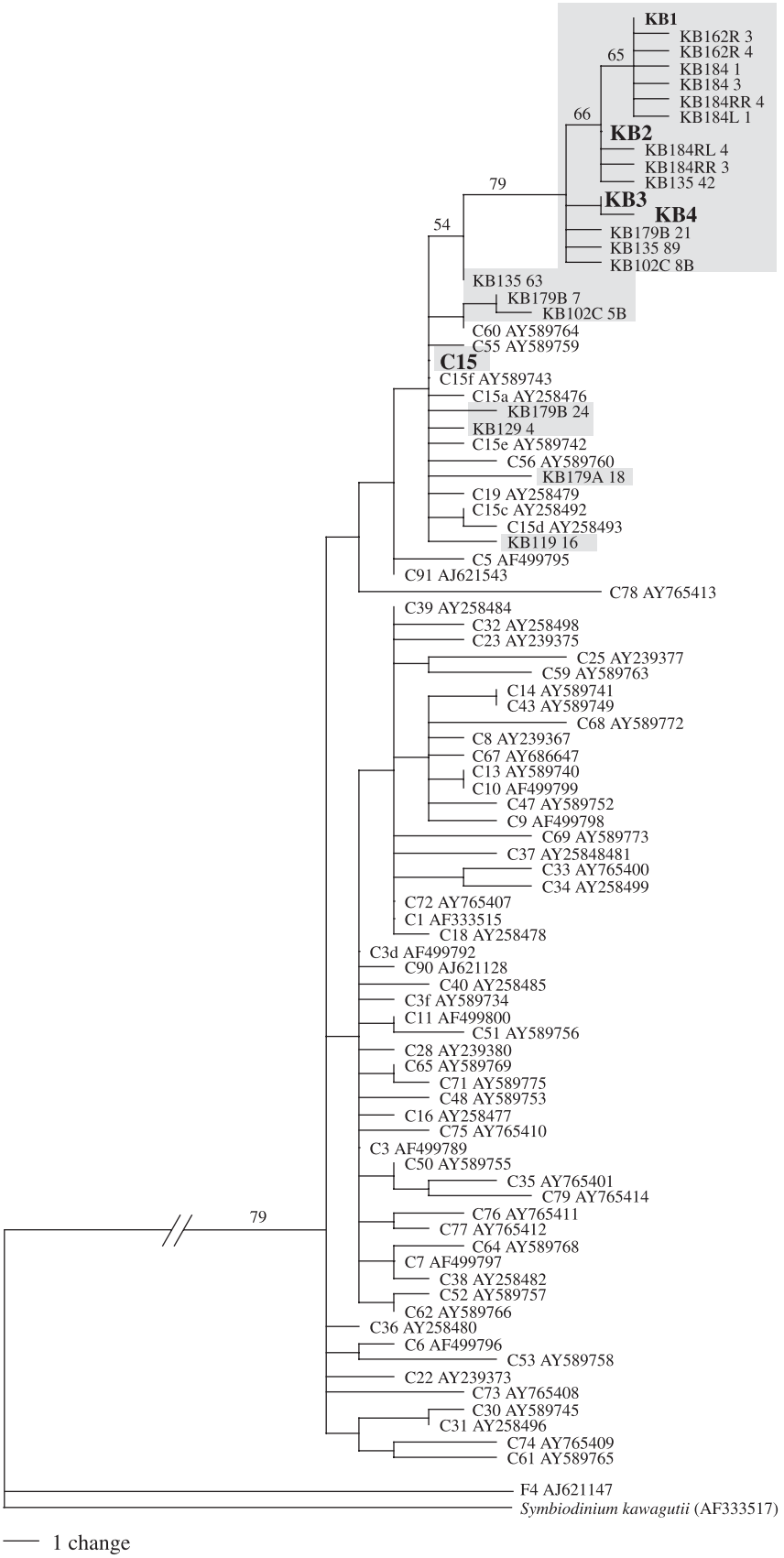

Fig. 3 Phylogenetic analysis based on parsimony of *Symbiodinium* ITS2 clade C with emphasis on KB clone library sequences. The 65 clade C sequences not found in this study are listed with GenBank Accession numbers, and sequences found in this study are highlighted in grey boxes with the most prominent types (KB1, 2, 3, 4 and C15) listed in larger, bold font. The outgroups are two clade F *Symbiodinium*. Numerals above the branches are bootstrap values for 600 replicates, and are only listed for branchings relevant to this study.

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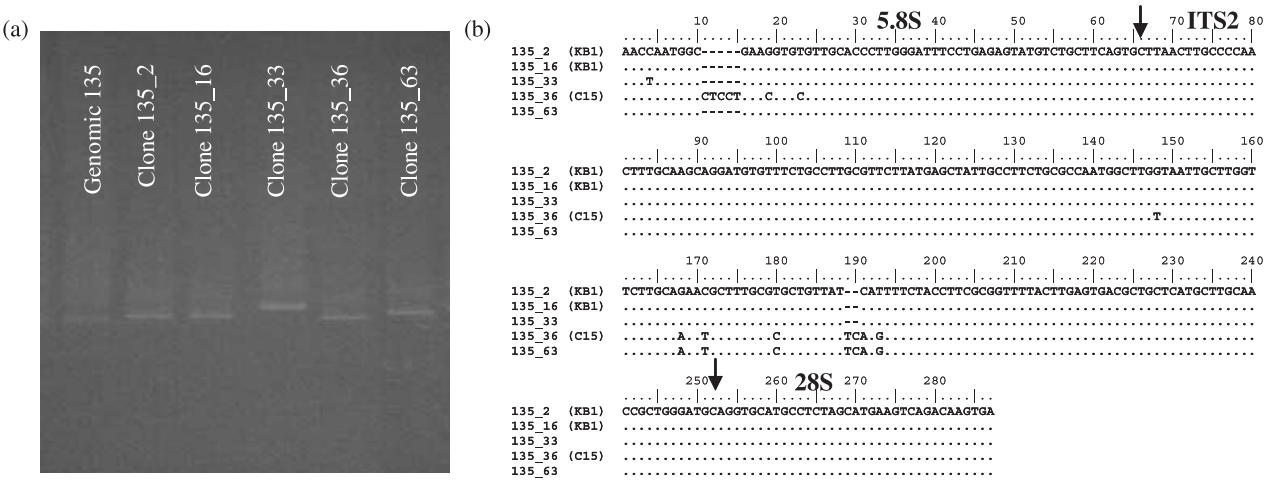

Fig. 4 DGGE profiles for ITS2 amplifications from a genomic DNA and from five ITS2 clones. (a) DGGE profiles represent ITS2 amplified from the genomic DNA of *Porites lobata* colony 135 (lane 1) and clones representing KB1 (135_2 and 135_16; lanes 2 and 3), 135_33 (lane 4), C15 (135_36; lane 5), and 135_63 (lane 4) isolated from the ITS2 clone library of *P. lobata* colony 135. (b) Alignments of the partial 5.8S, entire ITS2 (between arrows) and partial 28S for the cloned ITS2 sequences compared in DGGE. Sequences are aligned to KB1 (135_2) and invariant positions relative to KB1 are shown as a dot and gaps as a dash.

contain higher numbers of nucleotide differences, and error rates for the introduction of three or more substitutions for individual sequences are considerably lower (Kobayashi *et al*. 1999). For example, we found a difference of 15 basepairs between KB1 and C15 (Fig. 4), three differences between KB1 and KB2 and five differences between KB1 and KB3. While only one difference existed between KB3 and KB4, we still consider these nonartefactual because both types were detected in clone libraries generated for different colonies of two coral species sampled at different depths and locations within Kaneohe Bay. Examining a greater number of clones per library may help confirm the less abundant types we present here also as nonartefactual.

The likelihood of cloning errors present in the sequences found here is a difficult issue to address because one must separate PCR-generated errors, which many studies detail, from those generated from cloning, which no known studies address for mixed environmental samples. Therefore, in order to provide cloning-independent validation of the presence of KB1 in the symbiont communities harboured by *P. lobata* and *P. lutea*, we designed a specific primer to target a region in the 5.8S rDNA that is unique to this and other types of symbionts present in the KB1 lineage (large box in Fig. 3), a binding region not present in C15. These primers amplified bands of the correct size in all six of the genomics examined containing KB1, and from the KB1 plasmid, but not from the C15 plasmid. These results provide strong support that KB1 represents a novel symbiont taxon lineage that is an important component of the mixed endosymbiotic communities of *P. lobata* and *P. lutea*.

The functional implications of corals hosting different types of symbionts are encapsulated in the adaptive bleaching hypothesis (ABH), which posits that changing environmental conditions promote the loss of environmentally sensitive symbionts and repopulation by those more suited to the altered environment and capable of interacting with the coral host as a new more tolerant 'ecospecies' (Buddemeier & Fautin 1993; Berkelmans & van Oppen 2006). For the ABH, these symbionts are generally assumed to be culled from pools, free-living in the environment; however, if the complex symbiont communities we describe here also reflect functional diversity, it is plausible that a new 'ecospecies' could be achieved by changing the relative abundance of functional types within the symbiosis that are acquired during larval stages (Little *et al*. 2004). Such a scenario would circumvent the need for a chance encounter with a pool of environmentally robust free-living symbionts and would provide corals with considerable physiological scope and intrinsic flexibility to rapidly accommodate changes in their environment.

The inability of currently applied analytical techniques to resolve less dominant members of mixed *Symbiodinium* communities is well recognized (Fautin & Buddemeier 2004). However, the focus on the most abundant types is justified by the assumptions that the method for assigning dominance is accurate and that dominant symbionts are physiologically and ecologically more important to the corals and reef ecosystems than their less abundant counterparts. Our data demonstrate that DGGE protocols may not be a robust methodology for assigning dominance, and evidence from other systems and environments suggest that the latter assumption may also be flawed. For example,

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in open ocean surface waters, nitrogen-fixing bacterioplankton make a fundamental contribution to the transport of atmospheric nitrogen into the marine biogeochemical cycle (Montoya *et al*. 2004), yet they are rarely detected in 16S rDNA libraries (Giovannoni & Rappé 2000) due to their low abundance in nature relative to other bacterioplankton groups.

Molecular tools are seeing widespread use in the field of coral reef ecology (van Oppen & Gates 2006), and the assessment and validation of these techniques is critical to understanding data generated using these techniques. Here, we provide evidence that the complexity of coral dinoflagellate communities has been underestimated and that the assignment of dominance using DGGE profiling may be flawed. We demonstrate that a more comprehensive characterization of *Symbiodinium* diversity can be achieved with cloning and sequencing, and reveal a new taxonomic grouping of symbiont types in *P. lobata* and *P. lutea*. Detailed diversity studies using this approach will advance our framework for examining the functional capacity and physiological range of this important group. These data will also lay the foundation for developing methods to examine the temporal stability of symbiont communities in corals and evaluate how changes in community composition map onto the physiological characteristics of individual coral hosts. Ultimately, such analyses will resolve whether corals have the capacity to adjust the relative proportions of their mixed symbiotic communities as a means to rapidly accommodate changing environmental conditions and make a fundamental and important contribution to our understanding of how corals, and the reefs they sustain, will respond to global climate change.

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