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MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

The use of high-resolution melting analysis for genotyping *Symbiodinium* strains: a sensitive and fast approach

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Abstract

High-resolution melting (HRM) analysis is a closed-tube, rapid and sensitive technique able to detect DNA variations. It relies on the fluorescence melting curves that are obtained from the transition of double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA) as a result of temperature increase. In this study, we evaluated the effectiveness of HRM as a tool to rapidly and precisely genotype monotypic *Symbiodinium* populations using the internal transcribed spacer, region 2, ribosomal DNA (ITS2 rDNA). For this, *Symbiodinium* denaturing gradient gel electrophoresis (DGGE) profiles, where gel bands were excised and sequenced, were compared to HRM genotypes. Results showed that twenty cultures were correctly genotyped in <2 h using HRM analysis with a percentage of confidence >90%. Limitations of the technique were also investigated. Unlike other techniques used for genotyping *Symbiodinium*, such as DGGE and other fingerprint profiles, HRM is a technique of great advantage for field coral reef ecologists and physiologists as no expertise in advanced molecular methods is required.

Keywords: corals, denaturing gradient gel electrophoresis, genotyping, high-resolution melting, ITS2, *Symbiodinium*, symbiotic dinoflagellates

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Coral reefs are one of the most diverse and productive ecosystems on the planet, comparable to tropical rain forests (Connell 1978). This is largely caused by the symbiosis between scleractinian corals and dinoflagellates from the genus *Symbiodinium* (Freudenthal 1962). *Symbiodinium* is genetically classified into nine clades, designated with the letters A–I, with more than 50 types recognized (Coffroth & Santos 2005; Pochon & Gates 2010). Originally, it was thought that these dinoflagellates belonged to one, pandemic species, *Symbiodinium microadriaticum* (Taylor 1974). However, the introduction of molecular techniques opened a new avenue of research and gave light to the great existing diversity (Baker 2003; Coffroth & Santos 2005). Restriction fragment length polymorphism (RFLP) of PCR-amplified small subunit ribosomal RNA (SSU rRNA) was the first technique used (Rowan & Powers 1991). Further advances in the molecular identification of *Symbiodinium* include the use of denaturing gradient gel electrophoresis (DGGE) (LaJeunesse & Trench 2000), single-stranded conformation polymorphism (SSCP) (van Oppen *et al.* 2001), cloning (e.g. Apprill & Gates 2007), direct sequencing (e.g. Sampayo *et al.* 2009), real-time PCR (qPCR) (Ulstrup & van Oppen 2003; Mieog *et al.* 2007), and microsatellites (Santos & Coffroth 2003; Pettay & LaJeunesse 2007, 2009; Carlon & Lippé 2008; Andras *et al.* 2009; Bay *et al.* 2009). Ribosomal DNA (rDNA) is the molecular region that has been most widely used. Particular attention has given to the internal transcribed spacer, region 2, ITS2 rDNA, which has thus far been considered the most informative molecular region for identifying *Symbiodinium* types (e.g. LaJeunesse 2001). Given its variability and sensitivity, the majority of recent studies perform one of two post-PCR techniques: cloning or DGGE (Apprill & Gates 2007; Sampayo *et al.* 2009). The former technique has been criticized, because it may overestimate *Symbiodinium* diversity (Thornhill *et al.* 2007). DGGE, on the other hand, has been an alternative and successful approach used in the studies of microbial diversity, ecology and evolution (Muyzer 1999) and has been widely used to identify the genetic diversity of *Symbiodinium* (LaJeunesse & Trench 2000; LaJeunesse 2001, 2002). In DGGE, different banding profiles are obtained because of differences in the melting temperature, T_m, of double-stranded DNA (dsDNA) caused by a denaturant solvent, e.g. formamide and urea. However, this technique also possesses its limitations. It is time-consuming as it requires ~5 h

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for preparing the gel, between 10 and 15 h to run the electrophoresis, and ~1.5 h postelectrophoresis per gel. It is extremely labour-intensive with multiple steps including gel casting, visualization, excising bands, precipitation and re-amplification. Moreover, expertise is also required in several steps during DGGE analyses, such as skills in excising bands on the gel and, most importantly, in interpreting and comparing banding profiles from other studies/laboratories. In brief, while DGGE has been an effective technique in identifying *Symbiodinium* diversity, the mastering and, therefore, the use of this technique is an art.

Here, we use high-resolution melting (HRM) analysis as an alternative technique to rapidly and accurately genotype monotypic *Symbiodinium* populations. HRM is similar to DGGE in detecting mutations based on the melting properties of dsDNA. Different melting profiles are obtained from the transition of dsDNA to more denatured, single-stranded DNA (ssDNA) as a result of a gradual temperature increase after PCR amplification (Reed *et al.* 2007). All the processes of PCR amplification followed by HRM take place in the same tube during a real-time run in <2 h. The recent development of HRM can be attributed to the generation of new dyes designed for this technique and the technological improvements in real-time PCR instruments (Wittwer *et al.* 2003; Herrmann *et al.* 2007). HRM is considered the simplest method for genotyping and detecting mutations, because it is performed immediately after qPCR (Montgomery *et al.* 2007; Reed *et al.* 2007; Vossen *et al.* 2009). Previous studies have shown HRM as a more effective option compared to methods such as denaturing high-performance liquid chromatography (dHPLC), temperature gradient capillary electrophoresis (TGCE), mass spectroscopy and DGGE (Reed *et al.* 2007; Vossen *et al.* 2009). HRM has been used mainly for screening mutations linked to human diseases (e.g. Millat *et al.* 2009; Pineda-Álvarez *et al.* 2010) and for genotyping bacteria (e.g. Odell *et al.* 2005; Cheng *et al.* 2006; Stephens *et al.* 2008). To date, only one study has used HRM in wildlife populations. It demonstrated the ease and sensitivity of HRM for genetic studies of swordfish populations (Smith *et al.* 2010). To evaluate the potential of using HRM to genotype *Symbiodinium* strains based on the internal transcribed spacer, region 2, ribosomal DNA (ITS2 rDNA), HRM-based genotyping was compared to DGGE profiles, where gel bands were extracted and sequenced.

Symbiodinium cultures of clades A–E were obtained from Dr Scott Santos (Auburn University, USA; webpage: http://www.auburn.edu/~santosr/phplabware.htm) and transferred to new f/2 media (Sigma) on a monthly basis. They were maintained at a constant temperature of 25 °C and exposed to a photoperiod 12-h:12-h light:dark and a light intensity of ~80 photons/m^2 per second. Each culture was used for DNA extraction, and for the majority of the cultures, two replicates were used, for a total of 42 samples (Table 1). DNA extractions were carried out using DNeasy® Plant Mini kit (Qiagen) with a slight modification: additional cellular lysis was performed manually with the aid of a pestle. From there, the protocol was followed according to the manufacturer's guidelines. The ITS2 nuclear ribosomal region was amplified using the forward primer 'ITSintfor2' 5'-GAA TTG CAG AAC TCC GTG-3' (Invitrogen) and the reverse primer 'ITS2CLAMP' 5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GGG ATC CAT ATG CTT AAG TTC AGC GGG T-3' (Invitrogen) (LaJeunesse and Trench 2000). The underlined portion corresponds to the GC-clamp. PCR amplification had the following conditions: 1 μL of template at a concentration between 3 and 10 ng/μL, 10 μL of GoTaq Green Master Mix 1× (2× Green GoTaq reaction buffer, 400 μM each dNTP, 3.0 mM $MgCl_2$, GoTaq DNA polymerase, Promega), 0.25 μM forward primer, 0.75 μM reverse primer, and added Milli-Q water for a final volume of 20 μL per reaction. The touchdown protocol consisted of an initial denaturing step at 92 °C for 3 min, 21 cycles at 92 °C for 30 s, 62 °C for 40 s, and 72 °C for 30 s, decreasing each cycle 0.5 °C, followed by 15 cycles with a 52 °C annealing step and a final extension at 72 °C for 10 min. DGGE was carried out using a gradient of 45–80% using 8% acrylamide and 100% acrylamide, which consisted of 7 M urea and 40% deionized formamide. The electrophoresis was run for 14 h at a constant voltage of 100 V at 60 °C. Bands were excised, precipitated with EtOH and re-amplified. The re-amplification was carried out with 1.0 μL of template, 10 μL of GoTaq Green Master Mix 1× (Promega), 0.25 μM forward primer, 0.25 μM reverse primer (ITS2Rev, without clamp; Coleman *et al.* 1994), brought to a final volume of 20 μL with Milli-q water, and annealing temperature at 52 °C. PCR products were sent to the DNA Analysis Facility at Yale University for preparation and sequencing. Sequences were verified and edited with CodonCode Aligner (CodonCode Corp.), and their identities were determined using BLAST to search NCBI's GenBank. To further analyse nucleotide differences within the same clade, sequences were aligned using the program CLC Sequence Viewer v6.3 (CLC bio). Final edited sequences are available in GenBank (accession numbers HQ317737–HQ317756).

High-quality DNA for HRM was obtained using the QIAamp DNA Stool kit (Qiagen) from fresh cultures. Precipitation was carried out using 100% cold ethanol (200-proof Molecular Grade, Fisher) and 3 M sodium acetate to eliminate PCR inhibitors. HRM assays were performed using the Rotor-Gene 6000 (Qiagen) with the provided Rotor-Gene Q Series Software v1.7 (Qiagen). PCR was carried out using the Type-It HRM PCR kit (Qiagen), with

Table 1 Cultures used for genotyping with denaturing gradient gel electrophoresis (DGGE) and high-resolution melting (HRM). *Symbiodinium* strain type was identified in this study using ITS2-DGGE and confirmed with ITS2 sequence data

Culture	Invertebrate host	Geo location	Reps per culture	Clade*	ITS2-DGGE strain type	Corroboration of genotype by HRM (% confidence)
719§	*Pseudoplexaura porosa*	Caribbean	2	A	A4	A4†
2a§	*Plexaura kuna*	Caribbean	1	A	A4	A4 (96.65 ± 2.62)
Mf12.5f§	*Montastraea faveolata*	Caribbean	2	A	A3	A3†
13§	*Plexaura kuna*	Caribbean	3	B	B1	B1†
PeSc166‡	*Pseudoterogorgia elisabethae*	Caribbean	2	B	B1	B1 (97.93 ± 1.99)
Gv5.6c§	*Gorgonia ventalina*	Caribbean	3	B	B1	B1 (95.37 ± 5.22)
Mf1.5b§	*Montastraea faveolata*	Caribbean	2	B	B1	B1 (93.27 ± 7.40)
703§	*Plexaura kuna*	Caribbean	3	B	B2	B2†
PurPflex‡	*Plexaura flexuosa*	Caribbean	3	B	B3	B3†
Ap¶	*Aiptasia pulchella*	C. Pacific	3	B	B3	B3 (98.86 ± 0.73)
PtBr‡	*Briareum* sp.	Caribbean	2	C	C3	C3†
Pd45a§	*Porites astreoides*	Caribbean	1	C	C3	C3 (99.95 ± 0.03)
Pd44b§	*Porites divaricata*	Caribbean	2	C	C3	C3 (95.99 ± 4.46)
A014**	*Porites australiensis*	W. Pacific	1	D	D1a	D1a†
A001**	*Acropora* sp.	W. Pacific	2	D	D1a	D1a (94.19 ± 3.83)
A013**	*Porites annae*	W. Pacific	1	D	D1a	D1a (98.16 ± 0.14)
Mf10.8a§	*Montastraea faveolata*	Caribbean	2	D	D1a	D1a (84.09 ± 5.22)
Ap2‡	Unknown anemone	W. Pacific	2	D	D1	D1†
Ap37‡	Unknown anemone	W. Pacific	2	D	D1	D1 (84.48 ± 8.55)
CCMP421††	Acquired from CCMP	W. Pacific	3	E	E2	E2†

*Clade was identified by the culture provider; Dr Scott Santos (University of Auburn, USA) using 23S cprDNA and 28S nrDNA.
† Sample used as reference genotype for HRM, previously identified with ITS2-DGGE. ‡Isolated by Dr Scott Santos; §Isolated by Dr Mary Alice Coffroth; ¶Isolated by Dr Robert A. Kinzie; **Isolated by Dr Mishio Hidaka; ††Culture originally provided by The Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences.

1 μL of 1 ng/μL template, 1× HRM PCR Master Mix (2× HRM PCR Master Mix containing HotStartTaq *Plus* DNA polymerase, Type-it HRM PCR buffer (with EvaGreen dye), Q-solution, dNTPs; Qiagen), 0.7 μM of each primer (ITSintfor2 and ITS2rev), and adjusted with RNase-free water to a final volume of 10 μL. To check for precision, three technical replicates were performed per sample. The conditions of the qPCR consisted of an initial denaturing cycle at 95 °C for 5 min, 45 cycles at 95 °C for 10 s, 55 °C for 30 s and 72 °C for 10 s, acquiring dye (EvaGreen) emission at 530 nm for the 72 °C. HRM consisted of a temperature ramp between 78 and 90 °C, rising by 0.1 °C/2 s. Samples with the best amplification were used as reference controls for each genotype (Table 1). The rest of the samples were assigned to a genotype based on the percentage of confidence (Table 1). To calculate the percentage of confidence, an error is obtained from the square of the difference between the fluorescence of each reading of the sample and the reference genotype. This value is then added across all fluorescence readings and then incorporated in an algorithm executed by the Rotor-Gene Q Series Software v1.7 (Qiagen). The manufacturer technical support (Qiagen) recommends using a value above 85%. The accuracy of genotype detection by HRM was compared on the same samples genotyped using the DGGE technique.

Twenty cultured *Symbiodinium* isolates (~two replicates per culture) were successfully genotyped with PCR-DGGE (Table 1, Fig. S1, Supporting Information), confirming the previous identification carried out by Dr Scott Santos (Auburn University, USA) using 18S nrDNA and 23S cp-rDNA on the same cultures. Additionally, some of the cultures were previously genotyped using ITS2-DGGE, which also matches with the identification carried out in this study (Thornhill *et al.* 2007). Similarly, with the use of HRM analysis, we were able to detect and correctly identify the genotypes of all cultures with a percentage of confidence >90%, when compared to DGGE genotypes, except one genotype >84% (Fig. 1a, Table 1). The melting profiles of each clade were clearly distinctive. Moreover, different melting profiles were obtained within *Symbiodinium* clades (Fig. 1b). These differences were attributed to variation in the nucleotide composition. For example, *Symbiodinium* types A3 and A4 have 50 bp different when comparing their sequences from DGGE bands, while the difference between types D1a and D1 was only 2 bp. This distinction was clearly seen in their melting profiles (Fig. 1a).

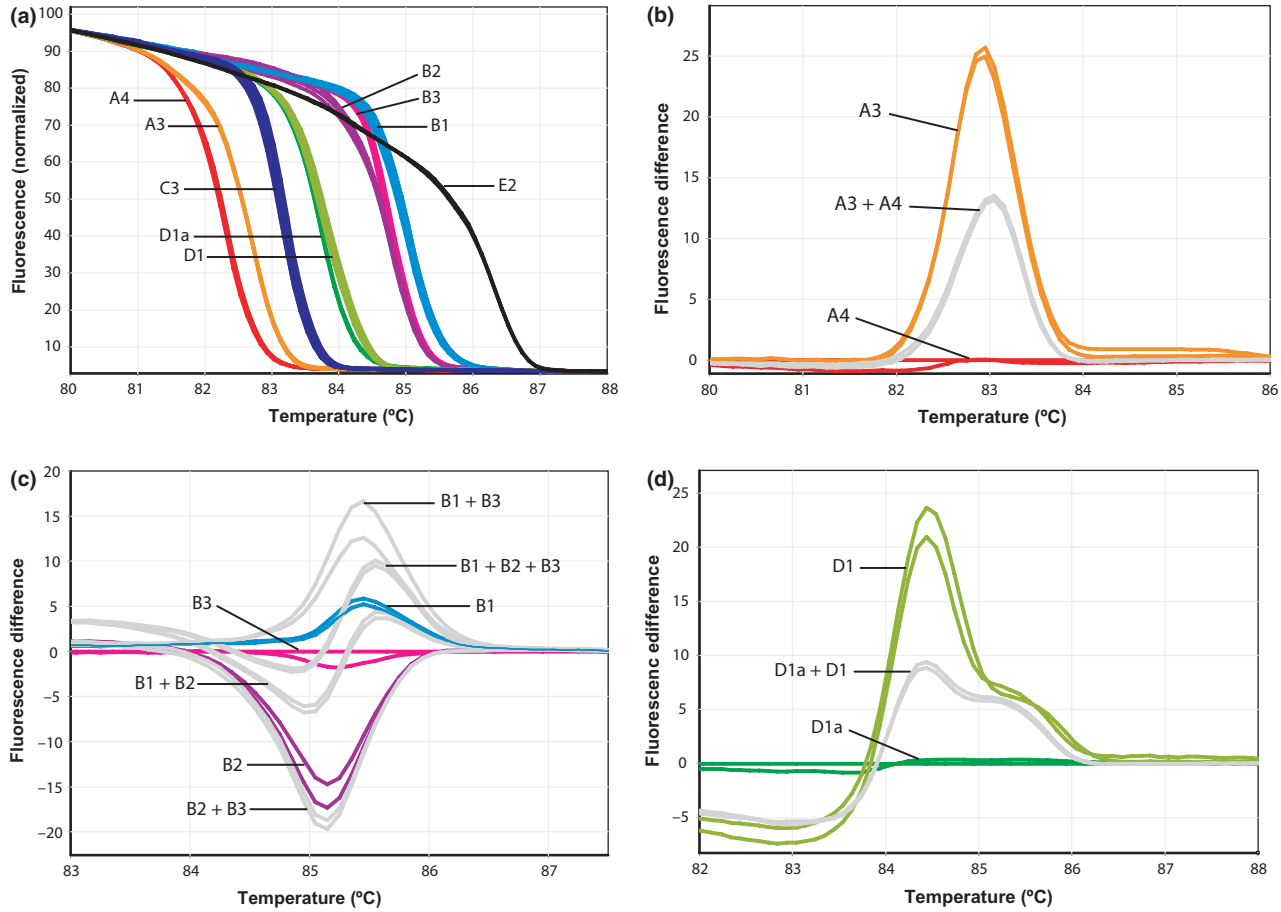

Fig. 1 High-resolution melting profiles showing the resolved *Symbiodinium* strain genotypes. (a) Normalized melting profiles of *Symbiodinium* strains with the internal transcribed spacer 2 (ITS2) type indicated. (b) Fluorescence difference plot for the pairwise combination test within clade A, using types A3 and A4. Reference genotype A4 was set as standard (red lines). Other reference genotype, A3 (orange lines), and combination (grey lines) are indicated. (c) Fluorescence difference plot for the pairwise combination test within clade B, using types B1, B2 and B3. Reference genotype B3 was set as standard (pink lines). Other reference genotypes, B1 (blue lines) and B2 (purple lines), and combinations (grey lines) are indicated. (d) Fluorescence difference plot for the pairwise combination test within clade D, using types D1a and D1. Reference genotype D1a was set as standard (dark green lines). Other reference genotype, D1 (olive green lines), and combination (grey lines) are indicated. For (b–d), the difference of the fluorescence between a chosen melting curve set as standard and each melting profile was plotted against temperature resulting in a 'fluorescence difference' plot.

To understand potential limitations of the HRM technique, three tests were conducted. The first test determined whether template concentration affected the accuracy of genotyping by HRM. For this test, two trials were carried out at low- and high-template concentration. The low-template concentration trial used 29 samples at two final concentrations in the PCR, 1 and 10 ng/μL, with three technical replicates per sample. The high-template concentration trial used two samples with the best quality from *Symbiodinium* types B1 and B2 and increased the concentration considerably, 15, 30 and 50 ng/μL (the maximum recommended by technical guidelines from Qiagen). For each genotype, one of the samples was used as the reference genotype, while the other was treated as an 'unknown.' Two technical replicates were carried out per sample. The low-template concentration trial resulted in a shift of ~3 C_t values, as expected, but this did not affect the correct genotyping by HRM (data not shown). Similarly, the high-template concentration trial resulted in a shift of the Ct values, but again genotyping was not affected (Fig. S2, Supporting Information). This was not surprising, as DNA concentration weakly affects the T_m of the template (Montgomery *et al.* 2007). However, when using the upper-limit concentration, e.g. 50 ng/μL, the correct genotype was identified with a lower percentage of confidence (Fig. S2, Supporting Information).

The second and third tests addressed real-case scenarios of mixed *Symbiodinium* populations. As previously shown, natural biological variation exists between the

coral and its symbiont, where more than one *Symbiodinium* clade or type could be hosted by a single colony (Baker 1999). A problem in these cases would be to call for a particular (single) genotype using HRM knowing that more than one *Symbiodinium* strain is present in the sample. The second test was carried out in a pairwise fashion by combining DNA from *Symbiodinium* types of the same clade. For this test, three clades were available: clade A with types A3 and A4, clade B with types B1, B2 and B3, and clade D with types D1a and D1. The only exception of a pairwise combination was clade B, where one mixture included all three types, B1, B2 and B3. A 25 μL reaction was assembled with equal concentrations of each template, and with the same HRM conditions and protocol as previously described. Each pairwise combination had two technical replicates. We found that the melting profiles of combined samples were different from the original reference genotypes used in the mixtures. Fluorescence difference plots clearly show the distinct profiles obtained for the mixtures (Fig. 1b–d, grey lines represent combinations). For the third test, pairwise combinations between all clades (A–E), including types within each clade, were performed. DNA of each *Symbiodinium* type was mixed in the PCR with DNA of other *Symbiodinium* type (e.g. A3 + B1, A3 + B2, A3 + B3, A3 + C3, and so on). PCR conditions and protocol were kept constant, and two technical replicates were used. The melting profiles obtained for the pairwise combinations were always intermediate between the two reference genotypes been mixed (Fig. S3, grey lines represent combinations, Supporting Information). Because combined samples display different melting profiles, false-positives (type-1 error) are not expected, and therefore, there is no concern of overestimating the abundance of a particular strain of *Symbiodinium* within a complex sample.

In this study, we showed that high-resolution DNA melting analysis (HRM) allows precise genotyping of *Symbiodinium* strains. There are several advantages of HRM, making it an attractive technique. HRM is a closed-tube technique that reduces cross-contamination and does not require the handling of hazardous materials, such as acrylamide, formamide and ethidium bromide. It is time-effective, requiring <2 h per run, which facilitates rapid turnover. This technique is sensitive, simple, non-destructive and of low cost. The software used reduces subjectivity by the researcher during the genotyping process. Moreover, instruments like the Rotor-Gene 6000 (Qiagen) are of suitable size to be brought to the field for genotyping during the sampling process. However, it is also important to understand its limitations. While currently all real-time PCR instruments are designed to detect the fluorescence of melting curves, not all of them are designed for HRM analysis (Herrmann *et al*. 2007). Though initial investment of acquiring the equipment could be high, the cost of the technique is up to ten times less than traditional screening, making it cost-effective in the long run (e.g. Cheng *et al*. 2006). One limitation of HRM analysis is that genotyping is restricted to the collection of available reference genotypes. The more *Symbiodinium* strains genotyped by the scientific community, the more power HRM will gain. Another limitation of HRM is that it is constrained to the detection of monotypic *Symbiodinium* populations. In our case, this limitation was proven when performing the pairwise combinations of *Symbiodinium* strains. Therefore, in cases where genotypes cannot be identified, these could be the result of either a lack of an appropriate reference genotype at the time of the analysis and/or the presence of a mixture of strains. Nonetheless, the conservative estimation of *Symbiodinium* diversity when using HRM contrasts with other methods that could overestimate abundance, such as solely cloning or DGGE. Cloning increases the estimation of diversity when assuming that intragenomic variation correlates with symbiont types (Thornhill *et al*. 2007). DGGE could potentially increase *Symbiodinium* diversity if PCR artefacts, especially heteroduplexes, or intragenomic variation, e.g. pseudogenes, are not correctly detected (Thornhill *et al*. 2007). Overall, HRM is an excellent alternative to easily, rapidly and accurately genotype monotypic populations of *Symbiodinium*. Moreover, this is of great advantage for field coral reef ecologists and physiologists as no expertise in advanced molecular techniques is required.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 (A–D) Gel-banding profiles of *Symbiodinium* sample cultures using PCR-DGGE of the ITS2.

Fig. S2 Melting profiles of high-template concentration using HRM.

Fig. S3 HRM curves for mixed *Symbiodinium* types.

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