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Rapid discrimination between four seagrass species using hybrid analysis

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ABSTRACT. Biological species are traditionally identified based on their morphological features and the correct identification of species is critical in biological studies. However, some plant types, such as seagrass, are taxonomically problematic and difficult to identify. Furthermore, closely related seagrass species, such as *Halophila* spp, form a taxonomically unresolved complex. Although some seagrass taxa are easy to recognize, most species are difficult to identify without skilled taxonomic or molecular techniques. Barcoding coupled with High Resolution Melting analysis (BAR-HRM) offers a potentially reliable, rapid, and cost-effective method to confirm species. Here, DNA information of two chloroplast loci was used in combination with HRM analysis to discriminate four species of seagrass collected off the southern coast of Thailand. A distinct melting curve presenting one inflection point was generated for each species using *rbc*L primers. While the melting profiles of *Cymodocea rotundata* and *Cymodocea serrulata* were not statistically different, analysis of the

normalized HRM curves produced with the *rpo*C primers allowed for their discrimination. The Bar-HRM technique showed promise in discriminating seagrass species and with further adaptations and improvements, could make for an effective and power tool for confirming seagrass species.

Key words: Seagrass; Species identification; DNA Barcoding; HRM analysis; *rbc*L; *rpo*C

INTRODUCTION

Seagrass is a vital component of marine ecosystems that produces oxygen and provides food and habitat for many aquatic species such as dugongs and green turtles (Orth, 2006; Short et al., 2007). To date, 60 seagrass species have been identified worldwide and of the 12 reported in Thai waters, 11 are found on the Andaman coast and one (*Ruppia maritima*) on the coast of the Gulf of Thailand (Poovachiranon, 1988; Poovachiranon and Chansang, 1994; Poovachiranon and Adulyanukosol, 1999; Terrados et al., 1999; Hine et al., 2005; Poovachiranon et al., 2006; Sakayaroj et al., 2010). The seagrass of Phuket and Krabi Provinces are of interest to us because of their importance to the native dugongs.

Seagrass reproduces asexually (Reusch et al., 1999) and, with the exception of *Enhalus acoroides*, sexually by generating flowers and pollinating in the water. Pollinated flowers become fruits and seeds, however, seagrass flowers are short-lived. This feature makes them difficult to observe and collect and thus challenging to accurately identify. In addition, some species such as *Halophila* spp share a close evolutionary relationship and similar features (Den Hartog and Kuo, 2006). To date, the accurate identification of seagrass species requires the comparison of plant cells to a known specimen by microscopy- and chemical-based techniques. However, these approaches are time-consuming and rely on expensive equipment and technical expertise. Furthermore, a lack in standards pertaining to equipment and species identification results in the high potential for misleading outputs.

To accurately identify seagrass species based on morphological characteristics, the examination of a number of features is required. However, many of these features have a range of phenotypes and can be missing altogether, thus making an accurate identification difficult. One potential method for resolving this issue is by using short orthologous DNA sequences referred to as DNA barcodes (Kress et al., 2005; Hollingsworth et al., 2009). Recently, Lucas et al. (2012) used DNA barcodes to identify seagrass species using single-locus and a combination of multiple loci analyses. Despite recommendations from the Consortium for the Barcode of Life (CBOL Plant Working Group, 2009), the *mat*K and *rbc*L loci were not sufficient to distinguish the samples tested by Lucas et al. (2012). Therefore, a three-locus analysis was performed and Lucas et al. (2012) concluded at the end that the *mat*K/*rbc*L combination was indeed a straightforward method for the DNA-based identification of seagrass in terms of simplicity and cost-effectiveness.

The aim of this research was to discriminate seagrass species using an approach that combines DNA barcoding with high-resolution melting analysis (BAR-HRM). BAR-HRM has been shown to offer great potential in several uses related to species identification (Ganopoulos et al., 2012; Faria et al., 2013; Sakaridis et al., 2013) and here we developed this hybrid method to aid in the rapid and accurate identification of seagrass species. Two regions of the chloroplast genome, *rbc*L and *rpo*C, were used. Importantly, this method can be used in samples that lack the morphological characteristics required for identification.

MATERIAL AND METHODS

Sample collection

Specimens (*Cymodocea rotundata*, *Cymodocea serrulata*, *Halophila ovalis,* and *Halodule uninervis*) were collected from mixed seagrass beds from Tungkhen Bay, Phuket Province (7°48.539'N, 98°24.692'E), and Laem Hangnak, Krabi Province (8°01.620'N, 98°46.420'E) on the southern coast of Thailand.

Selecting DNA regions

Several regions of the genome were selected to provide the species molecular data. Previous DNA sequencing analyses of molecular data (Newmaster et al., 2006, 2008; Kress and Erickson, 2007, 2008; Fazekas et al., 2008; Lahaye et al., 2008) suggested that several DNA regions were suitable for barcoding plants and based on these studies, the *rbc*L region was selected for this study. Although the *rpo*C locus has not yet been used to identify seagrass species, it has been shown to be a candidate barcode for plant identification (Kress and Erickson, 2007) and therefore, was also selected for this study. The seagrass *rbc*L and *rpo*C sequences were obtained from GenBank and subjected to rigorous processing to remove the low-quality sequences containing more than 1% "N" nucleotides. The processed sequences were aligned using ClustalW and corrected manually. Kimura's two-parameter distances were calculated using MEGA 5 package (Tamura et al., 2011). The distance values were plotted with the Gephi program (Bastian et al., 2009). The processed sequences were used for the HRM primer design.

DNA extractions

Total genomic DNA was isolated from leaf material using the DNeasy kit (Qiagen). Extracted DNA was stored in sterile microcentifuge tubes at -20°C. DNA was amplified in 25 µL reaction mixtures containing 1 U Taq Polymerase with 1X PCR Buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.5 mM of each primer, and 20 ng/µL template DNA.

HRM analysis

To determine the characteristic melting temperature (T_m) capable of distinguishing the four different seagrass species (*C. rotundata*, *C. serrulata*, *H. ovalis*, and *H. uninervis*), real-time PCR DNA amplification was performed using the Eco™ Real-Time PCR system (illumina®, San Diego, CA, USA). The 10-µL real-time PCR and HRM mixtures contained: 5 µL 2X THUNDERBIRD® SYBR qPCR Mix, 0.2 µM forward (HRM_*rbc*LF: 5'-TAGACCTTTTTGAAGAAGGTTCTGT-3' and reverse (HRM_*rbc*LR: 5'-TGAGGCGGR CCTTGGAAAGTT-3') primers for *rbcL*, 0.2 µM forward (HRM_*rpo*CF: 5'-CCSATTGTATG GGAAATACTT-3') and reverse (HRM_*rpo*CF: 5'-CTTACAAACTAATGGATGTAA-3') primers for *rpoC*, and 25 ng 1 µL DNA. SYBR fluorescence dye was used to monitor the accumulation of amplified products during PCR. High resolution melting was used to derive the T_m value. PCR protocol was conducted on a 48-well plate Helixis using an initial denaturing step at 95°C for 5 min followed by 40 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 20

s. The fluorescent data were acquired at the end of each extension step during the PCR cycles. Before HRM, the products were denatured at 95°C for 15 s and then annealed at 50°C for 15 s to form random DNA duplexes. For the HRM experiments, fluorescence data were collected every 0.1°C and the Eco™ software (version 4.0.7.0) was used to analyze the T_m. The negative derivative of fluorescence (F) over temperature (T) (dF/dT) curve displays the T_m while decreasing fluorescence *vs* increasing temperature is depicted in the normalized raw curve. To generate normalized melt curves and difference melt curves (Wittwer et al., 2003), pre- and post-melt normalization regions were set to define the temperature boundaries of the normalized and difference plots. *H. ovalis* was set as the reference species.

RESULTS AND DISCUSSION

Seagrass sequence data obtained from an online database were used to design HRM primers. The distance of all sequences was calculated and then plotted to show how they relate to each other (Figure 1).

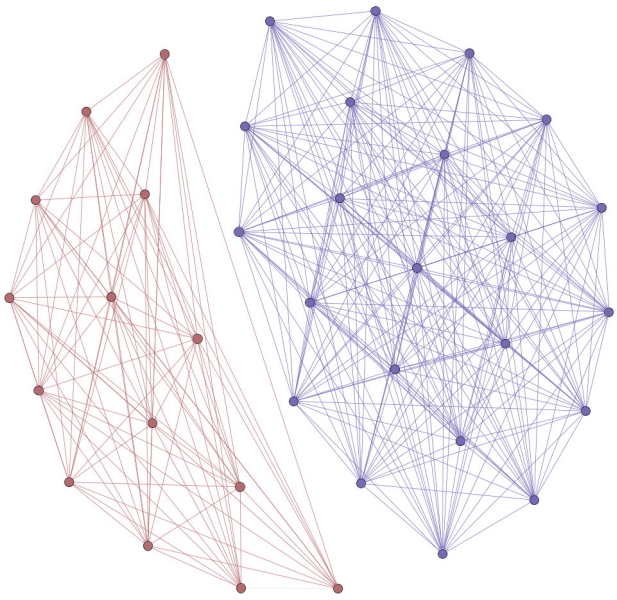

Figure 1. Calculated distance plot of seagrass sequences extracted from GenBank. Pairwise distance calculation was performed by the MEGA 5 program. Purple nodes correspond to *rbc*L sequences and red nodes correspond to *rpo*C sequences. All edge links refer to related sequences.

All seagrass *rbc*L sequences were grouped together, and in the same way, the *rpo*C sequences are also close to each other, as expected. In order to identify and/or discriminate the four seagrass species, two separate HRM were performed, in which the first one using *rbc*L primer pairs and then the *rpo*C primers were used in the second analysis. The HRM analysis of the *rbc*L barcoding region was evaluated first to identify the four seagrass species. The melting profiles of the *rbcL* amplicons of four different seagrass species (*C. rotundata*, *C. serrulata*, *H. ovalis*, and *H. uninervis*) are represented by peaks. The melting temperature peaks of all

four seagrass species, obtained from three replicates of HRM performed with *rbc*L primers are presented in Table 1. The T_m values are as follows: *H. ovalis* 81.13° ± 0.06°C, *H. uninervis* 80.75° ± 0.17°C, *C. rotundata* 79.83° ± 0.06°C, and *C. serrulata* 79.90° ± 0.00°C. Distinct melting curves presenting one inflection point were generated for each species (Figure 2A).

Table 1. T_m values with standard deviations obtained with the *rbc*L and *rpo*C primer pairs for each species.

Species	*rbc*L T_m (°C)	*rpo*C T_m (°C)
Cymodocea rotundata	79.83 ± 0.06	79.00 ± 0.00
Cymodocea serrulata	79.90 ± 0.00	79.53 ± 0.06
Halodule uninervis	80.75 ± 0.17	79.10 ± 0.00
Halophila ovalis	81.13 ± 0.06	79.70 ± 0.00

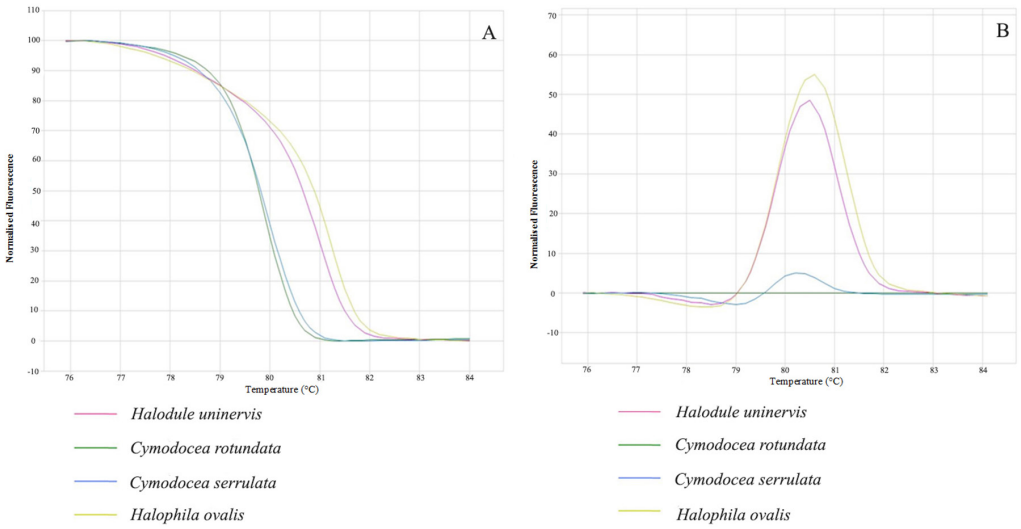

Figure 2. Melting curves obtained by HRM analysis of the *rbc*L specific amplicon when applied to the four seagrass species tested. **A.** Temperature-shifted melting curves of the *rbc*L amplicons. **B.** HRM difference plot of the samples amplified by *rbc*L primers.

However, the melting profiles of *C. rotundata* and *C. serrulata* were not statistically different (data not shown) and thus different specific primers are needed to distinguish these seagrass samples (Figure 2B). Analysis of the normalized HRM curves produced with the *rpo*C primer allows for the discrimination of *C. rotundata* and *C. serrulata* (Figure 3).

The use of HRM combined with the discriminating power of barcoding has been reported recently in the food industry (Ganopoulos et al., 2012; Faria et al., 2013; Sakaridis et al., 2013). Here, we describe the development of the Bar-HRM technique as a rapid, accurate, and economical molecular method to identify seagrass species. Although DNA barcodes alone are sufficient to discriminate species, a more detailed study covering global populations or a high-resolution system is needed for standard setting of seagrass identification (Lucas et al., 2012). The method described here offers great potential in the rapid identification and discrimination of species. The results from this study indicated that the designed primers were

inadequate, however, by modifying the primers and the primer design methods, the efficiency of this technique will be greatly improved. Furthermore, analysis of DNA sequences from more species, together with additional markers could yield suitable primers with better discriminatory power.

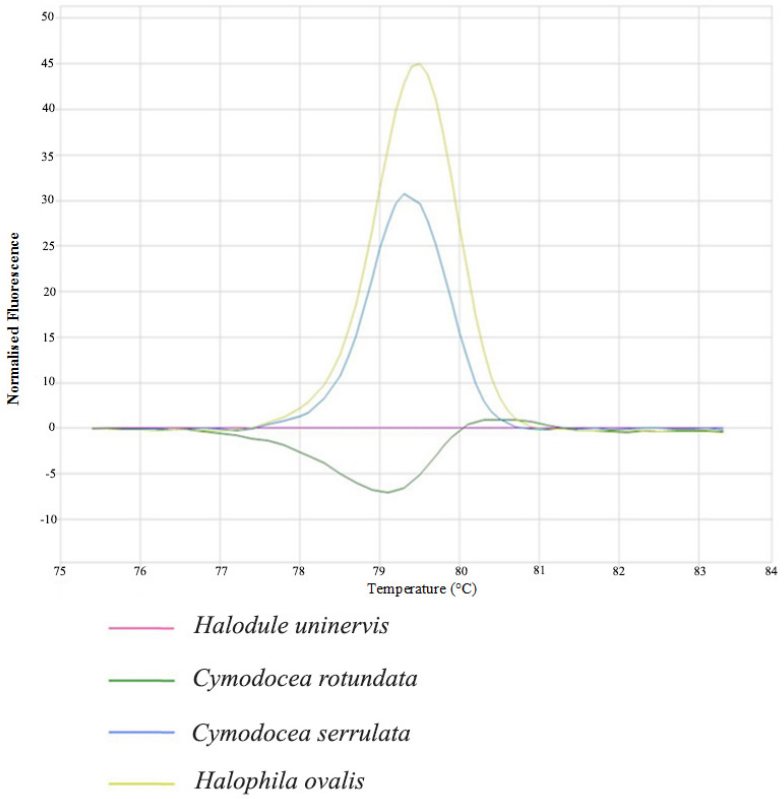

Figure 3. Normalized HRM curves for the amplicons from the four seagrass species based on HRM analysis with specific and *rpo*C primers.

CONCLUSIONS

The BAR-HRM analysis using the designed *rbc*L and *rpo*C primer sets demonstrated that it is a useful, simple, fast, and economical approach to discriminate between seagrass species. With continued improvements, BAR-HRM may be of great importance not only for seagrass plant taxonomy, but for related fields as well.

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