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Rapid analysis for the identification of the seagrass *Halophila ovalis* (Hydrocharitaceae)

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Seagrasses are considered as one of the most important species as they play key ecological roles in various types of ecosystems and also provide a food source for endangered animal species. There are two main characteristics of seagrasses that hinder efforts to correctly identify species based on conventional identification keys alone: i) the variability of morphological characteristics and ii) lack of needed morphological characters especially flowers. A taxonomically unresolved complex such as *Halophila* spp. is reported. Plant DNA barcoding regions (*rbc*L and *trn*H-*psb*A) were used to confirm species of collected seagrasses from the southern coast of Thailand. Small and big-leaved samples of *Halophila* spp. were analysed in this study. The big-leaved samples were identified on the field as *Halophila ovalis* whilst it was uncertain whether the small-leaved samples belonged to *H. ovalis*. DNA analysis revealed that the small-leaved samples could be *H. ovalis*. We also coupled PCR with high resolution melt (HRM) to more cost-effectively identify individuals of *H. ovilis* than using barcoding alone. Using HRM to resolve differences in the sequence of two genes showed that the two unknown seagrasses belonged to the same species as *H. ovalis.* In conclusion, using HRM proved to pose great potential in seagrass identification.

Key words: DNA barcoding, *Halophila ovalis*, *rbc*L, *trn*H-*psb*A, species identification.

INTRODUCTION

Seagrasses are flowering plants that are widely distributed along temperate and tropical coastlines of the world. There are 60 described seagrass species worldwide, with the majority of species found in the Indo-Pacific region (Den Hartog, 1970). Seagrasses play key ecological roles in many shallow, nearshore, marine ecosystems (Short et al., 2007; Orth et al., 2006). These plants provide protective shelter for many animals, including fish and amphipods, and provide a food to endangered manatees, dugongs and green turtles (Heck et al., 2003; Hine et al., 2005; Wongkamhaeng et al., 2009; Wilson et al., 2013). Twelve species of seagrass,

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from two families, have been reported in Thailand, including Andaman Sea and Gulf of Thailand coastlines. Only a few studies on seagrass in Thailand have been reported. Den Hartog (1970) reported five species: *Halophila ovalis* (R.Br.) Hook. f., *Halophila minor*, *Halophila decipiens*, *Halodule uninervis* and *Cymodocea rotundata*. Poovachiranon (1988) provided initial basic information on seagrass beds in Phangnga Bay, Andaman Sea of Thailand. Since 1988, several surveys revealed 12 seagrass species in Thailand (Poovachiranon, 1988; Poovachiranon et al., 1994; Poovachiranon and Adulyanukosol, 1999; Terrados et al., 1999; Hine et al., 2005; Poovachiranon et al., 2006; Sakayaroj et al., 2010). However, closely related species from *Halophila* spp., still form a taxonomically unresolved complex (Den Hartog and Kuo, 2006). The variability of morphological characteristics in seagrasses hinders efforts to correctly identify species based on conventional identification keys alone. Traditionally, biological species were classified according to some morphological features and still are the main basis of taxonomy (Heinrich, 2007).

In addition, accurate classification of individuals requires the expertise of an experienced professional taxonomist. In the case of seagrasses lacking the morphological character needed for identification, correct identification of species can be problematic and difficult. Although, some seagrasses taxa are relatively well known and easily identified, most of them are difficult to identify without specialised training. Two main characteristics of seagrasses contribute to identification difficulties: 1) they predominantly propagate by vegetative growth in units, with sexual reproduction occurring rarely due to irregular and infrequent flowering (Reusch et al., 1999) and 2) within the same species, morphological plasticity is commonly observed, with morphological acclimation to different environmental conditions (Bricker et al., 2011). The past decade has seen increasingly rapid advances in the field of molecular tools, which can be informative at many different levels of analysis. A method to identify plant species has been developed (Hollingsworth et al., 2009; Kress et al., 2005) using a short sequence region of DNA which is referred to as DNA barcoding. Yet the method is relatively expensive and is not suitable for a developing country with limited in-house sequencing capabilities like Thailand. Recently, DNA barcoding coupling with high resolution melting analysis (called Bar-HRM) has been applied to authenticate plant, meat and food products (Ganopoulos et al., 2012; Faria et al., 2013; Sakaridis et al., 2013).

H. ovalis shows morphological variability in the leaves in response to different environmental factors in various habitats. Three *H. ovalis* variants can be distinguished: small, intermediate and big-leaved. *H. ovalis* is a highly polymorphic taxon. Numerous studies pointed out the morphological variability of *H. ovalis* that relates to environmental factors (Duarte, 1991; Longstaff and Dennison, 1999; Ralph, 1999; Annaletchumy et al., 2005). Surveys, such as that conducted by Annaletchumy et al. (2005), concluded that the small-leaved *Halophila* specimen can either be *H. ovalis* or *H. minor*, requiring further study to resolve taxonomic uncertainty.

MATERIALS AND METHODS

Samples collection

Specimens of spoon grass (*H. ovalis*) were collected from mixed seagrass beds (three different locations) in 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 (Figure 1).

Molecular analysis

Selecting DNA regions

Several DNA regions were selected for providing molecular data of the species. 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) suggest that several DNA regions are suitable for barcoding plants. Based on these studies, three regions (*rbc*L, *mat*K and *trn*H-psbA) were chosen for this study.

DNA extractions, PCR conditions, DNA sequencing and accession numbers

Total genomic DNA was isolated from leaf material using DNEasy kits (Qiagen; Venlo, Limburg). 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 1 x PCR Buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl) and 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.5 mM of each primer, and 20 ng/µL template DNA. DNA barcodes were amplified by PCR using universal primers of *mat*K, *rbc*L and *trn*H-*psb*A (Supplementary Table 1) through 35 cycles of 94°C for 30 s, 55°C (both *rbc*L and *trn*H-*psb*A) and, 53°C (for *mat*K) for 1 min, and 72˚°C for 1 min. The PCR products were analysed by electrophoresis on 1.5% agarose gels (100 V, 40 mA), stained with ethidium bromide, visualised under UV. The specific DNA fragments were then purified. The amplicons were sequenced (First base, Malaysia) directly in both directions with the primers used for amplification. DNA sequences were then deposited in GenBank.

Sequence analysis

The obtained raw sequence data was analysed using Bioedit 7.0.9. The sequence files obtained were manually assembled to obtain a consensus sequence. The consensus sequence was subsequently analysed to verify the gene fragment and/or taxon. The sequences were blasted against the GenBank database to identify contaminant DNA sequences derived from fungal or other parasitic origins. After verification, the sequence was examined for the appropriate forward and reverse primer sequences. Sequences flanked by the specific primers were maintained. The resulting sequences were subsequently used for further analysis.

HRM analysis

To acquire the characteristic melting temperature (T_m) that was

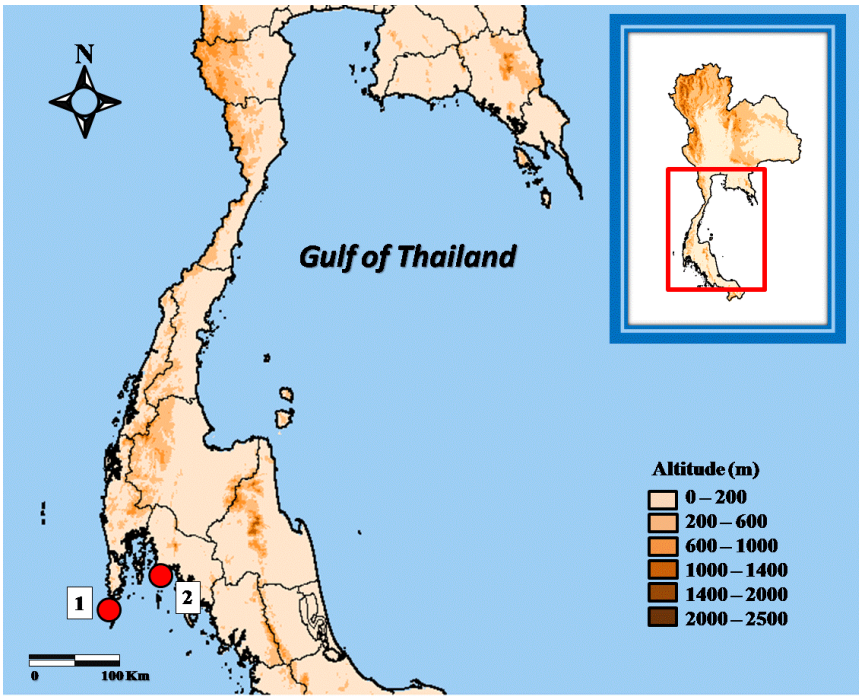

Figure 1. Samples collection map; Andaman sea from three different sites in Tungkhen Bay, Phuket Province (7˚48.539'N, 98˚24.692'E) (1), and Laem Hangnak, Krabi Province (8˚01.620'N, 98˚46.420'E) (2) on the southern coast of Thailand.

capable of distinguishing the different species of seagrass species; *Cymodocea serrulata*, *H. ovalis* and *H. uninervis*, DNA amplification using real-time PCR and DNA were performed using Eco^{TM} Real-Time PCR system (illumina®, San Diego, USA). The reaction mixture for real-time PCR and HRM analysis was done in 10 µl of total volume contained 5 µl of 2x THUNDERBIRD® SYBR qPCR Mix, 0.2 µM forward primer (HRM_rbcL3F: 5'-TAGACCTTTTTGAAGAAGGTTCTGT-3'), 0.2 µM reverse primer (HRM_rbcL3R: 5'-TGAGGCGGRCCTTGGAAAGTT-3') and 1 µl of 25 ng DNA. SYBR fluorescence dye was used to monitor the accumulation of amplified product during PCR and high resolution melting process to derive T_m value. PCR protocol was conducted in 48-well plate Helixis using an initial denaturing step at 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 20 s. The fluorescent data were acquired at the end of each extension step during PCR cycles. Before HRM, the products were denatured at 95°C for 15 s, and then annealed at 50°C for 15 s to randomly form DNA duplexes. For HRM experiments, fluorescence data was collected every 0.1°C. The EcoTM software (version 4.0.7.0) was used to analysis the Tm. The negative derivative of fluorescence (F) over temperature (T) (dF/dt) curve primarily displaying the T_m, the normalised raw curve depicting the decreasing fluorescence *vs.* increasing temperature. To generate normalised melt curves and difference melt curves (Wittwer et al., 2003), pre- and post-melt normalisation regions are set to define the temperature boundaries of the normalised and difference plot that were mainly used. *H. ovalis* was set as a reference species.

RESULTS

H. ovalis obtained from Tungkhen Bay, Phuket Province, Thailand showed morphological variability in leaf size and therefore were defined as small-leaved and big-leaved variant. The small-leaved variant has an average leaf length ≤12 mm whilst the big-leaved variant has an average leaf length ≥20 mm. The big-leaved samples were identified by an expert on the field as *H. ovalis* whilst there is uncertain whether the small-leaved samples are belonging to *H. ovalis*. Confusion in identification of *H. ovalis* variants has been long observed especially the small-leaved *H. ovalis*, which is commonly confused with *H. minor.* We only found the small and big-leaved samples although the intermediate *H. ovalis* have been observed in the area. Producing DNA data or short length sequences of chloroplast DNA known as DNA barcodes is seem to be a promising method to confirm *H. ovalis* species identification. In this study, three loci were initially chosen for analysis according to the Consortium for the Barcode of Life (CBOL) recommended primer pairs for the amplification of *mat*K, *rbc*L and *trn*H-*psb*A. However, only *rbc*L *and trn*H-*psb*A primer pairs worked well and led to successful or reproducible amplification. The lengths of the two successful DNA barcodes were 599 bp for *rbc*L and 295 bp for *trn*H-*psb*A. The selected nucleotide sequences obtained from the *rbc*L region (seqRs; sample of small-leaved variant and seqRb; sample of big-leaved variant) and the *trn*H-*psb*A region (seqTs; sample of small-leaved variant and seqTb; sample of big-leaved variant) were uploaded to a database (NCBI: www.ncbi.nlm.nih.gov) with accession numbers JX306023(seqRs),

Table 1. Genetic variation in 599 bp of partial ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) gene of *Halophila* species. Position based on sequence of GenBank Accession Number JN225349.

Species	GenBank acession numbers	Identity (%)[a]	Nucleotide differences at position									
			78	255	267	336	357	363	429	528	543	582
Halophila ovalis (Thailand: small-leaved)	JX306023	100	C	T	T	C	C	C	C	A	C	T
Halophila ovalis (Thailand: big-leaved)	JX306025	100	●	●	●	●	●	●	●	●	●	●
Halophila ovalis (India)	JN225349	100	●	●	●	●	●	●	●	●	●	●
Halophila decipiens	JN225340	100	●	●	●	●	●	●	●	●	●	C
Halophila minor	JN225347	99.83	●	●	●	●	●	●	●	●	●	C
Halophila stipulacea	JN225356	99.5	●	●	C	●	●	G	T	●	●	●
Halophila beccarii	JN225339	98.5	T	C	C	T	T	A	●	C	T	C

[a]The values are percentage of nucleotides identities for 599 bp calculated from pairwise alignment.

Table 2. Genetic variation in 259 bp of *psbA-trnH* intergenic spacer sequences of *Halophila* species. Position based sequence of GenBank Accession Number GU906229.

Species	GenBank acession numbers	Identity (%)[a]	Nucleotide differences at position					
			59	88-90	154	164-165	169	241
Halophila ovalis (Thailand: small-leaved)	JX306024	100	A	TCC	G	--	T	-
Halophila ovalis (Thailand: big-leaved)	JX306026	100	●	●	●	●	●	●
Halophila ovalis (Australia)	GU906229	99.61	●	●	●	●	●	T
Halophila ovalis (India)	JN225316	98.81	●	GGA	●	●	●	●
Halophila ovata	JN225315	98.81	●	GGA	●	●	●	●
Halophila decipiens	JN225318	98.81	●	GGA	●	●	●	●

[a]The values are percentage of nucleotides identities for 259 bp calculated from pairwise alignment.

JX306025(seqRb), JX306024(seqTs) and JX306026(seqTb), respectively. The nucleotide sequences were then analysed using the rapid identification tool BLAST (Nucleotide BLAST: www.ncbi.nlm.nih.gov) to find regions of local similarity between sequences.

The BLAST results, taking the first three BLAST hits into account, showed that seqRs and seqRb have a similar 100% maximum identity to *rbc*L gene of *H. ovalis* (AB004890, JN225348, JN225349). A close relationship to *H. minor* (JN225347) and *H. decipiens* (JN225340) was observed as shown in Table 1 whereas SeqTs and SeqTb have a similar 99.61% maximum identity to *H. ovalis* (GU906229) (Table 2), confirming that the analysed samples (small-leaved and big-leaved samples) are both, in fact, *H. ovalis*. Based on the searches, both regions offered discrimination at the species level. The DNA barcode results indicated a potential use of both *rbc*L and *trn*H-*psb*A in aiding identification of *H. ovalis* variants. Yet, some limitations of the approach exist. These limitations are included time-consuming and costly method which incurred in the sequencing step. Many laboratories in developing countries like Thailand, commonly do not own sequencing facilities so sequencing works have to be done by outsources and in many cases are done by companies aboard. An expected time to get sequences back is varied from two to five weeks. Thus, we employed a new fast and cost-effective application of DNA barcoding coupled with HRM analysis (Bar-HRM). The use of HRM in confirming the two suspected variants of *H. ovalis* was examined using one universal chloroplast region as marker to amplify polymorphic products from samples. It is appeared that the two variants belonged to the same species according to an analysis of the normalised (Figure 2A) and difference (Figure 2B) HRM curves with the barcode marker *rbc*L. The T_m of each seagrass species derived from HRM curves were shown in Table 3. The curve profiles of *H. ovalis* and both big- and small-leaved variants gaining the T_m value were similar, and could therefore not be visually differentiated, whereas the other tested species (*H. uninervis*, and *C. serrulata*) gave different curve profiles from the two variants. In addition, *H. ovalis* HRM curve was included as reference and the small differences among *H. ovalis* and the two variants melting curves showed in normalised plot. This indicated that the two variants could be *H. ovalis* and was in good agreement with barcodes results (Table 1).

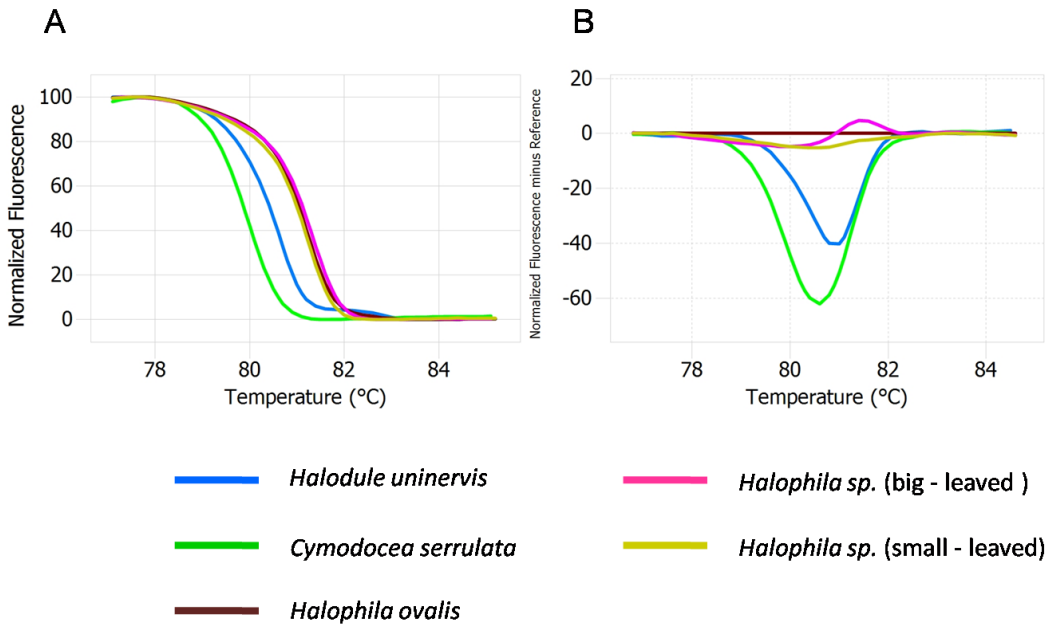

Figure 2. Bar-HRM on three seagrasses species (*Halodule univervis*, *Cymodocea serrulata* and *Halophila ovalis*) and questioned samples of *Halophila* sp. using HRM analysis with *rbc*L chloroplast marker. **(A)** Normalised melting profiles of seagrasses species with the two questioned samples. **(B)** Difference graph of all three species using *Halophila ovalis* as reference genotype. Colour code table with the species used.

Table 3. The melting temperature (T_m) profile of each seagrass species derived from HRM curves of *rbc*L DNA barcoding.

Species	Peak of T_m (°C)
Halodule uninervis	80.75 ± 0.17
Cymodocea rotundata	79.83 ± 0.06
Cymodocea serrulata	79.90 ± 0.00
Halophila ovalis	81.13 ± 0.06
Halophila sp. (big-leaved)	81.10
Halophila sp. (small-leaved)	81.20

DISCUSSION

Many species of seagrasses described by Den Hartog (1970) were identified based on vegetative characteristics, because reproductive structures were often not present. *H. ovalis* was included in a taxonomically unresolved complex given the great variety of different leaf morphologies that is the result of its response to different environmental factors in various habitats. In particular, the small-leaved *H. ovalis* and *H. minor* are frequently confused and difficult to distinguish between, because of several similarities in their morphological characteristics (Annaletchumy et al., 2005). Previous studies mentioned misidentification based on the morphology of seagrasses in the genus *Halophila*, especially *H. ovalis*, which shares considerable morphological plasticity with *H. decipiens* (Short et al., 2010; Lucas et al., 2012). Furthermore, other evidence shows that *H. decipiens* and *H. ovalis* have wide geographic distributions, separated by natural or "jump" dispersal via rafting or floating of vegetative fragments, and that *H. decipiens* is frequently found mixed with *H. ovalis* at 35 m sea depth. With jump dispersal, seagrasses can survive long distance dispersal across a range of different environments. Due to similar lifestyles, seagrass morphology is reduced and shares a number of similarities, even in the same species, but different distributions (Waycott et al., 2006; Short et al., 2010; Lucas et al., 2012). Because of a lack of lignified tissue, the structure of seagrasses is flexible and vulnerable to physical disturbance. Therefore, seagrasses resemble each other when encountered in similar environments, creating cryptic species (Carruthers et al., 2002). In addition, confusion may be caused by the morphology of *H. ovalis* and *H. ovata* not being completely different, as described in Lucas et al. (2012), and thus misidentification may occur between these two species. Thus, finding an accurate, rapid and reliable method for identifying these seagrasses is needed. DNA barcodes seem to be a useful method to support identification of seagrasses in the genus *Halophila* and therefore it used in this study. Here, we collected small and big-leaved variants of *H. ovalis* and then used molecular tools and traditional morphological taxonomic traits to classify

whether these variants belonged to the same or distinct species. They were defined as small or big-leaved variants according to their leaf length (≤12 mm = small-leaved variant and ≥20 mm = big-leaved variant) (Annaletchumy et al., 2005).

DNA barcodes; short DNA sequences, have been applied in the fields of taxonomy and molecular phylogeny in various groups of plants (e.g., Chase et al., 2005; Hajibabaei et al., 2007; Fazekas et al., 2008; Chen et al., 2010; China Plant BOL Group et al., 2011; Goldstein and DeSalle, 2011; Bhargava and Sharma, 2013). DNA barcoding was also reported as having a huge potential in many plants species and so chosen for this study. Several recent works have documented several seagrass DNA barcoding regions of either the nuclear or chloroplast genome (Table 3). However, there is no general agreement on the recommended DNA barcode region for seagrasses. One of the three regions (*mat*K, *rbc*L and *trn*H-*psb*A) chosen for DNA analysis in this study according to CBOL recommendation failed to obtain amplified fragments from the *mat*K; this amplification difficulty using the *mat*K universal primer pair has also been reported in other research (e.g., Hollingsworth et al., 2011). The *rbc*L and *trn*H-*psb*A (two successful markers) meet two of the major criteria of selecting an ideal DNA barcode – the ability of routine amplification using universal primer pair and the generation of unambiguous bidirectional DNA sequences with minimum manual editing (Hollingsworth et al., 2009). Although, several recent work on plant DNA barcoding recommended an informative region in nuclear genome, ITS (Internal transcribed spacer) which found to be effective for species identification of various plant groups (e.g., Li and Dao, 2011; Yang et al., 2012; Pang et al., 2012; Gu et al., 2013). The *rbc*L and *trn*H-*psb*A are effective to categorised the seagrass samples in this study.

The information gained from *rbc*L and *trn*H-*psb*A region analyses indicated that our samples (the two variants) are unequivocally *H. ovalis*. Interestingly, sequences of *rbc*L region from small-leaved variant showed a close-relationship to both *H. ovalis* and *H. decipiens* resulting from BLAST analysis whilst the sequences of *trn*H-*psb*A region confirmed that the samples are indeed *H. ovalis*. Although, we can confirmed now that these two studied variants are *H. ovalis*, the DNA barcodes method itself has some limitations as it is relatively expensive and take some time to produce data, especially when sequencing facilities are not available locally. It is a challenge to develop accurate and reliable methods for more rapid and inexpensive identification of species. Here we proposed, to our knowledge, the first development of HRM analysis coupled with universal chloroplast DNA barcoding region *rbc*L (Bar-HRM) for the rapid identification of seagrasses. Although, *Halophila minor* could not be obtained and used in the analysis, the result of melting curve and *rbc*L sequences were be able to indicate that the tested sampled are *H. ovalis.*

The potential identification power of this method can be observed from melting curves. The shape of the melting curves could be informative when compared difference species and to apply this information to confirm that the two seagrass samples with different average in leave size belong to the same species, other two species were included in the analysis. HRM curve analysis (Fig. 2A and 2B) revealed that the two questioned samples gave the same shape of melting curves as *H. ovalis* whilst other species (*H. uninervis*, and *C. serrulata*) in the analysis could easily be distinguished visually as can be seen from their different melting curves. Although, we could not definitely state that the two variants are not *H. minor* due to lack of the species samples, the results from this study do confirm the two are the same species and could be *H. ovalis*. DNA analysis is not a replacement for morphological identification, but helps to confirm its results and therefore in this case.

Conflict of interests

The authors have not declared any conflict of interest.

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Supplementary Table 1. Oligonucleotide primers used for DNA barcoding in this study.

Primer name	5'-->3'	Ta (˚C)
rbcL F	GTAAAATCAAGTCCACCRCG	
rbcL R	ATGTCACCACAAACAGAGACTAAAGC	55
matK F	CGCGCATGGTGGATTCACAATCC	
matK R	GTTATGCATGAACGTAATGCTC	55
trnH-psbA intergenic spacer F	ATACCCCATTTTATTCATCC	
trnH-psbA intergenic spacer R	GTACTTTTATGTTTACGAGC	55