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Assessment by microsatellite analysis of genetic diversity and population structure of *Enhalus acoroides* from the coast of Khanh Hoa Province, Vietnam

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Abstract

Seagrass beds degraded significantly since the last century on both, global and local scale. The seagrass species *Enhalus acoroides* (Linnaeus f.) Royle is a common species found in almost all marine ecosystems including bays, lagoons and around offshore islands in tropical regions of the West Pacific. It was shown that genetic diversity is an essential indicator of the conditions of ecosystems. In the present study, microsatellite markers were used to assess the genetic diversity and population structure of six distinct seagrass beds along the coast of the Khanh Hoa Province, Vietnam. The results indicate that the genetic diversity of the populations in the open sea is higher than in the lagoon. Seagrass beds occurring in disturbed sites show reduced genetic diversity. The fixing index value (F_{ST}) depicts a relatively high genetic structure among populations. Structure analysis clusters the populations into open sea and lagoon populations and cluster analysis and AMOVA indicate a significant difference between the two groups. There are low but non-significant positive correlations between geographic and genetic distances. The different habitats of the open sea and the lagoon are probably responsible for forming two groups.

Key words: *Enhalus acoroides*, genetic diversity, lagoon, open sea, population structure

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1 Introduction

Several theoretical and empirical studies indicated that genetic variation is the basis for populations adapting to changes in the environments (Carja et al., 2014; Frankham, 2005). Hughes and Stachowicz (2011) showed that populations with high genetic variation may allow for more rapid adaptation to climate change due to selection effects. The ability of different populations to adapt to their local environment results from the benefits of genetic variation, for example, having high probability to maintain advantageous genotypes (Lönn et al., 2008; Wang et al., 2012). On the other hand, lower variation decreases the resistance to diseases and adjustability in coping with environmental changes (Lacy, 1997). Milot et al. (2007) demonstrated that low genetic diversity indicated negative impacts on species viability which has been a central concern for conservation.

Natural disasters and/or human activities may cause reduction of genetic variability in natural populations, causing a phenomenon known as "genetic erosion" (Ungherese et al., 2010). With respect to seagrasses, the genetic diversity of *Halophila ovalis* (R. Brown) J D Hooker was ascertained based on the geographic barrier between the East Indian Ocean and West Pacific (Nguyen et al., 2014) or based on different salinities in different habitats (Nguyen et al., 2013). Recently, Jiang et al. (2014) suggested that human disturbances, which are common in coastal areas of China, can cause drastic changes in the population size and reduce the genetic diversity of *Halophila beccarii* Asch. There were significant positive correlations between genetic diversity and environment status for *Posidonia oceanica* (Linnaeus) Delile, a member of the Posidoniaceae (Jahnke et al., 2015). It is known that seagrass beds degraded worldwide and accordingly, the conservation of seagrass beds in particular and the marine ecosystem, in general, has attracted attention in the last decades (Waycott et al., 2009).

Seagrasses are important coastal ecosystem engineers that can act upon the physical environment through their morphological structures such as the leaf, shoot, rhizome and root. In addition, seagrass beds can reduce waves and current energy as water passes through the densely packed seagrass meadows. They also act as a biological filter by trapping the fine sand and particles that are suspended in the water column and therefore increase water clarity. However, seagrass meadows are fragile ecosystems in the coastal zone. Global assessment revealed that the area of seagrass beds are decreasing at a rate of 110 km^2/a since 1980 (Waycott et al., 2009). Human-induced disturbances may cause degradation of seagrass beds worldwide (Short and Wyllie-Echeverria, 1996). *Enhalus acoroides* is a large seagrass in size,

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native to coastal waters of the tropical Western Pacific and Indian Ocean, and it is commonly found in various habitats. Recently, it was demonstrated that genetic diversity seems to be an essential tool to evaluate the health of ecosystems (Lacy, 1997). The aim of this study is to assess the genetic diversity of the seagrass species *Enhalus acoroides* in distinct seagrass beds in Vietnam.

Vietnam is located in the West Pacific that is considered the hot spot of marine biodiversity. *Enhalus acoroides*, a member of the Hydrocharitaceae family, is a common species found in almost all marine ecosystems in bays, lagoons and around offshore islands in Vietnam (Dai et al., 1998). Like other marine ecosystems, *Enhalus acoroides* meadows in Vietnam are under threats due to both human activities and natural disasters. Remarkable degradation of *Enhalus acoroides* beds was recorded at My Giang (MG) Island and the Thuy Trieu (TT) Lagoon (Khanh Hoa Province) due to various anthropologic activities, for example by fishing gears and aquaculture activities (Quang et al., 2017; Pham et al., 2006). Recently, high Cu concentrations were identified as contamination in the surface sediment of some areas along the coastline of the Khanh Hoa Province (Dung et al., 2014). Our previous study also indicated that Cu and Pb concentrations in the surface sediment of seagrass beds at MG and TT locations were much higher than in other locations at the coast of the Khanh Hoa Province (Nguyen et al., 2017b). Production of heavy metal-binding peptides from *Enhalus acoroides* showed significant differences between different populations at Khanh Hoa Province (Nguyen et al., 2017a).

This study aims to detect the genetic diversity and population structure of *Enhalus acoroides* collected at six different meadows along the coast of the Khanh Hoa Province, Viet nam, based on microsatellite markers. The data can serve as a basis for recommendations for the conservation of seagrass meadows.

2 Materials and methods

2.1 *Sample collection*

Samples from six seagrass meadows were collected from the Khanh Hoa coast. Seagrass meadows at Tuan Le (TL), My Giang (MG) and Song Lo (SL) belong to the northern part of the province, and are located in the open sea. The seagrass beds are situated on the coarse sand or death coral substratum and are exposed to the air during low tide. The other sampling spots, Thuy Trieu (TT), Cam Phuc Nam (CPN) and Cam Thinh (CT), are located in the lagoons (Fig. 1). Environmental conditions of the three locations in the northern part are very similar with a water temperature between 29.3°C and 31°C, pH in the range of 7.4–7.6 and salinity of 31–32 (Nguyen et al., 2017c). The total suspended solid (TSS) values are 10.2–16.7 and 3.73–8.94 mg/L in the dry and the rainy season, respectively (Le and Le, 2009). In the southern part, average salinity and TSS show high fluctuations between the dry and the rainy season. Water temperatures are between 28.3°C and 31.8°C, with the pH in the range of 7.7–8.2. The salinity concentrations in the southern part are between 26 and 33 and 31 and 34 in the rainy and the dry season, respectively (Chen et al., 2016). The TSS values are 1.75–52.0 and 0.95–25.7 mg/L in the dry and the rainy season, respectively (Quang et al., 2017). *Enhalus acoroides* produces high amounts of biomass and forms dense beds on the muddy bottom. These seagrass beds are submerged all the time, even during low tide. At each location, ten different plants were randomly collected across the beds with a distance of 15 to 20 m between specimens. From one plant, the young leaves were selected. Seagrass samples were

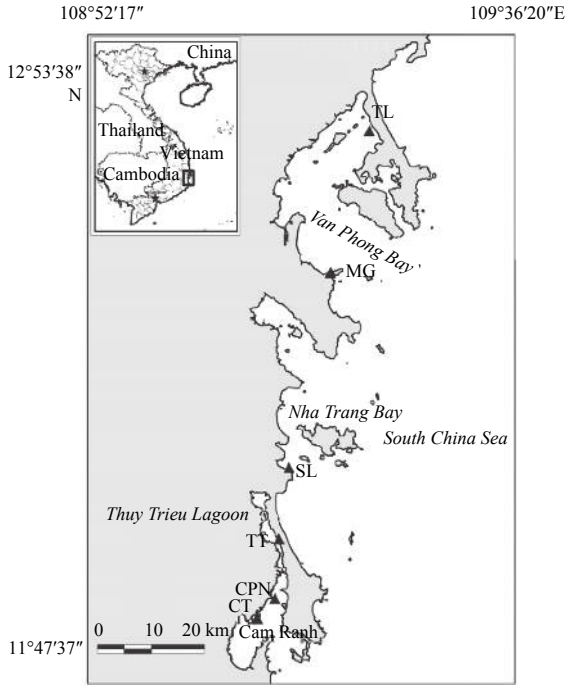

Fig. 1. The map of Khanh Hoa coast and sampling sites. TL represents Tuan Le, MG My Giang, SL Song Lo, TT Thuy Trieu, CPN Cam Phuc Nam, CT Cam Thinh (Source: The National Oceanic and Atmospheric Administration (NOAA), USA, public domain data). See Table 1 for more information. The map was processed by MapInfor ProTM, Version 12.5.5 (Pitney Bowes Software Inc., NY, USA).

washed with sea water to remove the sediment and epiphytes that are commonly attached to the leaves. Samples were placed separately in polyethylene bags and transported to the laboratory under constant cooling in an icebox. Coordinates of sites and description of the habitats are presented in Table 1. In the laboratory, samples were desiccated in silica gel and shipped to the Institute of Botany, Leibniz University Hannover, Germany, for further analysis.

2.2 *DNA extraction and SSRs genotyping*

Young leaves of each individual plant were ground in a bead mill (22 Hz, 2 min) (Retsch, Technology GmbH, Haan, Germany), and 100 mg of the finely powdered plant material was used for DNA extraction. DNA was extracted by the Plant Nucleospin II Kit (Macherey & Nagel, Düren, Germany), following manufacturer's instruction with a few modifications according to Lucas et al. (2012). DNA quality was checked on agarose gels stained with ethidium bromide. DNA concentration was quantified using a microplate reader with micro-volume plates (Synergy Mx Multi-Mode, BioTek, Germany). Sixty individuals collected from six populations along the coast of the Khanh Hoa Province were used for the analysis. Details of sample size, locations and coordinates are shown in Table 1. Among 10 primer pairs suggested by Nakajima et al. (2012), we used five primer pairs resulting in highly polymorphic bands (Eaco_009, Eaco_050, Eaco_051, Eaco_054, and Eaco_055) for PCR. Thirty ng of template DNA was used in each 15 μL PCR including 1x Williams buffer (10 mmol/L Tris/HCl pH 8.3, 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.001% gelatin), 0.2 mmol/L dNTPs, 1 U Dream*Taq* DNA polymerase (Thermo Fisher Scientific, Dreieich, Germany), and 1 pmol

Table 1. Genetic diversity of *Enhalus acoroides* collected in six different seagrass beds in both the northern and the southern part of Khanh Hoa Province, Vietnam

Locations	Abbreviation	Coordinates	Substratum	Species	Genetic diversity			
					H_O	H_E	A	F_{IS}
Northern part								
Tuan Le	TL	12°45′N, 109°21′E	muddy sand	Ea*	0.38±0.11	0.63±0.06	8.6±1.0	0.43
My Giang	MG	12°29′N, 109°17′E	dead coral, muddy sand	Cs, Cr, Ea*, Th, Ho	0.50±0.11	0.53±0.09	9.4±0.6	0.01
Song Lo	SL	12°09′N, 109°12′E	dead coral, gravel, sand	Ea*, Th, Hu, Cr	0.52±0.08	0.62±0.06	8.8±0.6	0.16
Southern part								
Thuy Trieu	TT	12°02′N, 109°11′E	mud	Ea*, Th, Hu, Ho	0.50±0.13	0.64±0.07	9.6±0.4	0.26
Cam Phuc Nam	CPN	11°56′N, 109°11′E	mud	Ea*, Th	0.33±0.10	0.52±0.13	8.8±1.0	0.22
Cam Thinh	CT	11°53′N; 109°09′E	sandy mud	Ea*, Th	0.72±0.06	0.69±0.04	9.8±0.2	0.45

Note: Cr represents *Cymodocea rotundata*, Cs *Cymodocea serrulata*, Ea *Enhalus acoroides*, Ho *Halophila ovalis*, Hu *Halodule uninervis*, Th *Thalassia hemprichii*, * Dominant species, H_O observed heterozygosity, H_E expected heterozygosity, A allele richness, and F_{IS} inbreeding coefficient. Data was calculated by GenAlEx Version 6.503 (Peakall and Smouse, 2012).

primer each. The PCR was performed in a PTC 200 thermocycler (Biozym-Diagnostik GmbH, Hessisch Oldendorf, Germany) under the following conditions: initial denaturation for 5 min at 94°C followed by 25 cycles of denaturation for 30 s at 94°C, primer annealing for 30 s at 58°C and extension for 35 s at 72°C, and terminated by a final hold at 10°C for all loci (Nakajima et al., 2012). 200 μL of dye (98% formamide, 10 mmol/L EDTA, 0.05% pararosaniline) was added to each sample. Reactions were heated up to 72°C for 6 min before loading onto 6% microsatellite gels (Sequagel XR, National Diagnostics, Hull, England). For running a microsatellite gel on the 4300 DNA Analyzer (LI-COR, Biosciences, Germany) manufacturer's instruction was followed. Base pair lengths obtained from the visual analysis was resolved with previously published allele lengths (Nakajima et al., 2012).

2.3 *Statistical analysis*

Micro-Checker software, Version 2.2.3 (Van Oosterhout et al., 2004) was used to check for microsatellite scoring errors and null alleles. GenClone software, Version 2.0 (Arnaud-Haond and Belkhir, 2007) was used to test the number of multilocus genotypes (MLGs) for each population. Clonal diversity (R) was estimated following the method of Dorken and Eckert (2001): $R = (G–1)/(n–1)$, where G represents the number of genotypes and n is the number of ramets.

Genetic diversity was determined for each site using the indices described by Williams and Orth (1998). These indices include: expected heterozygosity (H_E) under Hardy-Weinberg equilibrium = (Σ expected frequency of heterozygotes at each locus)/(total number of loci); observed heterozygosity (H_O) = (Σ frequency of heterozygotes at each locus)/(number of individuals), the inbreeding coefficient (F_{IS}) based on Weir and Cockerham's (1984) method and allele richness (A) = (Σ number of alleles at each locus)/(total number of loci) were assessed by GenAlEx Version 6.503 (Peakall and Smouse, 2006, 2012). Deviation from Hardy-Weinberg proportion was tested using a Markov-chain algorithm developed by Guo and Thompson (1992) and implemented in the Arlequin 3.5 software (Excoffier and Lischer, 2010). Linkage disequilibrium among all pairs of loci for each population and for all populations were also tested by Arlequin 3.5 (Excoffier and Lischer, 2010).

For the population structure, Wright's F-statistics (F_{ST}) was calculated. F_{ST} measures the degree of inbreeding in the subpopulation relative to the total population, and is frequently used to assess population differentiation. The software FSTAT Version 2.9.3.1 (Goudet, 1995, 2001) was also used for calculation. Population genetic structure was inferred from microsatellite data using structure Version 2.3.4 (Pritchard et al., 2000). Determination of the number of possible clusters was assessed by both methods of Rosenberg et al. (2001) and Evanno et al. (2005). Cluster analysis was carried out by the unweighted pair group (UPGMA) method in TFPGA software, Version 1.3 (Miller, 1997). Significant differences among groups (F_{ST}) and among populations within groups (F_{SC}) were tested by AMOVA (analysis of molecular variance). This analysis was carried out by Arlequin 3.5 (Excoffier and Lischer, 2010). Pairwise distances were calculated from a number of different alleles data using the distance method described by Weir and Cockerham (1984) in Arlequin 3.5 (Excoffier and Lischer, 2010). Geographic distances (km) among populations were determined from the National Oceanic and Atmospheric Administration (NOAA) digital map. The genetic-geographic distance matrix was statistically tested for correlation using the Mantel test (Mantel, 1967). This test was carried out by Microsoft® Excel 2017.3/XLSTAT©-Pro (Addinsoft, Inc., Brooklyn, NY, USA).

3 Results

3.1 *Scoring of genotypes and determination of clonal and genetic diversity*

Initially, allele scoring errors were tested. The results of Micro-Checker showed that neither null alleles nor scoring errors are present in the dataset. Among these shoots, 100% MLGs were identified as genets that were raised from different sexual events based on P_{sex} values calculated at each site. Shoots belonging to the same MLG were not observed within identical populations and among populations (data not shown). The clonal diversity (R) was 1.0 for all populations. The mean number of alleles per locus was 4.03. The highest number of the allele was found at the locus Eaco_055 counting 11 alleles. The second highest numbers of alleles were found at loci Eaco_054 and Eaco_051 with 9 and 10 alleles, respectively. The lowest numbers of alleles were found at loci Eaco_050 and Eaco_009 with 6 and 7 alleles, respectively. Both genetic diversity indices including allele richness (A) and

expected heterozygosity (H_E) were higher in the southern part than in the northern part. Among the seagrass beds in the northern part, the allele richness was highest at MG whereas the expected heterozygosity was highest at TL. For the population in the southern part, the highest values of both allele richness and expected heterozygosity were recorded at CT. Among the locations, the mean allele number over all loci (4.4) was highest at CT while CPN showed the lowest value (3.2). The mean expected and observed heterozygosities (H_E and H_O) over loci and populations were 0.60 and 0.49, respectively. Within populations, the highest H_E was recorded at CT, whereas this value was lowest at CPN (Table 1).

3.2 *Population structure*

The related low genetic difference was detected among populations with an overall F_{ST} value of 0.25. The pairwise genetic differentiation depicted that the highest genetic differentiation was found between MG and SL (F_{ST} = 0.47, p-value<0.01) whereas these values were lowest among populations growing in lagoon including the TT, CPN and CT sites (pairwise F_{ST}<0.06, p-value>0.1) (Table 2). The results of structure analysis indicated that six populations formed two clusters, one including the open sea populations (TL, MG and SL: open sea cluster 1) and one including the lagoon populations (TT, CPN and CT: lagoon cluster 2) (Fig. 2, Panel A) by both methods. The largest variation of value L (K) was observed at K=2 according to the method of Rosenberg et al. (2001) and the highest ΔK was also found at K=2 according to the method of Evanno et al. (2005) (data not shown). The cluster analysis also showed that six populations separated into two main clades including open sea (TL, MG and SL) and lagoon (TT, CPN and CT) (Fig. 2, Panel B). The results of AMOVA showed 9.5% (p-value<0.01) of variation between the two clusters. The percentages of variation among populations within populations were 15.6 (p-value<0.01) and 74.2% (p-value>0.1), respectively (Table 3). The isolation-by-distance parameter resulting from pairwise plotting of F_{ST} values against the geographic distance (km) was generated using the Mantel test for all regions. The Mantel test showed no significant isolation by distance at a significance level of 95%. The mantel r statistic of 0.41 indicates that there is a relatively low positive correlation between genetic and geographic distance. However, these results are not statistically significant at α = 0.05 (Fig. 3).

Table 2. Matrix of genetic distances (F_{ST}) among populations of *Enhalus acoroides* calculated by using Arlequin 3.5 (Excoffier and Lischer, 2010)

	TL	MG	SL	CT	CPN	TT
TL	0.00					
MG	0.24*	0.00				
SL	0.17*	0.47*	0.00			
CT	0.13*	0.38*	0.14*	0.00		
CPN	0.23*	0.38*	0.26*	0.05	0.00	
TT	0.14*	0.39*	0.14*	0.04	0.05	0.00

Note: * Significant different at α = 0.05, p-value<0.01. See legend in Fig. 1 for the abbreviation of the locations.

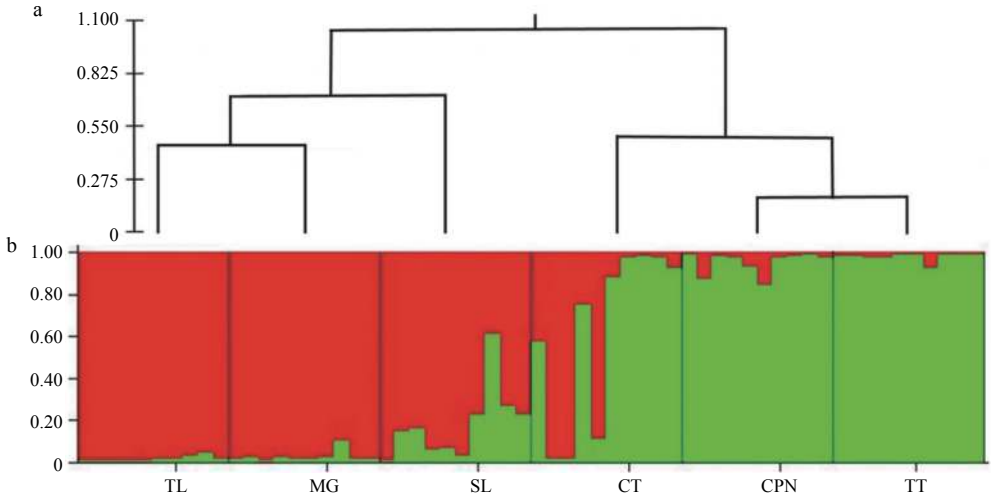

Fig. 2. The result shows the cluster analysis by the UPGMA method in the TFPGA software, Version 1.3 (Miller, 1997) (Panel A) and the analysis of two groups with burn-in periods of 10 000 iterations and 1 000 000 repetitions. The histogram shows the structure analysis at the model with K=2 (highest ΔK). Each smallest vertical bar represents one individual. The data was processed by Structure software, Version 2.3.4 (Pritchard et al., 2000) (Panel B). See legend in Fig. 1 for the abbreviations of the locations.

Table 3. AMOVA (Analysis of MOlecular VAriation; Excoffier et al., 1992) results for microsatellite variation at six collection sites of *Enhalus acoroides*

Source of variation	Degrees of freedom	Sum of squares	Variance of components	Percentage of variation	Probability
Among groups	1	11.7	0.12	9.5	<0.01
Among populations within groups	4	18.8	0.19	15.6	<0.01
Within populations	114	103.9	0.91	74.8	>0.1

Note: Group 1 consists of three populations including TL, MG and SL (open sea) in the northern part, and Group 2 consists of three populations including TT, CPN and CT (lagoon) in the southern part of the province. The data was processed by Arlequin 3.5 (Excoffier and Lischer, 2010).

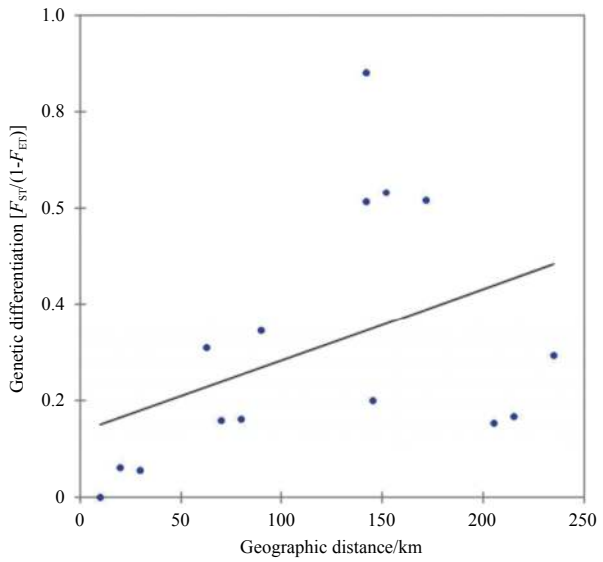

Fig. 3. Isolation-by-distance results from pairwise $F_{ST}/(1-F_{ST})$ values (Slatkin, 1995) plotted against geographic distance (km) using the Mantel test (Mantel, 1967). R^2=0.17, p-value=0.12. The data analysis was conducted in Arlequin 3.5 (Excoffier and Lischer, 2010).

4 Discussion

The genetic diversity of populations in the northern part of the Khanh Hoa Province is higher compared to the southern part. Three populations in the northern part occur on various substrata such as muddy sand at TL or dead coral reef/coarse gravel in MG and SL in the open sea. Sinclair et al. (2014) indicated that the levels of genetic diversity in meadows occurring in the open waters were higher compared to the inshore sites that face strong prevailing winds at the time of seed dispersal, or that have little water movement as was shown for other species investigated, namely *Posidonia australis* J D Hooker. For the mangrove species *Avicennia schaueriana* Stapf & Leechman ex Moldenke, the genetic diversity is higher in populations near the riverside than in the inner part of the salt marsh (Lira-Medeiros et al., 2015). Among the three populations in the northern part of the Province, MG showed lower genetic diversity than the two remaining populations. It is well demonstrated that heavy industrial activity, such as the shipyard industry, was the main cause for degradation of the seagrass beds (Pham et al., 2006). The total daily deposition measured with sediment traps at MG was very high [266.5 g/(m²·d)], and in fact much higher than in other seagrass beds in Southeast Asia (Gacia et al., 2003). Our recent study on heavy metal accumulation indicated that the use of Cu slag as an abrasive material for removing rust from the surface of the ships at the shipyard company nearby was the source of the Cu contamination in the seagrass bed at MG (Nguyen et al., 2017b). Another study on the genetic diversity of *Zostera marina* L. also indicated that an intertidal population from a highly disturbed habitat showed much lower genetic diversity than an intertidal population from an undisturbed site (Alberte et al., 1994). Among the three populations in the southern part of the Khanh Hoa Province, TT showed lower genetic diversity than the two remaining populations. At the TT location, higher environmental stresses were observed than at other locations. High turbidity and a high fluctuation of salinity are the main characteristics of the environment in the lagoon. In a recent study, it was shown that the Pb contamination and phytochelatin content in the root tissue of *Enhalus acoroides* collected at T were much higher than in roots collected at other locations (Nguyen et al., 2017a). For the terrestrial plant, *Sedum alfredii* Hance, a significant reduction of genetic diversity was detected when collected in extremely high concentrations of Zn, Cd and Pb (Deng et al., 2007). Hence, for the genetic diversity of *Enhalus acoroides*, the result showed lower genetic diversity in disturbed seagrass beds like MG and TT than in other seagrass beds along the coast of Khanh Hoa Province.

In this study, the mean fixing index value F_{ST} = 0.25 indicated a relatively high genetic differentiation among populations. Structure analysis showed two clusters with three populations in the open sea and three populations in the lagoon. It was supported by the AMOVA results with significant differences in the percentage of variation between the two clusters. The low positive correlation between genetic and geographic distance that was also statistically not significant indicated that genetic differentiation was not affected by the geographic distance.

The specific reproductive cycle may explain the wide dispersal of *Enhalus acoroides*. For pollination, the entire *Enhalus acoroides* male flowers break off, and pollen grains are discharged onto the surface of the water and settle on female plants (Horgarth, 2015). Floating male flowerlets of this species carrying pollen disperse at the water surface with a mean velocity of around 13 km per day (Lacap et al., 2002). The seeds which are dispersed by both biotic and abiotic factors are produced underwater (Papenbrock, 2012).

By structure analysis of *Enhalus acoroides*, Nakajima et al. (2014) recently showed that significant genetic differentiation among most populations of *Enhalus acoroides* in the Philippines, China and Japan exists; however, Japan and northeast Philippines populations were genetically similar, despite being separated by ~1 100 km (Nakajima et al., 2014). For *Cymodocea rotundata* Ascherson & Schweinfurth it was suggested that the area of the Philippine populations are hotspots for tropical seagrass and perhaps the origin of the populations in the Ryukyu and Hainan Island (Arriesgado et al., 2016). For *Thalassia hemprichii* (Ehrenberg) Ascherson, another member of the Hydrocharitaceae, molecular analysis revealed a strong genetic structuring among populations in eastern and southwestern Indonesia and the Indian Ocean; the genetic diversity was generally higher in eastern Indonesia and decreased in southwest Indonesia and the Indian Ocean (Hernawan et al., 2017). The structure discovered in our study showed two clusters that occurred in two different habitats: muddy substratum in the lagoon and dead coral/coarse gravel/sand in the open sea. Another study on *Cymodocea serrulata* (R. Brown) Ascherson & Magnus indicated that there are several factors acting as barriers, such as water temperature and reduced habitat area (Arriesgado et al., 2015). *Zostera marina* populations within the coastal lagoons (distinguished to island populations) possessed a signature of genetic admixture and strong gene flow among populations (Kim et al., 2017). However, the high level of genetic differentiation observed in the study of Sherman et al. (2016) suggested limited gene flow between the north and south meadows of *Zostera muelleri* Irmisch ex Asch. within the small scale (9–16 km between populations) in Lake Macquarie, Australia. The different salinities may also act as an environmental barrier to genetic differentiation of *Halophila ovalis*, in this case in India (Nguyen et al., 2014). For this purpose, more sampling sites in a larger area, more samples per site and deeper seagrass genetics are needed for a further step into the population genetics of the seagrass *Enhalus acoroides* from north to south of Vietnam.

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