

## Article

# Strong Genetic Structure and Limited Gene Flow among Populations of the Tropical Seagrass *Thalassia hemprichii* in the Philippines

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**Abstract:** Seagrasses are marine angiosperms, and seagrass beds maintain the species diversity of tropical and subtropical coastal ecosystems. For proper understanding, management and conservation of coastal ecosystems, it is essential to understand seagrass population dynamics. Population genetic studies can cover large geographic scales and contribute to a comprehensive understanding of reproductive dynamics and potential dispersal among locations. The clonal and genetic diversity and genetic connectivity of *Thalassia hemprichii* in the Philippines were estimated by a population genetics approach. The geographic scale of this study has a direct distance of approximately 1600 km. Although high clonal diversity was found in some sites ( $R = 0.07\text{--}1.00$ ), both sexual and asexual reproduction generally maintains separate populations. Genetic diversity is not definitely correlated with latitude, and genetic differentiation is significant in all pairs of sites ( $F_{ST} = 0.026\text{--}0.744$ ). Complex genetic structure was found in some regions, even at a fine geographic scale. The migration of fruits and seedlings was elucidated as an infrequent and stochastic event. These results suggest the necessity for the conservation of this species due to a deficiency in migrants from external regions.

**Keywords:** clonality; genetic diversity; geographic isolation; microsatellite; population genetics; reproduction

## 1. Introduction

Recently, rapid anthropogenic disturbances of coastal ecosystems have been occurring at global and local scales [1,2]. Such disturbances could cause the extinction of coastal organisms and affect many ecosystem services for humans, such as maintenance of tourism, fisheries, erosion control, water purification and carbon sequestration [3,4]. Seagrasses are coastal marine angiosperms, and the species richness of seagrasses in Southeast Asia is the highest in the world [5]. Estimating the geographic population structure and connectivity based on biogeographic research is essential for the proper understanding, management and conservation of coastal ecosystems. Estimates of the genetic composition and diversity

at each location, as well as the genetic structure and connectivity among populations, are crucial for understanding the ecological characteristics of coastal organisms, including seagrasses.

Population genetic studies can encompass large geographic scales and contribute to a comprehensive understanding of the genetic structure and connectivity of seagrasses. Genetic studies have been conducted in some seagrass species, and significant genetic differentiation in seagrasses has been detected among regions based on large geographic analyses at distances of greater than 1000 km (e.g., *Cymodocea nodosa* [6], *Posidonia oceanica* [7], *Thalassia testudinum* [8], *Zostera marina* [9–11]). These analyses have suggested potential long-distance dispersal among regions. Long-distance dispersal is stochastic but important for maintaining connectivity among populations and intraspecific homogeneity. Nevertheless, population genetic studies of seagrasses have seldom been conducted in the Indo-West Pacific region, despite the high species richness in these regions. Recently, the isolation of microsatellites and population genetic studies have been conducted for seagrass species in the region (*Enhalus acoroides* [12–14], *Cymodocea serrulata* [15,16], *Cymodocea rotundata* [14,17,18] and *Syringodium isoetifolium* [19,20]). Overall, those population genetic studies of seagrasses showed high genetic differentiation among populations.

The genus *Thalassia* is composed of two species, *Thalassia hemprichii* and *T. testudinum*. *Thalassia hemprichii*, known as turtle grass, is found in the Indo-West Pacific region in the tropical and subtropical zones and is one of the most widely distributed and dominant seagrass species in the Northwest Pacific [5]. *Thalassia hemprichii* is a representative species in Blue Carbon ecosystems for carbon storage in tropical and subtropical regions of the Indo-West Pacific [21,22]. This genus is distributed over vast shallow coastal areas, and can spread by extending its underground rhizomes and forming dense meadows. Although flowering events of *T. hemprichii* occur at a local scale, meadows of this species are primarily maintained by vegetative propagation [23,24]. van Dijk and van Tussenbroek [25] reported a high rate of clonal expansion by *T. testudinum* in the Caribbean Sea, with the longest genet estimated to reach up to 230 m. Nevertheless, there is no evidence of inbreeding via sexual reproduction due to the high extent of kinship within local populations of *T. testudinum* [26]. *Thalassia hemprichii* fruits could theoretically travel 73.5 km over a maximum of 8 days [27]. Successful germination of fruits floating for 19 days was reported in *T. hemprichii* [28]. Potential long-distance dispersal of fruits in *T. testudinum* ranged from approximately 190 to 720 km over 10 days, and this long dispersal distance appears to maintain the gene flow among sites (approximately 350 km) [8]. The dispersal period is shorter than *E. acoroides* [27] and longer than *Halophila ovalis* [28]. However, the dispersal cannot be observed and verified in actual seagrass meadows.

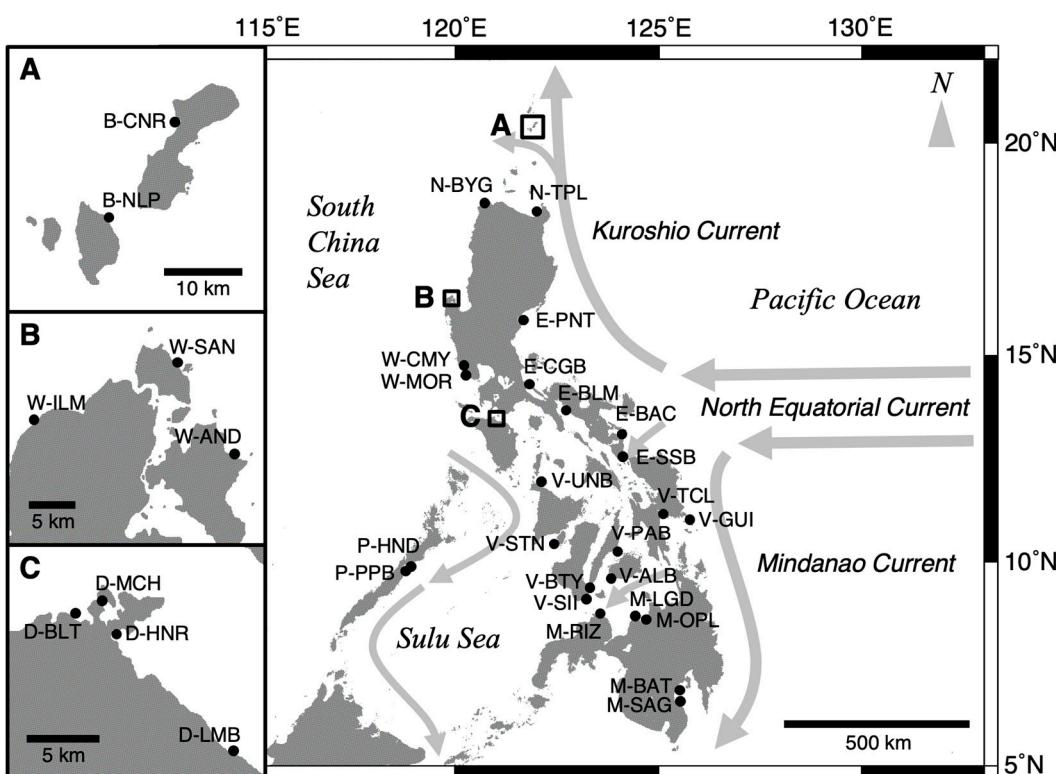
In this study, microsatellite markers were used to investigate the clonal diversity, genetic diversity, genetic structure and connectivity of populations of *T. hemprichii* in the Philippines to determine the reproductive dynamics and potential dispersal mechanisms among sites. Long-distance dispersal of fruits by ocean currents may contribute to maintain the genetic connectivity among sites in *T. hemprichii*. However, if long-distance dispersal is rare and stochastic, genetic differentiation and structure will be confirmed among sites. In this study, we verify whether *T. hemprichii* maintains connectivity by gene flow owing to frequent dispersal or is genetically separated among sites due to rare and stochastic dispersal in that region.

## 2. Materials and Methods

### 2.1. Study Sites and Sample Collection

Seagrass sampling sites were located in the Philippines (33 sites; Figure 1, Table 1). Initial letters in the population codes indicate the region: B, Batanes; N, North Luzon; W, West Luzon; D, Mindoro; E, East Luzon; P, Palawan; V, Visayas; M, Mindanao (see Table 1). The maximum distance between sites, from Chanarian (B-CNR) to San Agustin (M-SAG), was approximately 1600 km. Major surface ocean currents are also shown in Figure 1, following the map by Ravago-Gotanco and Juinio-Meñez [29]. A strong westward

current, the North Equatorial Current, flows from the Pacific Ocean to the eastern coast of the Philippines, through the study region. The current bifurcates into two currents, and the northward-flowing branch, the Kuroshio Current, is a particularly strong current. The southward current, the Mindanao Current, flows toward the southern Philippines through the eastern coastal area. Other currents flow into the inland sea within the Philippine Islands, with southward currents flowing from the South China Sea to the Sulu Sea. We collected samples from approximately  $200\text{ m} \times 300\text{ m}$  sites in the intertidal or subtidal zones, which were less than approximately 3 m in depth. We maintained a distance of more than 10 m among shoot samples collected within a site to unify the sampling strategy with other studies in this region [13,16,18,20]. A young leaf from each shoot was collected, dried with silica gel and preserved at room temperature until use.



**Figure 1.** Map of the 33 collection sites in the Philippines. A–C are enlarged views at Batanes, West Luzon and Mindoro. The map was produced using the Generic Mapping Tools (GMT) ver. 4.5.5 [30]. Major surface currents are drawn following the map by Ravago-Gotanco and Juinio-Meñez [29].

**Table 1.** Population genetic parameters for each site sampled.

No.	Code	Site	N	$N_{MLG}$	G	R	$N_A$	$A_R$	$P_A$	$H_O$	$H_E$	$F_{IS}$	Bottleneck (IAM/TPM)
1	B-CNR	Chanarian	44	36	36	0.81	2.56	2.25	0	0.207	0.225	0.217	0.406/0.656
2	B-NLP	Nalipang	43	41	41	0.95	3.78	3.19	0	0.425	0.430	0.007	0.064/0.150
3	N-TPL	Tapel	42	27	31	0.73	2.89	2.39	0	0.176	0.175	-0.012	0.922/0.969
4	N-BYG	Bayog	45	17	17	0.36	2.89	2.89	0	0.261	0.271	0.013	0.500/0.922
5	W-AND	Anda Island	40	39	39	0.97	4.89	4.16	0	0.382	0.399	-0.002	0.180/0.410
6	W-SAN	Santiago Island	42	40	40	0.95	4.78	4.00	0	0.328	0.363	0.041	0.410/0.633
7	W-ILM	Ilog-Marino	43	42	42	0.98	4.67	3.94	0	0.368	0.388	0.047	0.545/0.875
8	W-CMY	Camayan Beach	44	39	39	0.88	4.33	3.59	3	0.496	0.458	-0.082	0.180/0.850
9	W-MOR	Morong	44	44	44	1.00	3.22	2.67	0	0.429	0.394	-0.091	0.150/0.455
10	D-BLT	Baletero	39	37	38	0.97	3.33	3.04	0	0.313	0.330	0.135	0.344/0.656
11	D-MCH	Manila Channel	37	36	37	1.00	3.33	2.87	0	0.288	0.360	0.226	0.191/0.473
12	D-HNR	Hondura Bay	23	21	22	0.95	2.44	2.36	0	0.232	0.287	0.127	0.289/0.531

**Table 1.** Cont.

No.	Code	Site	N	$N_{MLG}$	G	R	$N_A$	$A_R$	$P_A$	$H_O$	$H_E$	$F_{IS}$	Bottleneck (IAM/TPM)
13	D-LMB	Lumang Bayan	30	26	26	0.86	2.89	2.86	0	0.372	0.360	0.078	0.180/0.367
14	E-PNT	Puntian	44	36	36	0.81	2.33	2.20	0	0.306	0.323	0.142	0.148/0.188
15	E-CGB	Cagbalete Island	44	41	41	0.93	3.56	2.90	0	0.333	0.350	0.016	0.191/0.527
16	E-BAC	Bacon	23	19	20	0.86	2.22	2.15	0	0.261	0.265	0.042	0.078/0.500
17	E-SSB	San Sebastian	24	24	24	1.00	3.89	3.59	0	0.361	0.388	0.242	0.674/0.752
18	E-BLM	Bahao Libmanan	31	30	30	0.97	2.89	2.62	0	0.356	0.329	-0.070	<b>0.039</b> /0.219
19	P-HND	Honda Bay	41	38	38	0.93	5.78	4.49	9	0.348	0.431	0.159	0.230/0.578
20	P-PPB	Puerto Princesa Bay	44	4	4	0.07	1.78	—	0	—	—	—	—
21	V-UNB	Union Beach	33	32	32	0.97	4.22	3.69	0	0.347	0.422	0.187	0.156/0.473
22	V-STN	Sitio Nabinbinan	34	34	34	1.00	4.33	3.81	0	0.395	0.433	0.078	0.150/0.326
23	V-SII	Sit Bay	42	37	37	0.88	4.00	3.51	0	0.312	0.361	0.102	0.674/0.918
24	V-BTY	Bantayan	43	43	43	1.00	3.67	3.12	1	0.292	0.341	0.164	0.629/0.963
25	V-ALB	Alona Beach	44	41	41	0.93	4.33	3.44	0	0.374	0.430	0.182	0.191/0.371
26	V-PAB	Palm Beach	37	37	37	1.00	4.33	3.73	1	0.417	0.484	0.124	<b>0.014</b> /0.326
27	V-TCL	Tacloban	38	13	22	0.57	1.67	1.62	0	0.172	0.145	-0.127	0.438/0.563
28	V-GUI	Guiuan	36	36	36	1.00	2.78	2.62	1	0.386	0.397	0.022	<b>0.008</b> / <b>0.027</b>
29	M-OPL	Opol	31	27	30	0.97	2.89	2.58	0	0.352	0.367	0.059	<b>0.027</b> /0.156
30	M-LGD	Laguindingan	41	31	31	0.75	4.56	3.90	2	0.466	0.468	-0.006	0.102/0.674
31	M-RIZ	Rizal	37	29	31	0.83	4.44	3.95	1	0.384	0.427	0.074	0.455/0.898
32	M-BAT	Bato	39	14	17	0.42	2.67	2.67	0	0.471	0.417	-0.080	<b>0.014</b> / <b>0.020</b>
33	M-SAG	San Agustin	39	22	22	0.55	3.56	3.41	1	0.359	0.368	0.059	0.527/0.680

Initial letter in the code indicates the region: B, Batanes; N, North Luzon; W, West Luzon; D, Mindoro; E, East Luzon; P, Palawan; V, Visayas; and M, Mindanao.  $N$  is the number of samples analysed.  $N_{MLG}$  is the number of multilocus genotypes.  $G$  is the number of multilocus genotypes, including identical genotypes resulting from sexual reproduction by chance, as estimated by  $P_{SEX}$  values for each site calculated using GenClone and an index of clonal diversity  $R = (G - 1)/(N - 1)$ .  $N_A$  is mean number of alleles for nine microsatellite loci.  $A_R$  is allelic richness, the mean allele number for nine loci using FSTAT.  $P_A$  is the number of private alleles for all loci.  $H_O$  and  $H_E$  are mean observed and expected heterozygosities for nine loci, respectively.  $F_{IS}$  is mean inbreeding coefficients for nine loci.  $N_A$ ,  $A_R$ ,  $P_A$ ,  $H_O$ ,  $H_E$  and  $F_{IS}$  were calculated using GenAIEx. Bottleneck IAM and TPM are probability values of the infinite allele mutation model and two-phase model in the analysis by BOTTLENECK, respectively (bold:  $p < 0.05$ ). The values of  $N_A$ ,  $A_R$ ,  $P_A$ ,  $H_O$ ,  $H_E$  and  $F_{IS}$  per locus for each site are shown in Table S1.

## 2.2. DNA Extraction and Microsatellite Analysis

Silica-gel-dried leaves from each shoot were ground using TissueLyser (Qiagen, Hilden, Germany). Genomic DNA was extracted using a modified cetyltrimethylammonium bromide method [31]. After extraction, genomic DNA was stored at  $-30\text{ }^{\circ}\text{C}$  until Polymerase chain reaction (PCR). We determined the microsatellite genotypes of all samples collected in those regions using nine polymorphic loci (Themp030, Themp041, Themp047, Themp048, Themp049, Themp066, Themp103, Themp107 and Themp193) isolated from *T. hemprichii* and characterised by Matsuki et al. [32] (Table S2). PCR was performed using a Multiplex PCR Kit (Qiagen) in a 6  $\mu\text{L}$  reaction containing 20–200 ng template genomic DNA, 2  $\times$  Multiplex PCR Master Mix and 0.2  $\mu\text{M}$  (final concentration) primer that is fluorescently labelled with FAM, VIC, NED or PET (Table S2). The PCR settings were 15 min at  $95\text{ }^{\circ}\text{C}$ , followed by 30 cycles of 30 s at  $94\text{ }^{\circ}\text{C}$ , 90 s at  $56\text{ }^{\circ}\text{C}$  and 60 s at  $72\text{ }^{\circ}\text{C}$ , with a final extension of 30 min at  $60\text{ }^{\circ}\text{C}$ . The fragment length of PCR products was analysed with an automated capillary DNA sequencer (ABI 3130xl Genetic Analyzer; Thermo Fisher Scientific, Waltham, MA, USA) and GeneMapper software ver. 4.1 (Thermo Fisher Scientific).

## 2.3. Genotypic Variation and Clonality

The number of multilocus genotypes ( $N_{MLG}$ ) among the nine loci was counted using GenAIEx ver. 6.503 [33] for each site. Whether two shoots belong to the same genet or to two different genets was determined based on the probability of identical multilocus genotypes being derived from distinct sexual reproduction events by chance ( $P_{SEX}$ ) and calculated using GenClone ver. 2.0 [34].  $P_{SEX}$  was calculated by considering the inbreeding coefficient ( $F_{IS}$ ) estimates in the dataset and was used to test for clonal identity and clonal propagation [34,35]. To remove duplicate clonemates from further analysis, identical multi-

locus genotypes at a given site were considered clonal replicates ( $P_{\text{SEX}} < 0.01$ ). However, we did not evaluate the probability with a low number of multilocus genotypes, due to the low statistical power under such conditions. The index of clonal diversity  $R$  proposed by Dorken and Eckert [36] was calculated with the formula  $R = (G - 1)/(N - 1)$  for each site, where  $G$  represents  $N_{\text{MLG}}$  accounting for  $P_{\text{SEX}}$  and  $N$  indicates the number of shoots successfully genotyped. To estimate the resolution level of the microsatellites, the combined probability of identity ( $P_{\text{ID}}$ ) was calculated for each site using GenAlEx.

#### 2.4. Genetic Diversity and Recent Bottleneck Effect

The mean number of alleles per locus ( $N_A$ ) and allelic richness ( $A_R$ ) across all the loci at each sampling site were estimated using GenAlEx and FSTAT ver. 2.9.3.2 [37], respectively.  $A_R$  is the mean number of alleles per locus and standardised to the site with the smallest number of genets. The values of observed and expected heterozygosity ( $H_O$  and  $H_E$ , respectively) and the number of private alleles ( $P_A$ ) at each site were also calculated with GenAlEx. To estimate the effects of latitude on genetic diversity, the significance of the correlation between genetic diversity ( $A_R$  and  $H_E$ ) and latitude was tested using regression analyses. In addition, we evaluated whether populations had experienced a recent bottleneck event using BOTTLENECK ver. 1.2.02 [38]. We performed Wilcoxon's signed-rank test for significant deviations from population mutation-drift equilibrium using 1000 replications under assumptions of both the infinite allele mutation model (IAM) and the two-phase model (TPM) (30% IAM and 70% stepwise mutation model).

#### 2.5. Genetic Differentiation, Structure and Connectivity among Sites

Genetic differentiation within and among populations was estimated using a hierarchical analysis of molecular variance (AMOVA) [39]. The genetic differentiation index (pairwise  $F_{\text{ST}}$ ) between sites was calculated using GenAlEx with 999 permutations to test the significance of each  $F_{\text{ST}}$  value. The Mantel test was performed using 999 random permutations in GenAlEx for the significance of the correlation between pairwise  $F_{\text{ST}}/(1 - F_{\text{ST}})$  values [40] and direct geographic distances between paired sites. Furthermore, we estimated the patterns of genetic differentiation derived from pairwise  $F_{\text{ST}}$  values among sites via principal coordinates analysis (PCoA) using GenAlEx. Population genetic structure was inferred from microsatellite data using STRUCTURE ver. 2.3.4 [41]. STRUCTURE analysis employs a Bayesian clustering algorithm to assign genotypes to clusters while minimising the Hardy–Weinberg equilibrium and linkage disequilibrium. Ten replicate runs without prior information were conducted for each  $K$  between 1 and 20 using the admixture model and assuming correlated allele frequencies [42]. Each run consisted of 1,000,000 Markov chain Monte Carlo replications after a burn-in period of 100,000 iterations. The optimal  $K$  was determined using the  $\Delta K$  method of Evanno et al. [43] as implemented in STRUCTURE HARVESTER [44]. Run data were merged using the CLUMPAK server [45]. The Q-matrix of each cluster was plotted onto a map in R ver. 4.1.2 [46] using the POPS script ver. 1.2 [47]. Furthermore, discriminant analysis of principal components (DAPC) was conducted in R using the package adegenet ver. 2.1.8 [48], to represent the distribution patterns for each multilocus genotype as plots. This clustering method does not assume a particular model. Therefore, it is free from assumptions regarding Hardy–Weinberg equilibrium and linkage disequilibrium. We allowed 60 principal components to be retained for discriminant analysis. To infer the extent of gene flow and direction, BayesAss ver. 3.0.3 [49] was used to calculate the migration rate between sites per generation over the last few generations. The following settings were used: number of iterations, 10,000,000; burn-in, 1,000,000; and sampling frequency, 1000.

### 3. Results

#### 3.1. Scoring of Genotypes and Common Genotypes between/among Sites

Of 1339 shoots sampled, 1261 (94.2%) were successfully genotyped at nine loci. Of these 1261 shoots, 1033 (81.9%) multilocus genotypes were detected at each site and 1058

(83.9%) were identified as genets that were raised from different sexual events based on  $P_{\text{SEX}}$  values calculated for each site. The combined  $P_{\text{ID}}$  of each site varied between  $9.3 \times 10^{-6}$  (P-HND) and  $1.5 \times 10^{-2}$  (N-TPL). The clonal diversity ranged from 0.07 to 1.00, with a mean of  $0.84 \pm 0.04$  SE (Table 1). For one site (P-PPB) with the lowest  $R$  value for clonal diversity,  $P_{\text{SEX}}$  correction was not applied due to the low resolution of this value with a low number of multilocus genotypes (see Materials and Methods). Five multilocus genotypes were shared among multiple sites: one between W-MOR and D-MCH (147.7 km), one between E-PNT and E-CGB (169.3 km), one between E-PNT and E-SSB (440.4 km) and two between E-CGB and E-BAC (275.6 km). Clonal replicates within each site were removed from further analysis, while replicates between sites were preserved for subsequent analysis.

### 3.2. Genetic Diversity and Bottleneck Event

Allelic richness was standardised based on the minimum number of  $G$ , except for site P-PPB. The mean number of alleles ( $N_A$ ) and allelic richness ( $A_R$ ) per site ranged from 1.67 (V-TCL) to 5.78 (P-HND) and from 1.62 (V-TCL) to 4.49 (P-HND), respectively. Nineteen private alleles ( $P_A$ ) were detected from eight sites, and nine of these nineteen alleles were detected in P-HND. The observed and expected heterozygosities ( $H_O$  and  $H_E$ ) ranged from 0.172 to 0.496 and from 0.145 to 0.484, respectively. Inbreeding coefficients,  $F_{IS}$ , varied between  $-0.127$  and  $0.242$  (Table 1). The values of  $N_A$ ,  $A_R$ ,  $P_A$ ,  $H_O$ ,  $H_E$  and  $F_{IS}$  per locus for each site are shown in Table S1. Correlation analysis showed that genetic diversity index values had no definite correlation with an increase in latitude ( $A_R$ :  $R^2 = 0.044$ ,  $p = 0.248$ ;  $H_E$ :  $R^2 = 0.151$ ,  $p = 0.028$ ). Some sites appear to have undergone a recent bottleneck, resulting in a significant excess of heterozygosity compared to that expected at mutation drift equilibrium (V-GUI and M-BAT) under both IAM and TPM assumptions ( $p < 0.05$ ; Table 1).

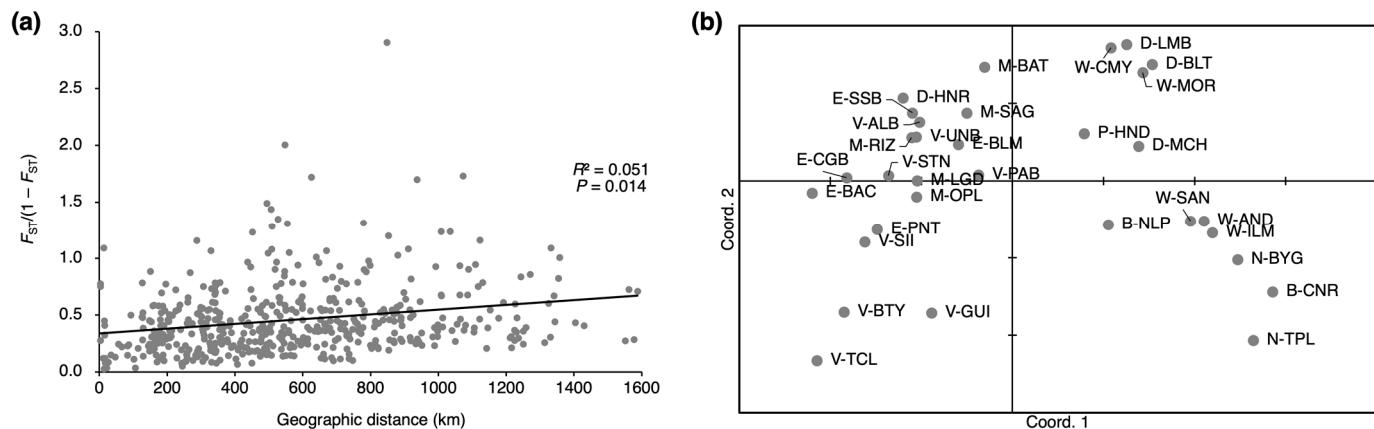
### 3.3. Genetic Differentiation, Structure and Connectivity among Sites

Hierarchical AMOVA detected considerable genetic differentiation among regions and among sites (Table 2). The proportion of genetic variance was significantly partitioned among sites ( $F_{ST} = 0.291$ ,  $p = 0.001$ ). Pairwise  $F_{ST}$  values ranged from 0.026 (between W-AND and W-SAN) to 0.744 (between N-TPL and V-TCL) (mean  $F_{ST} = 0.289 \pm 0.005$  SE), and all combinations showed significantly genetic differentiation (all  $P_s = 0.001$ ) (Table S3). These results indicate high levels of genetic differentiation in *T. hemprichii* among sites. Moreover, analysis of isolation-by-distance revealed a significant correlation between geographic distance and genetic differentiation (Mantel test:  $p = 0.014$ ) (Figure 2a). PCoA plots were not completely separated by the regions; unexpected complex distribution patterns were found in  $F_{ST}$ -based data (Figure 2b). The results of STRUCTURE analysis indicated the existence of a strong population genetic structure (Figure 3 and Figure S1). The  $\Delta K$  value was greatest at  $K = 2$  ( $\Delta K = 2782.92$ , mean  $\text{LnP}(D) = -20,796.93$ ). When  $K = 2$ , Batanes, North Luzon, West Luzon, Mindoro (excluding D-HNR) and Palawan were mainly assigned as the same cluster. Furthermore, V-SII, V-BTY, V-TCL and V-GUI in the Visayas formed a separate cluster at  $K = 3$  (Figure 3). In regions such as Mindoro and the Visayas, the main clusters at each site differed despite the geographic proximity due to the delimited borders of genetic clusters (Figure 3). DAPC also identified continuous genetic clusters, indicating genetic differentiation among sites (Figure 4). In this analysis, V-SII, V-BTY, V-TCL and V-GUI in the Visayas composed isolated clusters. V-GUI was particularly far removed from the clusters of the three sites. More specific clusters were detected visually by removing the clusters of these four populations. In that case, D-HNR was closer to the Visayas compared to the other three populations in Mindoro. Most sites showed a migration rate of less than 0.01 (Table S4). A relatively high migration rate (greater than 0.1) between sites was detected in eight pairs of sites, and the geographic distance ranged from 13.7 km (W-AND <- W-SAN) to 402.2 km (E-BAC <- E-PNT). The highest rate between sites was 0.197, and the distance was 169.3 km (E-CGB <- E-PNT).

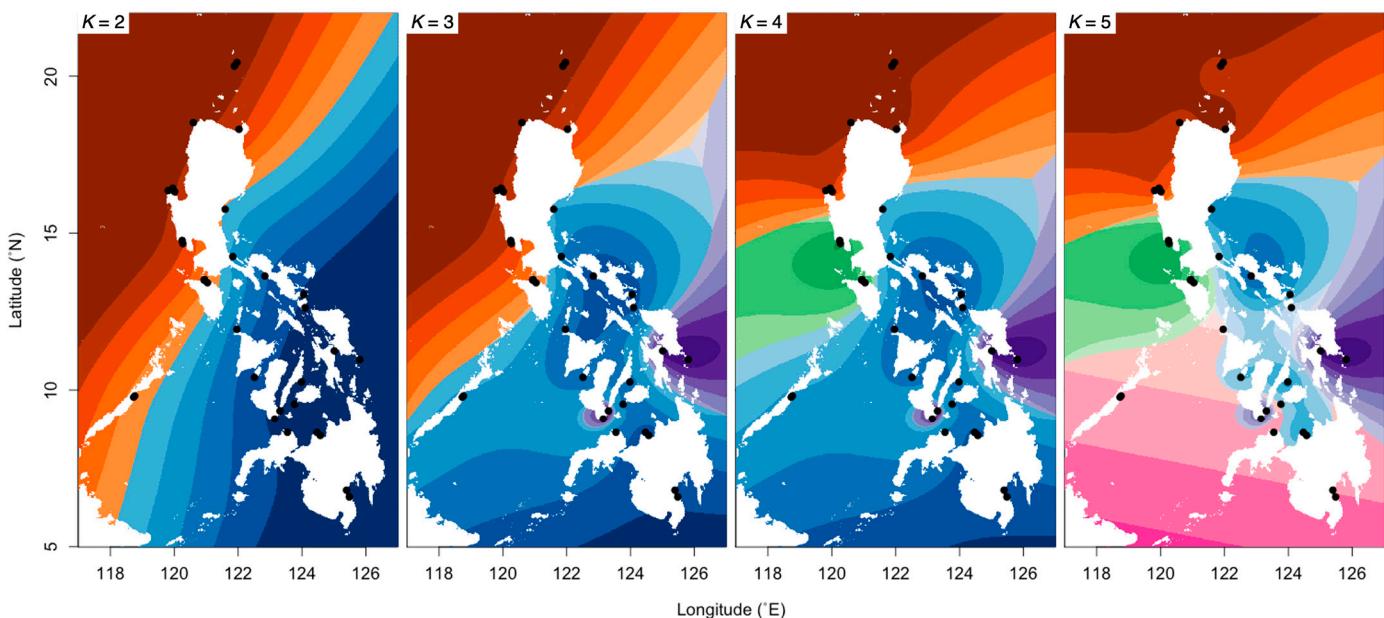
**Table 2.** Analysis of molecular variance (AMOVA) showing degrees of freedom (d.f.), sum of squares (SS), variance component (Variance), percentage of variance (%) and F-statistic among sites.

Source	d.f.	SS	Variance (%)	F-Statistic	p Value
Among sites	31	1463.426	0.692 (29%)	$F_{ST} = 0.291$	0.001
Within sites	2076	3499.762	1.686 (71%)		
Total	2107	4963.188	2.378 (100%)		

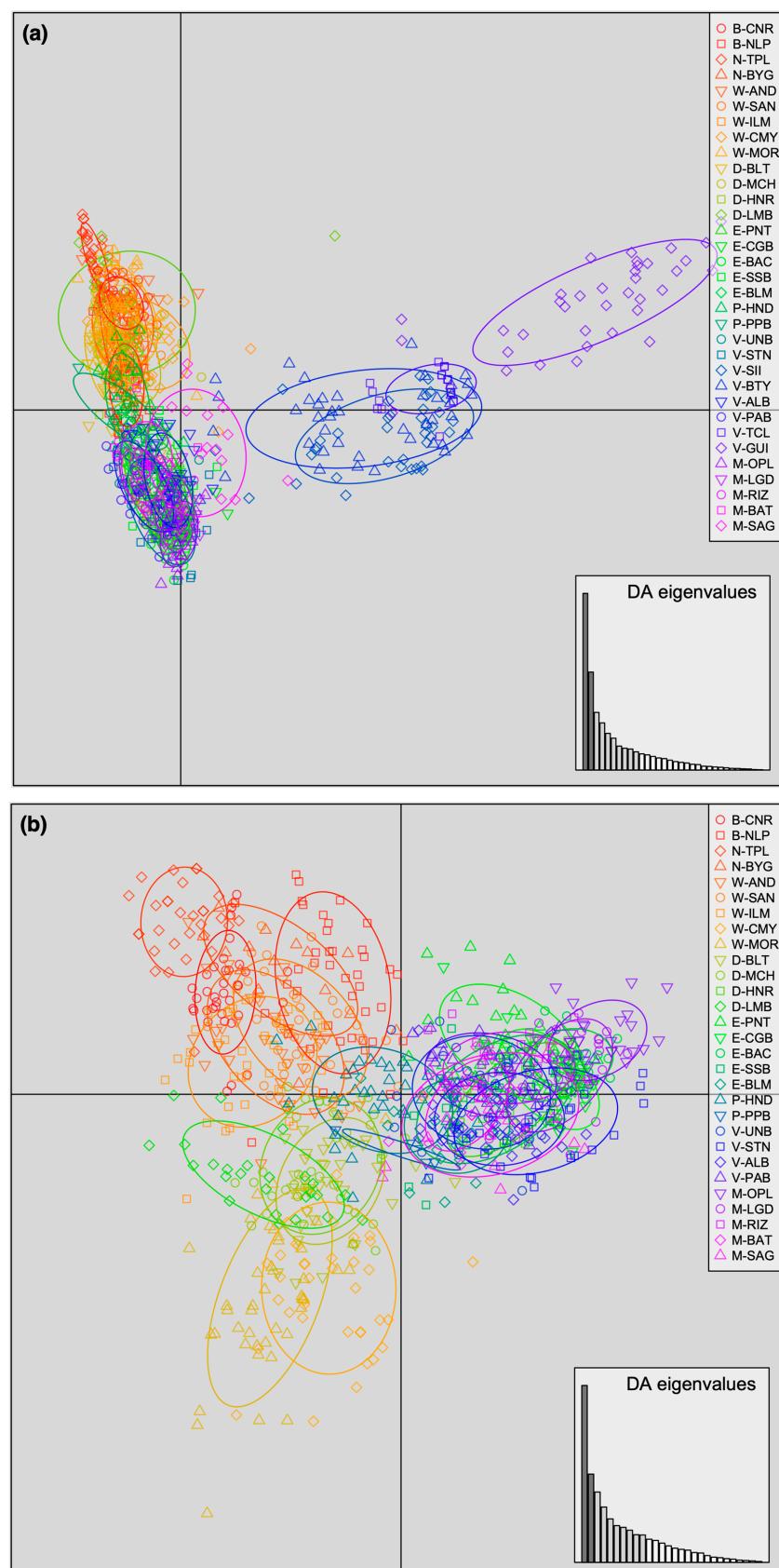
P-PPB was removed due to low sample size with a large number of clonal replicates ( $N_{MLG} = 4$ ,  $R = 0.07$ ).



**Figure 2. Figure 2.** (a) Isolation-by-distance for all sites except for P-PPB based on pairwise  $F_{ST}/(1 - F_{ST})$  values plotted against geographic distance (km) using the Mantel test. (b) Principal coordinates analysis (PCoA) from the covariance matrix based on pairwise  $F_{ST}$  values except for P-PPB. The first two axes explained 35.97% of the variation (the first axis explained 24.14%, the second axis, 11.83%). The values of pairwise  $F_{ST}$  are shown in Table S3.



**Figure 3.** Map showing the frequency of Q-matrices from Bayesian clustering analysis implemented in STRUCTURE using clustering without prior information under the admixture model and assuming correlated allele frequencies. K values of these panels are  $K = 2$  to  $K = 5$ .



**Figure 4.** Scatter plots of discriminant analysis of principal components (DAPC) results using the R package adegenet. **(a)** All populations. **(b)** Twenty-nine populations after removal of four populations (V-SII, V-BTY, V-TCL and V-GUI).

#### 4. Discussion

We concluded that populations of *T. hemprichii* have been maintained by both sexual and asexual reproduction, as the index of clonal diversity  $R$  ranged from 0.07 to 1.00 with a mean value of  $0.84 \pm 0.04$  SE. The index of clonal diversity  $R$  was never low, even in high-latitude sites, where clonal plants could have lower seed production and dominant asexual reproduction [36]. The value of clonal diversity was slightly lower than that for *E. acoroides* in the Philippines (mean  $R = 0.88$ ) [13], which was collected using the same procedure employed in this study. On the other hand, it is higher than values for *C. serrulata* (mean  $R = 0.62$ ) [16], *C. rotundata* (mean  $R = 0.66$ ) [18] and *S. isoetifolium* (mean  $R = 0.38$ ) [20]. Large genets in some populations with low clonal diversity (e.g., P-PPB) suggest that seagrass meadows formed as the result of ecological processes over a long time scale. Although meadows of *T. hemprichii* are primarily maintained by vegetative propagation [23,24], sexual reproduction also contributes to the persistence of populations, even in high-latitude areas. Along with sexual reproduction, asexual reproduction methods, such as clonal expansion of *T. hemprichii* through elongation of the horizontal rhizome within a meadow or drifting of shoots between sites, may contribute to population maintenance. Three and seven genotypes were detected in *E. acoroides* and *S. isoetifolium*, respectively, which were common among sites in this region [13,20]. *Cymodocea serrulata* also exhibited shared multilocus genotypes, with patterns found in the Ryukyu Archipelago [16]. In addition, five common multilocus genotypes were found among the sites in this study for *T. hemprichii*. There was no clonal replication between distant sites in an extremely large-scale geographic analysis of *T. hemprichii* (see Hernawan et al. [50] and the corresponding microsatellite genotypic data deposited in DRYAD). A monoclonal seagrass meadow of this species does not appear to be present in our focal area. Because the clonal growth of seagrasses is also influenced by environmental conditions such as nutrients, temperature, water salinity, light availability and other physical factors [25,51,52], variation might reflect differences among environments, even in high-latitude areas.

Genetic diversity did not clearly decrease in high-latitude areas, and the central region of this species' habitat distribution has been considered a hotspot of diversity for various organisms including seagrass species [5]. This result is similar to the result for *E. acoroides* in the Philippines [13]. This result implies that seagrass populations in the Philippines are close to the centre of the distribution area, and genetic diversity does not distinctly decrease with latitude in relation to the temperature. Meanwhile, genetic diversity appears to decrease in high-latitude areas and populations at the periphery of the distribution ranges of some tropical coastal organisms [53,54]. In the case of seagrass species, the Coral Triangle represents a hotspot. The Coral Triangle region is important from the viewpoint of species and genetic diversity. In *T. hemprichii*, high genetic diversity has been maintained in Indonesia, in the southern part of the Coral Triangle, although it decreases toward the southwest [50]. Further collection and analyses that are standardised among sites will provide detailed data on the clonal distribution and genetic diversity among sites in subtropical areas of the Northwest Pacific region. Spatial genetic information is needed to estimate the genetic diversity and allele distribution for local or fine-scale geographic analysis. In addition, the genetic diversity of seagrasses may play a role in the maintenance of species diversity in organisms that live in seagrass beds [55].

The genetic differentiation of *T. hemprichii* among sites is generally large, even between nearby sites. Pairwise  $F_{ST}$  values and migration rates indicated that gene flow is highly restricted and successful recruitment occurred primarily to the natal habitat. Previous studies have found evidence of lower genetic differentiation in the Northeast Pacific reef region for broadcast-spawning invertebrates [56–59]. In broadcast-spawning invertebrate species, mean larval duration ranged from several days to a few weeks and some of the larvae can survive for a few months [60]. Meanwhile, seagrass species usually maintain high genetic differentiation among sites, even at local or regional geographic scales [61,62]. The pattern of genetic structure appears to have been influenced by the North Equatorial Current (see Figure 1). Such complex genetic structures have been confirmed in other

seagrass species in the Philippines. For example, a complex and irregular genetic structure was confirmed in a regional study of *E. acoroides* in Visayas [63]; populations in central Visayas (V-SII and V-BTY) were assigned to the cluster found in eastern Visayas (V-TCL and V-GUI). Meanwhile, a complex genetic structure at the local scale may be caused by differences in environment or historical fluctuation of populations between sites. As an example of variation in environment, differences in sea bottom (soft bottom and hard bottom) may be influenced in populations of *T. hemprichii* [64]. However, the physiological mechanism of habitat selection has not yet been elucidated. On the other hand, historical fluctuation of populations may influence genetic structure. *T. hemprichii* populations in Lesser Sunda and the Indian Ocean exhibited irregular genetic structures unrelated to location [50], as did *T. testudinum* along the Mexican Coast [8]. This disagreement may be influenced by historical fluctuations in coastlines and basin boundaries since the Pleistocene. Sea levels during the Pleistocene were ~120 m lower than at present in our focal regions [65]. Repeated historical events may have caused complex genetic differentiation and structures. Adaptive reproductive strategies and biological characteristics may also lead to genetic background differences in some areas.

Although most of pairwise migration rates were less than 0.01, the higher migration rate (greater than 0.1) was confirmed in eight pairs of sites which widely ranged from 13.7 km to 402.2 km. Such long-distance dispersal of larvae and repeated stepping-stone dispersal events will contribute to the maintenance of genetic connectivity among populations at ecological time scales (a few generations). Due to ecological and historical migration, the long-distance dispersal of this species may be particularly stochastic, as it has a short maximum floated-fruits-dispersal period of eight days [27]. Similarly, the common multilocus genotypes among sites (147.7 to 440.4 km) may be caused by the rare and stochastic dispersal of floated seedlings, which can survive for over 12 weeks in *T. hemprichii* [28]. Seagrass species appear to have the potential for long-distance dispersal of clonal propagules at the regional scale [66]. The habitat range of *T. testudinum* has extended over a long time frame due to stochastic flow [8]. The range of *T. hemprichii* in the Northwest Pacific could have been extended by repeated fragmentation events and historical stepping-stone dispersal of clonal propagules and seeds or fruits from sexual reproduction, as suggested by continuous clusters observed with DAPC. Moreover, clarification of genetic connectivity at the local scale within this region is needed for appropriate population management [63]. Proper management depends upon a comprehensive understanding of the relationship between genetic connectivity and the reproductive and biological characteristics of seagrasses species. The specific floating processes of seagrass species need to be investigated further with a focus on hydrodynamic approaches for a better understanding of population connectivity. Successful dispersal rate would need to be analysed using an ocean circulation model due to the extraordinary low probabilities involved. In addition, not only ocean currents but also biotic vectors must be considered simultaneously, because some propagules, seeds or fruits of seagrasses can be transported by manatees, dugongs, turtles, ducks [61,62] or other birds [67,68]. Whether propagules, seeds or fruits of *T. hemprichii* in this region can be carried or swallowed by these vertebrates should be determined.

## 5. Conclusions

We conclude that *T. hemprichii* is genetically separated among sites due to its uncommon and stochastic dispersal in the Philippines. These results suggest the necessity of conservation due to a deficiency in constant migrants from external regions at the contemporary time scale and a difficulty in rapid recovery of the population with high genetic diversity after disturbances. The relationship between environmental factors and reproductive characteristics of seagrasses is also a question that warrants future study. Data from the field of genetics could be used to maximise the effects of conservation efforts for seagrass meadows by providing useful information for retaining the function of carbon

storage in Blue Carbon ecosystems [69], contributing to ecological monitoring of the health of seagrasses [1,61].

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/jmse11020356/s1>. Figure S1: Panels output from STRUCTURE analysis of 1058 genets at 33 sites after removing clonal duplicates; Table S1: Population genetic parameters per locus for each site; Table S2: Characteristics of nine polymorphic microsatellite loci used in this study: locus name, repeat motif, forward and reverse primer sequences, allele size range, combination of loci for multiplex PCR, fluorescent label and GenBank accession number; Table S3: Pairwise  $F_{ST}$  values (below diagonal) and geographic distance (km) (above diagonal) between sites; Table S4: Pairwise migration rates for 32 sites using BayesAss.

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