Genetic structure and connectivity of *Thalassia testudinum* in oceanic and continental environments of the Colombian Caribbean

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To my mother in heaven, my father, my siblings, my wife, and all who have always been there: thank you for your love and constant motivation, helping me achieve this goal despite the difficulties.


Foto: Santiago Estrada

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Abstract

Title: Genetic structure and connectivity of Thalassia testudinum in oceanic and continental environments of the Colombian Caribbean

Thalassia testudinum, a critical ecosystem engineer in the Caribbean, provides essential habitat and supports vital ecosystem services. However, likewise other seagrass species globally, its populations face increasing anthropogenic pressures, leading to significant declines in coverage and functionality. Understanding the historical and contemporary population dynamics of this species, which exhibits both sexual and clonal reproduction, is crucial for effective conservation and management strategies. This study investigated the genetic diversity and connectivity of *T. testudinum* populations across oceanic and continental environments in the Colombian Caribbean, integrating data across the species' range and considering potential anthropogenic impacts. Microsatellite analysis of 240 samples from eight Colombian populations, compared with a previously developed regional dataset encompassing 32 additional populations, revealed a complex interplay of geological, oceanographic, biological, and anthropogenic factors shaping population structure. While relatively high genetic diversity and gene flow were observed at national and regional scales, distinct patterns emerged between oceanic and continental environments. These findings highlight (i) the identifiable population structure of the species comprising five genetic clusters in Colombia (ii) the remarkable (re)colonisation capacity of *T. testudinum* facilitated by long-distance dispersal, clonality, and inbreeding avoidance; (iii) the role of stochastic processes in dispersal and the lack of strong isolation-by-distance at certain scales; and (iv) the vulnerability of populations to short-term anthropogenic disturbances. This study identifies five Independent Management Units and underscores the need for area-specific conservation strategies to safeguard the long-term resilience of *T. testudinum* and the invaluable ecosystems it supports.

Keywords: Conservation genetics, seagrasses, anthropogenic impacts, connectivity, oceanic islands, continental environments, Caribbean.

Resumen

Título: Estructura genética y conectividad de *Thalassia testudinum* en ambientes oceánicos y continentales del Caribe colombiano

Thalassia testudinum, una especie clave para la ingeniería del ecosistema de pastos marinos en el Caribe, proporciona un hábitat esencial y sustenta servicios ecosistémicos vitales. Sin embargo, al igual que otras especies de pastos marinos a nivel mundial, sus poblaciones se enfrentan a crecientes presiones antropogénicas, lo que ha conducido a disminuciones significativas en su cobertura y funcionalidad. Comprender la dinámica poblacional histórica y contemporánea de esta especie, que exhibe tanto reproducción sexual como clonal, es crucial su efectiva conservación. Este estudio investigó la diversidad genética y la conectividad de las poblaciones de *T. testudinum* en entornos oceánicos y continentales del Caribe colombiano, integrando datos a lo largo del rango de distribución de la especie y considerando los posibles impactos antropogénicos. El análisis de microsatélites de 240 muestras de ocho poblaciones colombianas, en comparación con un conjunto de datos regional previamente desarrollado que abarca 32 poblaciones adicionales, reveló una compleja interacción de factores geológicos, oceanográficos, biológicos y antropogénicos que dan forma a la estructura poblacional. Si bien se observó una diversidad genética y un flujo génico relativamente altos a escala nacional y regional, surgieron patrones diferenciados entre los entornos oceánicos y continentales. Estos hallazgos resaltan (i) la identificable estructura poblacional de la especie, que comprende cinco clusters genéticos en Colombia; (ii) la remarcable capacidad de (re)colonización de *T. testudinum,* facilitada por la dispersión de larga distancia, clonalidad y la prevención de la endogamia; (iii) el papel de la estocasticidad en la dispersión y la falta de significancia del aislamiento por distancia en ciertas escalas; y (iv) la vulnerabilidad de las poblaciones a las perturbaciones antropogénicas de corto plazo. Este estudio subraya la necesidad de estrategias de conservación específicas para cada área, a fin de salvaguardar la resiliencia a largo plazo de esta especie y el invaluable ecosistema que soporta.

Palabras clave: Conservación genética, pastos marinos, impactos antrópicos, conectividad, islas oceánicas, ambientes continentales, Caribe.

Contents

Figure list

Pág.

Table list

Pág.

List of Symbols and Abbreviations

Symbols with Latin letters

Symbol	Term
°C	Centigrade
A	Mean allele number per locus
A_r	Allelic richness
Chi^2	Pearson's chi-squared test
F_{is}	Inbreeding coefficient
F_{st}	Fixation index
G	Genets
H_e	Expected heterozygosity
H_o	Observed heterozygosity
K	Probable number of populations
Ln P(X|K)	Log likelihood of each value of K
m2	Square meter
N	Number of individuals
N_a	Number of alleles
Nei	Nei genetic distance
ng	Nanograms
P_a	Private alleles
P_{gen}	probability of a clonal identity per population
P_{sex}	Probability of obtaining the same genotype by a sexual event
R	Clonal richness
Ta	Annealing temperature
uH_e	Unbiased expected heterozygosity
uNei	Unbiased Nei genetic distance

Symbols with Greek letters

Symbol	Term
µL	Microlitres
ΔK	Change in probable number of populations

Abbreviations

Abbreviation	Term
Agr	Agriculture
AMOVA	Analysis of Molecular Variance
Bio	Biodiversity loss
bp	Base pairs
BR	Biosphere Reserve
CCC	Caribbean Central Current
Cov%	Average coverage
CPG	Colombia-Panama Gyre
DAPC	Discriminant Analysis of Principal Components
Den	Average shoot density
EM	Expectation maximisation algorithm
Fih	Fishing
HWE	Hardy Weinberg equilibrium
IBD	Isolation by distance
IC	Island Cays
ICS	Islas Cayos del Sur
Ind	Industry
IRSB	Islas del Rosario y San Bernardo
LD	Linkage disequilibrium
LDD	Long distance dispersal
LGP	Last glacial period
MCMC	Markov Chain Monte Carlo
Mec	Mechanical disturbances
MedMeaK	Median of means
MedMedK	Median of medians
MLG	Multilocus genotype
MPA	Marine Protected Area
Mya	Million years ago
PCA	Principal Component Analysis
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
SD	Standard deviation
Sea	Seaports
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeat
Tou	Tourism
TPM	Two-phase model
Tur	Turbidity due sedimentation
Was	Waste management/deposition
ya	Years ago

Introduction

Thalassia Banks & Sol. ex K. D. Koenig is a genus of seagrasses distributed through tropical and subtropical environments worldwide. It comprises two species: *Thalassia hemprichii* (Ehrenb. ex Solms) Asch. in the Indo-Pacific and *T. testudinum* Banks ex König in the Caribbean and Gulf of Mexico (Larkum et al., 2006; van Tussenbroek et al., 2006; Cox & Tomlinson, 1988). As aquatic angiosperms, these seagrasses can form extensive meadows spanning from kilometres to hundreds of kilometres, facilitated by both sexual and clonal reproduction (Orth et al., 2006). A single genet of *Thalassia* can extend over 750 meters and live over 1500 years (van Dijk et al., 2018). The multi-scale dispersion of these species, and many other seagrasses, depends on their vegetative structures such as rhizomes, pollen, seeds, and propagules, which can travel from centimetres to hundreds of kilometres through clonal growth, sediment and water column movements, surface currents and animal vectors (Kendrick et al., 2012; McMahon et al., 2014; Ruiz-Montoya et al., 2012).

Thalassia testudinum, commonly known as turtle grass, is the primary structural species of seagrass ecosystems in the Caribbean Sea. It provides various services, including primary production, habitat for numerous invertebrate and vertebrate species, support to fisheries (particularly for juvenile fish), nutrient cycling, sediment stabilisation, and blue carbon capture and sequestration (Apostoloumi et al., 2021; Serrano et al., 2021; Cullen-Unsworth et al., 2014; Waycott et al., 2009). These seagrass meadows are closely associated with coral reefs and mangroves, creating strategic links that enhance life cycles, coastal protection, and other ecosystem services (Cox & Tomlinson, 1988; Guerra-Vargas et al., 2020).

The coverage of seagrass meadows, including *T. testudinum*, has significantly declined since the last century due to human activities, with losses occurring at a rate of 111 km^2 per year (Waycott et al., 2009). As sessile organisms, seagrasses cannot escape localized pressures such as contamination, mechanical disturbances, and rising temperature, making them more susceptible to local extinctions (Costanza et al., 1997; Waycott et al., 2006).

While ecological approaches provide crucial information for the management of seagrasses and related ecosystems (Díaz et al., 2003; Kaldy & Dunton, 1999; Rose & Dawes, 1999), they have serious limitations in explaining population structure (Waycott, 1998). Genetic tools significantly enhance our understanding of basin-level phylogeography, landscape-scale connectivity, and local-scale events within the populations, including the effects of diversity, clonality and mating system (Procaccini et al., 2007) in the resilience of the marine sessile organisms (Hughes & Stachowicz, 2004, 2009). Therefore, this geo-spatial genetics approach (Kershaw et al., 2021) enhances the knowledge needed for effective marine spatial planning implementation and appropriate science-policy approach for conservation (Riginos & Beger, 2022).

Even though genetic diversity has been proven to correlate with environmental status (Jahnke et al., 2015; Reusch, 2006), understanding the impacts of anthropogenic and natural disturbances on Colombian seagrass ecosystems requires knowledge of the differential characteristics of both, oceanic and continental environments. These two differ, *a priori*, from the geographical conditions that made oceanic places more remote and less connected. However, specific ecological and genetic differences between these two groups remain largely unknown (Hernawan et al., 2017; van Dijk et al., 2018).

Over the past 20 years, genetic studies of seagrass populations worldwide have increasingly utilized microsatellite markers, or simple sequence repeats (SSR), due to their co-dominant nature and high sensitivity to polymorphisms (Arnaud-Haond et al., 2005; D'Esposito et al., 2012; Migliaccio et al., 2005; van Dijk et al., 2007). These features have sustained the usefulness of microsatellites in seagrass studies, as they complement new techniques such as single nucleotide polymorphisms (SNPs) (Hernawan et al., 2017). Although next-generation sequencing and genomics can provide broader insights into the ecology of these sexual/clonal species, microsatellites remain the most common and cost-effective technique, facilitating comparisons between different studies.

The risk of extinction is high for seagrass species, in the Caribbean and worldwide, due to factors such as lack of resiliency, loss of heterozygosity, and inbreeding (Campanella et al.,

2015) as their ecosystems are exposed to rapid coastal changes driven by human development. Genetic diversity, which reflects the evolutionary potential of seagrass species, can be influenced by their biological characteristics, such as sexual or clonal reproduction (Campanella et al., 2015; Kendrick et al., 2012; Waycott, 1998), as well as by external factors like anthropogenic stress (Waycott et al., 2009). While ecological approaches provide valuable qualitative information on ecosystem health through analysis of shoot density, spatial extent, height, biomass, temperature, and salinity, they cannot measure genetic diversity or serve as an early warning system for pre-emptive and adaptive management.

The health of *Thalassia testudinum* is crucial for the well-being of other coastal ecosystems and species (Gómez-López & Alonso, 2016). Therefore, it is essential to understand the diversity patterns and connectivity levels they exhibit across their entire distribution range, including Colombian populations, to ensure the effectiveness of conservation policies and strategies at different scales.

This study assessed the genetic structure and connectivity of *T. testudinum* populations from four oceanic islands and four continental islands in the Colombian Caribbean. A mid-range scale approach was used covering a diagonal distance of 720 km and an area of 305.000 km^2 approximately, with the entire species distribution range serving as the large-scale reference (van Dijk et al., 2018). Microsatellite markers were employed to provide a deeper view of the species' recent migration processes. The hypothesis tested was that *T. testudinum* exhibits different population dynamics between oceanic and continental islands, influenced by environmental and anthropogenic factors such as oceanographic currents, geographic distance, colonization and recruitment opportunities (gene flow), as well as human activities. Based on this baseline scenario, we discussed the main natural and anthropogenic dynamics that may affect the meadows. Finally, we calculated the relative health status of the Colombian turtle grass meadows by comparing allelic richness (A_r) and observed heterozygosity (H_o) with previous studies that used similar loci (Campanella et al., 2015; Larkin et al., 2006; van Dijk et al., 2018; van Dijk & van Tussenbroek, 2010; van Dijk et al., 2009). The results were used to provide local and national management recommendations for conservation.

1. Methods

1.1 Study area

The Colombian Caribbean, located between 8° - 16°N and 69° - 82°W, encompasses the main tropical marine and coastal ecosystems (Andrade, 2015; Gómez-Cubillos et al., 2015), including seagrass meadows. In this region, *Thalassia testudinum* is the dominant species, with its populations influenced by factors such as depth (0-12 m), sediments type (favouring fine sediments or sludge), turbidity (accumulated sediments between the roots), and nutrient availability, among others (Barrios & Gómez, 2001; Díaz et al., 2003).

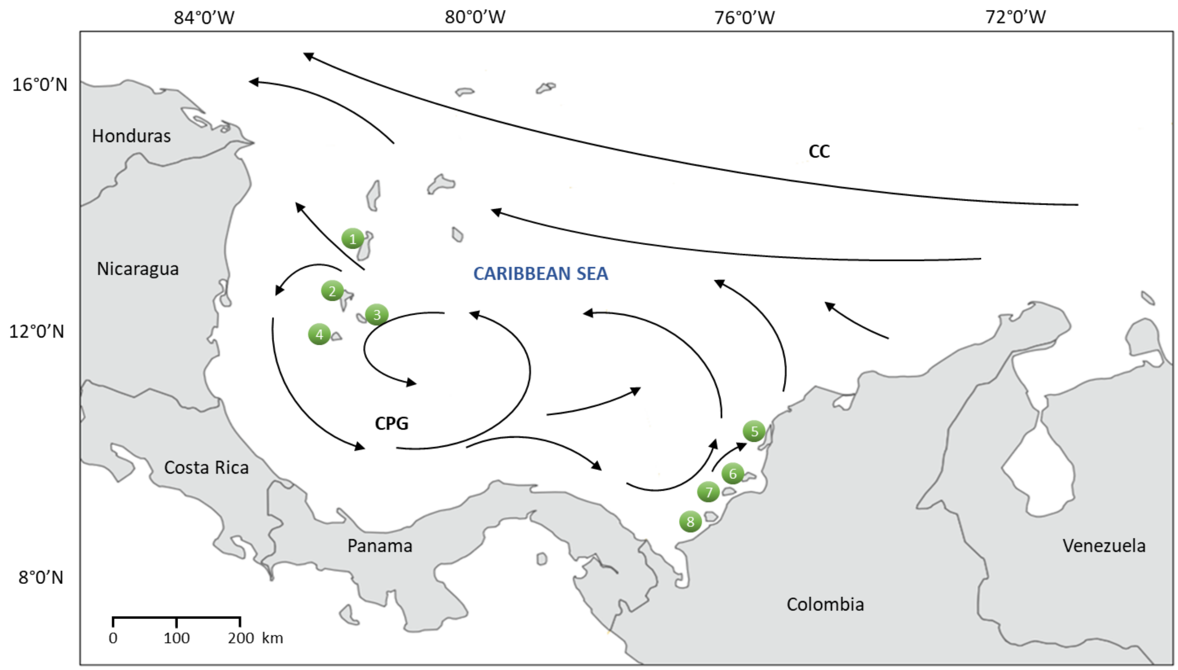
Thalassia testudinum grows along the whole Colombian Caribbean continental coast, from Puerto Lopez, on the border with Venezuela, to Capurganá, on the border with Panama (Díaz et al., 2003; Gómez-Cubillos et al., 2015). Within this area, the meadows growing around continental islands and located up to 10 km from the mainland face limitations in expandability due to coralline sediments, shallow water availability, and more oligotrophic waters compared to mainland meadows, despite being affected by coastal dynamics (Barrios & Gómez, 2001; Galeano et al., 2016). None of the selected continental islands is part of established Marine Protected Areas (MPAs), but they are located near the Islas del Rosario y San Bernardo MPA, which includes the Rosario and San Bernardo National Park and the Deep Corals National Park (Parques Nacionales Naturales de Colombia, 2023) (locations 5 to 8, **Figure 1-1**).

On the other hand, *T. testudinum* oceanic meadows of the Seaflower Biosphere Reserve (locations 1 to 4, **Figure 1-1**) grow around most of the Archipelago’s islands, including

Serrana, Roncador, Old Providence, San Andrés, East Southeast (Bolívar) and South-Southwest Cays (Albuquerque) (CCO, 2015; Díaz et al., 2003; Vides et al., 2016). The structural characteristics of these meadows are influenced by the same limiting factors described for the continental islands. However, the influence of coastal dynamics on the oceanic ecosystems is significantly reduced, and the seed and fruit dispersal stochasticity may be higher. All these islands are located within the Seaflower MPA and the Seaflower Biosphere Reserve (CCO, 2015; CORALINA-INVEMAR, 2012).

Both oceanic and continental meadows in the Colombian Caribbean islands have evolved around geological formations of coral reefs (Díaz et al., 2000; Geister & Díaz, 2007; Idárraga-García et al., 2021; Vargas, 2004). In the case of this study, it allows the comparison between these two environments regarding the influence of the continental ecology on the gene flow, diversity, and population structure while maintaining the similar characteristics of small-scale meadows. Despite the general similarities, differences arise from the geological formation time of the islands. Old Providence originated in the early Miocene (23-15 million years ago (mya)), while San Andres, East Southeast, and South Southwest formed between the middle Miocene and Pliocene (15-2.5 mya) (Carvajal-Arenas & Mann, 2018; Geister & Díaz, 2007; Geister, 1972, 1992). Additionally, the continental islands (Barú, Isla Palma, Isla Fuerte e Isla Tortuguilla) date back to the late Pliocene (5 Mya) (Díaz et al., 2000; López-Victoria et al., 2000). Therefore, the current existing reefs in the oceanic Archipelago date from around 20.000 ya, before the ending of the Last Glacial Period (LGP) (100.000 – 12.000 ya), while the current reefs of the continental islands date from 5.000 to 3.000 ya, in the Holocene (Cendales et al., 2002; Díaz et al., 2000; Díaz et al., 1996).

Figure 1-1. Sampling locations of *Thalassia testudinum*.



(1-4) and continental islands (5-8). 1) Old Providence 2) San Andrés 3) East Southeast Cays 4) South Southwest Cays 5) Barú 6) Isla Palma 7) Isla Fuerte 8) Isla Tortuguilla. Black arrows indicate the main surface currents directions (Andrade, 2015, 2001; Centurioni & Niiler, 2003). CCC: Caribbean Central Current, CPG: Colombia-Panama Gyre.

1.2 Data and sample collection

Ecological and anthropogenic characteristics

Qualitative and quantitative features of each locality were assessed to identify differential ecological and anthropogenic characteristics of the meadows. Qualitative data including fishing pressure, agriculture and sedimentation influences on turbidity, biodiversity loss, waste deposition and tourism pressure, were evaluated through social surveys, field observations, and literature reviews (Barrios & Gómez, 2001; Galeano et al., 2016; Gómez-López et al., 2020; Gómez-López & Alonso, 2016) and categorised on a scale from 1 (low) to 5 (high). Quantitative and semi-quantitative data included shoot density (shoot number per m^2 with a 50 x 50 cm quadrat), coverage (percentage, measured as presence/absence of shots in 100 sub quadrats of 5 x 5 cm), visibility (Secchi disc measurements) and mechanical disturbances (number of holes within 10 x 10 m area of the meadow). The latter data were collected in the field and corroborated with existing reports (Barrios & Gómez, 2001; Gómez-López et al., 2020). Principal Component Analysis (PCA) was conducted using the Ade4 Package (Dray & Dufour, 2007) in the software R (R Core Team, 2022) to analyse all these variables and features collectively and to understand the relationship among the sampling locations.

Genetics

Sampling for genetic analyses was conducted in eight selected localities encompassing both continental and oceanic islands, as shown in **Figure 1-1**. At every station, a minimum of 30 samples were collected through snorkelling along random transects, extending up to 100 m from the coastline. Sampling depths ranged between 1 m to 5 m, with an average distance of 10 m between samples or ramets (Nakajima et al., 2023), which is an increase of the 5 m employed in previous studies to prevent clone collection (Campanella et al., 2015; van Dijk et al., 2009; van Dijk & van Tussenbroek, 2010). Upon collection, samples were rinsed with fresh water and preserved in falcon tubes with 96% ethanol. In the laboratory, each leaf was cleaned of epiphytes, air-dried with paper towels for two hours and individually stored in Ziploc bags containing silica gel at room temperature.

1.3 DNA extraction, microsatellites amplification, and genotyping

Dry tissue samples (0.2 g of each sample) were disrupted using a BeadBug homogenizer with ceramic beads (Benchmark, Sayreville, NJ, USA), prior to extraction. DNA extraction followed the DNeasy Plant Mini kit® protocol (Qiagen, Hilden, Germany), with a five-ten minutes extension in incubation times. To assess the quantity and quality of the products, electrophoresis was performed using 2-3% agarose gels in 1X TBE buffer solution and 2µL of Gel Green. Then, 3µL of the sample was mixed with 1µL of 1000x loading buffer before loading. Electrophoresis was conducted at 75 V – 45 minutes. Additionally, DNA quantity and quality were evaluated using a Nanodrop 2000 spectrophotometer testing (ThermoFisher Scientific, Waltham, MA, USA).

Twelve microsatellite loci for *T. testudinum* were selected based on previous research (van Dijk et al., 2007), as detailed in **Table 1-1**. Initially, each locus was individually PCR-amplified to optimise conditions before multiplexing. PCR was conducted using the Taq PCR Master Mix Kit ® protocol (Qiagen) with the following cycling conditions: initial denaturation at 94°C for 3 min, followed by 28-30 cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Each reaction had a final volume of 20uL with approximately 10-20 ng of DNA. The PCRs were carried out in an Eppendorf Thermocycler. Forward primers were fluorescently labelled (6-FAM, ROX, TAMRA, and JOE) by Macrogen to distinguish amplified fragments (see Table 2). Two separate multiplex reactions were designed according to Table 2 using the Type-it ® microsatellite PCR Kit protocol (Qiagen). Cycling conditions for multiplex PCR included initial activation at 95°C for 5 min, 32 cycles of denaturation at 95°C for 30 sec, annealing at 58°-60°C for 90 sec, extension at 72°C for 30 sec, and a final extension step at 60°C for 30 min. Each multiplex reaction had a final volume of 10 uL and 30-50 ng of DNA. Loci TTMS-GT112 and TTMS-CA180 were excluded from subsequent analyses due to unsuccessful multiplexing attempts.

Table 1-1. Primers isolated by van Dijk et al. (2007) employed in this study.

	Locus	Repeat motif	Size range	Ta(C°)	H_o	H_e	GenBank Accession No.	Multiplex
1	TTMS-GA6	(GA)19[AG](AC)2	125-153	t 62–52	0.692	0.833	DQ512877	1-JOE
2	TTMS-GA8	(GA)18 **	234-274	t 64–54	0.810	0.859	DQ512878	1-TAMRA
3	TTSM-GA12	(GA)18	153-168	t 63–53	0.355	0.399	DQ512879	1-FAM
4	TTSM-TGA39	(GGA)6(TGA)12	219-231	t 65–55	0.656	0.671	DQ512880	2-ROX
5	TTMS-TCT58	(GAA)15 (GAT)4(GAA)3	158-196	t 63–53	0.761	0.796	DQ512881	2-TAMRA
6	TTMS-GGT59	(GGT)11 [GGAGGGTGA] (GGT)5	221-242	59	0.656	0.741	DQ512882	2-JOE
7	TTMS-GA72	(GA)16	224-232	58	0.843	0.609	DQ512883	2-FAM
8	TTMS-GT77	(GT)16	100-110	t 65–55	ND	ND	DQ512884	2-FAM
9	TTMS-GT104	(GT)15	183-189	t 69–59	0.821	0.548	DQ512885	2-FAM
10	TTMS-GT112	(GT)3[TT](GT)12 *	202-212	t 69–59	0.410	0.436	DQ512886	1-FAM
11	TTMS-CA180	(CA)15 *	148-166	t 64–54	0.449	0.763	DQ512887	1-ROX
12	Th1 MS	(CT)15	163-181	56	0.624	0.607	DQ837163	2-JOE

Locus, repeat motif, size range, annealing temperature (Ta), observed heterozygosity (H_o), expected heterozygosity (H_e) and GenBank accession number are reported by van Dijk et al. (2007). Multiplex: number refers to the multiplex created in this study and their assigned dye (JOE, TAMRA, FAM, ROX). *Removed from analyses after unsuccessful amplification. ** removed from analyses after null allele and HWE tests.

The fluorescence-labeled PCR products were forwarded to the SSIGMOL; Sequencing Facility of the Genetics Institute, Universidad Nacional de Colombia. Fragment lengths were determined using an ABI 3500 analyser (Applied Biosystems) with a mix containing 9 uL of formamide, 0.5uL of Liz 600 size standard, and 0.5 uL of each sample. The denaturation step was carried out at 95°C for 3 minutes followed by immediate cooling on ice for 5 min. Subsequently, fragment analysis was performed and scored using GENEMAPPER (Applied Biosystems) and the open-source software OSIRIS 2.13.1 (National Library of Medicine). Manual verification of the peaks was conducted to ensure accurate interpretation, considering the specific range of each microsatellite. Picks outside the typical range (e.g, less than 100 bp or longer than 600 bp) were identified as non-specific and disregarded in the analysis. For instance, if a microsatellite dyed with FAM was expected to range from 180 to 200 bp, a peak at 260 bp would be recognized as an artifact rather than a genuine microsatellite allele (Pompanon et al., 2005; Bonin et al., 2004). The resulting genotype data from different loci and individuals served as the input file for the genetic and statistical analysis.

1.4 Data analysis

The dataset was processed using the software GenClone (Arnaud-Haond & Belkhir, 2007) to remove duplicate multilocus genotypes (*MLGs*), and to calculate clonal richness (*R*). Additionally, this software calculated the probability that these replicates are part of the same clone (P_{gen} and P_{sex}), based on the inbreeding coefficient (F_{is}) (Arnaud-Haond et al., 2007; Arnaud-Haond & Belkhir, 2007). The presence and frequencies of null alleles were assessed using the expectation-maximization (*EM*) algorithm with 20.000 bootstraps in FreeNA (Chapuis & Estoup, 2007) and confirmed with MICROCHECKER 2.2.3 (van Oosterhout et al., 2004). Linkage disequilibrium (*LD*) between pairs of loci and deviations from Hardy–Weinberg proportions (*HWP*) were assessed with the web version of GENEPOP (Raymond & Rousset, 1995) and compared with ARLEQUIN 3.5 (Excoffier & Lischer, 2010), employing 100.000 steps Markov chain permutation with 10.000 dememorization steps. Microsatellites that exhibited null alleles and did not conform to

HWP and LD expectations after Bonferroni correction were discarded, and the remaining set was re-evaluated.

Several genetic diversity metrics were calculated to understand the genetic complexity of *Thalassia testudinum* across its geographic range. Observed heterozygosity (*Ho*), unbiased expected heterozygosity (*uHe*) and its standard deviation (*SD*), mean allele number per locus (*A*) and the fixation index (*Fst*) were determined using GENALEX 6.503 (Peakall & Smouse, 2012). The inbreeding coefficient (*Fis*) was estimated using the web version of Genepop (Raymond & Rousset, 1995). Allelic richness (*Ar*) for each population was calculated with Hp-Rare (Kalinowski, 2005), applying rarefaction to fifteen (15) genets (Kalinowski, 2004).

A hierarchical analysis of molecular variance (AMOVA) was carried out in GENALEX to estimate the differentiation within and among populations. Genetic differentiation indexes between sites (pairwise F_{st}) and the Nei's unbiased genetic distance (*uNei*) were also calculated using GENALEX. Additionally, to test the isolation by distance (IBD) hypothesis, a Mantel test was conducted using 999 random permutations to evaluate the significance of the correlation between Log-transformed geographical distances (1 + geographic distance) and both direct Nei genetic distance and F_{st}. Patterns of genetic differentiation were further assessed through a Principal Coordinates Analysis (PCoA) in GENALEX, derived from the covariance matrix with data standardisation values (pairwise F_{st}) among sites.

To detect potential population subdivisions, the individual-based clustering Bayesian method was applied in STRUCTURE 2.3.4 (Hubisz et al., 2009; Pritchard et al., 2000). This software assumes that the loci are unlinked and in linkage equilibrium within populations, and it optimizes the number of clusters based on the assumption of Hardy-Weinberg equilibrium (Pritchard et al., 2000). This involved fifteen (15) runs of 1,000,000 Markov chain Monte Carlo iterations, with a burn-in period of 200,000 for each K value (with K ranging from 1 to 8), using the admixture model and assuming correlated allele frequencies. The admixture model allows for the analysis of recent or current gene flow, enabling individuals to have ancestors from more than one population, and correlated allele frequencies are expected to occur in populations with a common origin (Pritchard et al., 2000). To determine the optimal number of K clusters, two methodologies were implemented (Stankiewicz et al., 2022): the ΔK method, which measures the change of Ln

P(X|K) (Evanno et al., 2005), the median of means (MedMeaK) and the median of medians (MedMedK) methods (Puechmaille, 2016). ΔK was computed in STRUCTURE HARVESTER (Earl & VonHoldt, 2012), while the median of means and median of medians were visualised in CLUMPAK (Kopelman et al., 2015), incorporating the nine viable loci.

A Discriminant Analysis of Principal Components (DAPC) was conducted using the Adegenet package in R (Jombart et al., 2017), to analyse and visualise genetic differences among populations. This method was chosen because it does not rely on assumptions such as Hardy-Weinberg equilibrium or linkage equilibrium. The first two components were plotted, and a membership probability graphic was also obtained to detect the admixture and visualise the inter and intra-population diversity. Cross-validation was applied to determine the optimal number of PCs to retain.

Complementary to previous analyses, gene flow was inferred using BAYESASS 3.0.4 (Wilson & Rannala, 2003) which calculates the migration rate among populations over recent generations. The parameters used were 10 MCMC random seeds, 5,000,000 iterations, a Burn-in of 500,000, and a sampling interval of 500. In addition, to evaluate if populations have suffered recent bottleneck, a two-phase model (TPM) Wilcoxon's test was deployed in BOTTLENECK 1.2.02 (Piry et al., 1999), using 10,000 replications with a probability of 70% stepwise mutation model.

1.5 Regional context of Colombian in the Caribbean

Although a regional study of the Great Caribbean was developed recently (van Dijk et al., 2018), it did not include data from Colombian meadows. Therefore, it was not possible to integrate this dataset into a regional analysis, as the microsatellites were amplified using different methodologies and fragment length analysis, and scoring can be subjective. However, specific comparisons of allelic richness, unbiased expected heterozygosity, clonality, observed heterozygosity, and F_{is}, were carried out to provide insights into the genetic and ecological status of the present meadows in the regional context. Both studies employed nine microsatellite loci, differing only in one locus (TTSM-TGA39 used in this project and TTMS- GA8 in van Dijk et al. (2018)).

2. Results

2.1 Socio-ecological characterisation of the sampling meadows

Ecological analysis provided a general idea of the differences and similarities of the studied populations **(table 2-1).** Significant differences between continental and oceanic islands were spotted, mainly related to ecological aspects such as visibility and shoot density. Tortuguilla exhibited divergent characteristics even within continental islands, where visibility, seagrass coverage per quadrant and density were the lowest among all populations. The island possesses coarse sediment that hinders the expansion of seagrass meadows, coupled with strong pressures from biodiversity loss that were evidenced in the developed surveys, which positioned it as the most affected by human activities and pressures, followed by Barú and San Andrés.

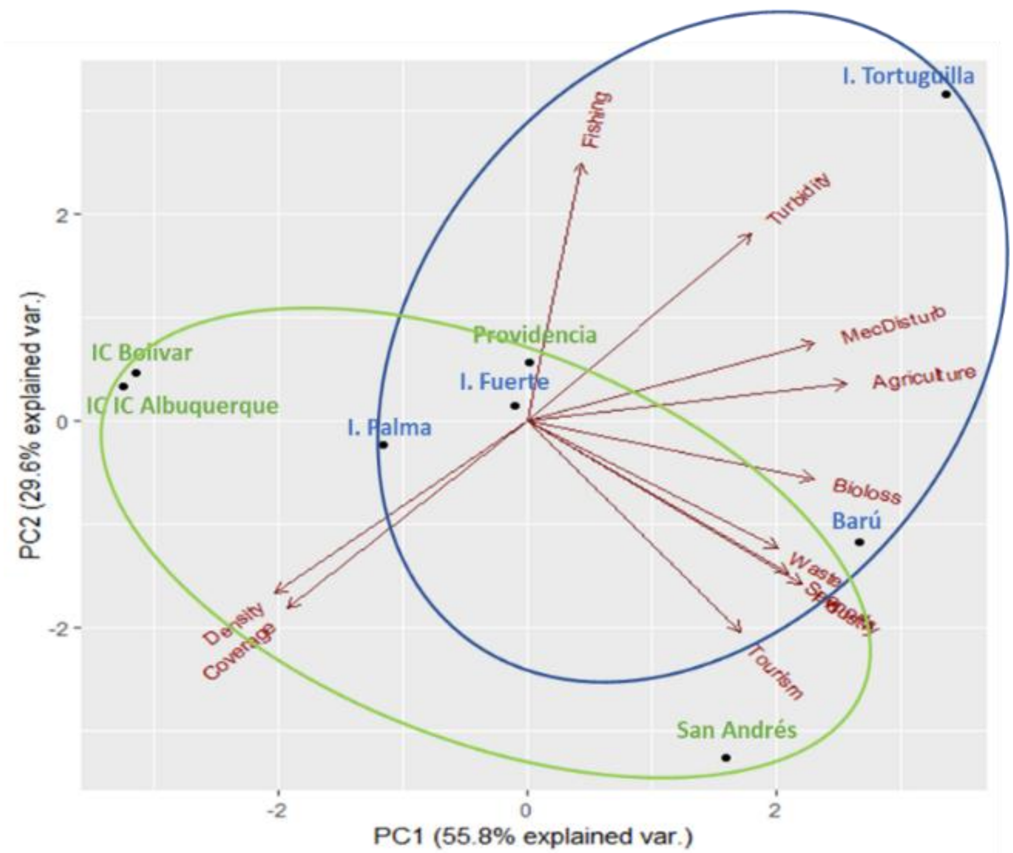
Table 2-1. Ecological characteristics, human activities, and pressures.

Location	Environment	Structure		Human Activities						Pressures		
	Vis (m)	Cov %	Den (#/ m2)	Tou	Fih	Sea	Ind	Agr	Was	Mec	Tur	Bio
Old Providence	6.0	70	247	3	4	2	2	2	3	3	2	2
San Andrés	10.5	72	248	5	2	4	3	2	4	3	1	3
IC Bolívar	18.5	74	265	1	3	1	1	1	2	2	1	2
IC Albuquerque	22.7	79	271	1	3	1	1	1	2	2	1	2
Isla Barú	8.6	62	204	4	3	4	3	3	3	3	2	3
Isla Palma	4.2	77	260	3	3	2	2	2	2	2	2	2
Isla Fuerte	7.4	58	192	3	3	2	2	2	3	2	2	2
Isla Tortuguilla	3.5	22	90	2	4	2	2	3	3	4	3	3

Vis: Average visibility (secchi disc) of five measurements, Cov: Average coverage of five measurements, Den: Average shoot density of five measurements. Human activities and pressures are calculated in a scale of 1-5, where 1 is the lowest and 5 is the highest. Tou, Tourism; Fih, Fishing; Sea, Seaports, and commerce; Ind, Industry; Agr, Agriculture (based on the extension of the activity in the different areas of study and use of pesticides and fertilisers); Was, Waste management systems/waste deposition; Mec, Mechanical disturbances; Tur, turbidity due sedimentation; and Bio, Biodiversity loss.

The PCA analysis **(Figure 2-1)** indicated no clear differentiation between oceanic and continental islands, suggesting that ecological features and anthropogenic pressures are not solely dependent on their proximity to the mainland. The most significant pressures impacting the ecological characteristics of turtle grass, specifically coverage and shoot density, were water turbidity resulting from river sediment deposition, and mechanical disturbances such as anchoring, improper fishing practices, and removal. While tourism alone did not appear to have negative effects on seagrass meadows, the removal of meadows to maintain "clean" and sandy beaches contributed to mechanical disturbances and, consequently, biodiversity loss. The data variability was 85.4% explained by the first two components.

Figure 2-1. Principal Component Analysis (PCA) of the Table 1-2 data.



Components 1 and 2 describe 85.4% of data variability.

2.2 Genetic diversity and population structure

Ten out of 12 microsatellite selected loci **(Table 1-1)** were successfully amplified in two multiplex PCRs and, therefore, the loci TTMS-GT112 and TTMS-CA180 were removed from the analyses after subsequent unsuccessful multiplexes. The locus TTMS-GA8 was excluded from further analyses due to the presence of null alleles, Hardy-Weinberg proportions (HWP), and linkage disequilibrium (LD), which differed significantly after Bonferroni corrections (Supplementary material S2-S4). Of the 240 ramets collected across the eight locations **(Table 2-2)**, 161 MLGs were included in the population genetics analysis after removing 79 clones. A total of 88 alleles were genotyped across the eight populations based on nine microsatellite loci. Allelic richness (*Ar*) among the populations ranged from 3.3 to 7.2. Clonal richness (*R*) varied significantly among meadows, with values ranging from 0.172 to 0.966. The probability of obtaining the same genotype by a sexual event (P_{sex}) was generally low across both the oceanic and continental islands, but was notably higher in San Andres and Albuquerque, due to the elevated number of clones. Despite the predominance of clonal reproduction in these two populations, the observed heterozygosity (*Ho*) was higher than the expected heterozygosity (*He*) and the unbiased *He* (*uHe*), showing negative F_{is} and no evidence of inbreeding. Overall, genetic diversity was high across all the populations (*Ho* ranging from 0.627 to 0.796), although it was lower in the oceanic islands **(Table 2-2)**. The F_{is} values in all cases were close to zero (0) and mainly negative, indicating a low probability of inbreeding. Private alleles were most prevalent in Providencia and Isla Palma, which also exhibited the highest allelic richness.

Old Providence presented similar genetic characteristics to those of the continental islands in almost all the indicators and emerged as the most genetically rich population of the Seaflower Biosphere Reserve. In contrast, San Andrés showed lower allelic richness and the highest clonality with 24 out of the 30 ramets sharing the same multi-locus genotype (MLG), meaning that this individual is larger than 400m and expanded perpendicularly. Despite this, both uH_e and H_o were high in San Andrés. Bolívar and Albuquerque islands, unpopulated small oceanic atolls, also showed high clonality (low *R*), and the lowest values for A_r and uH_e of the studied meadows (0.605 and 0.565 respectively). Furthermore, Bolívar population characteristics such as excess of heterozygotes (H_o>uH_e but not significant Chi-square test), low number of alleles (N_a=34) and A_r (3.8), negative F_{is} (0.037), and significative results from the two-phase model (TPM) Wilcoxon's test for bottlenecks, suggested a possible founder effect **(Table 2-2)**.

The features of the continental islands were quite similar among themselves, in terms of *R* (range from 0.793 to 0.996), P_{gen} max and P_{sex} max, and H_o (range from 0.641 to 0.736). However, Isla Tortuguilla had a significantly lower number of alleles (N_a=41 vs 60 overall), A_r (4.6 vs 6.5 overall), and a significant TPM Wilcoxon's test for bottlenecks **(Table 2-2)**.

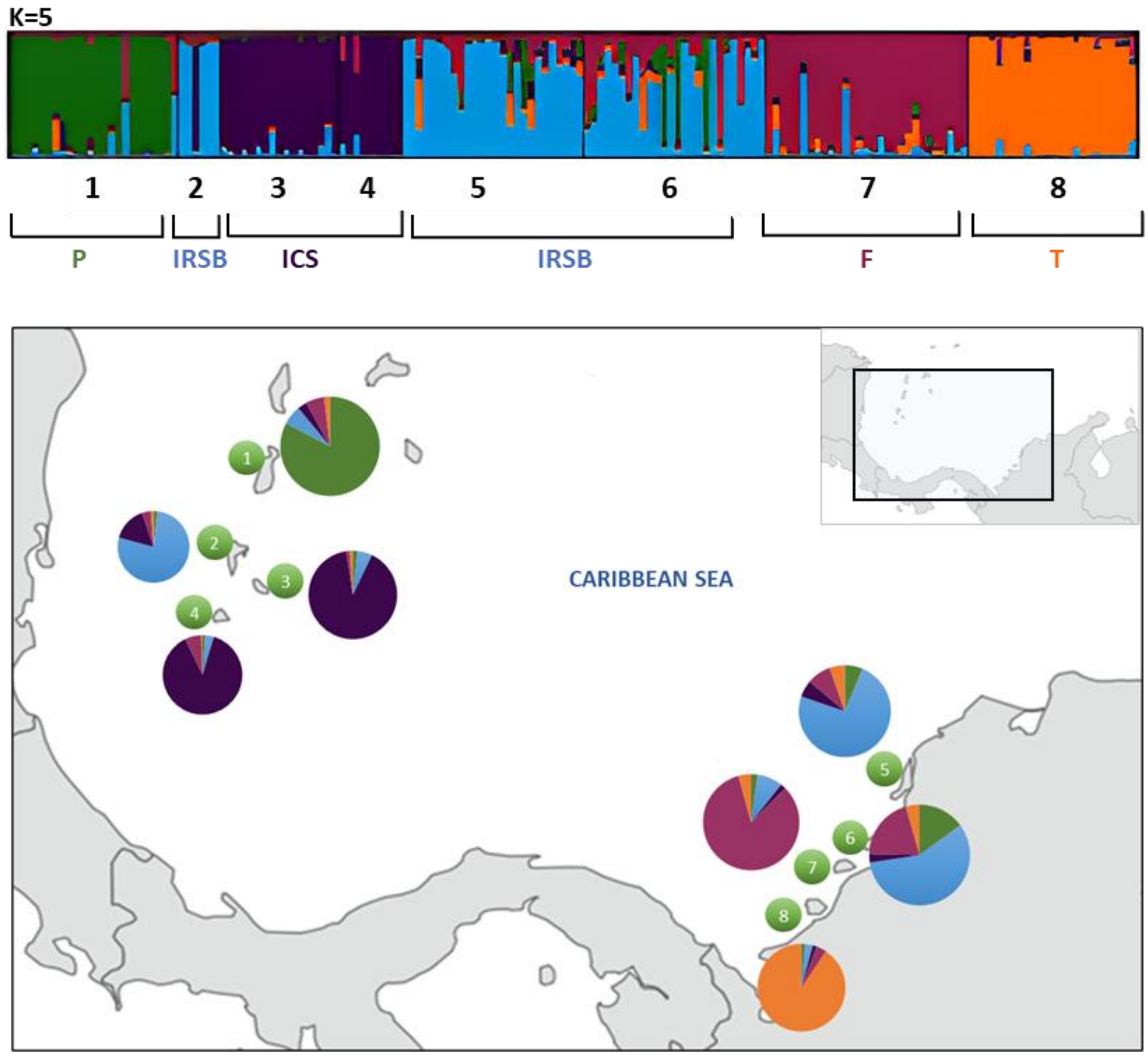
Table 2-2. Genetic Diversity of *T. testudinum* in the Colombian Caribbean based on nine microsatellite loci.

#	Location	N	G	R	P_{gen} max (F_{is})	P_{sex} max (F_{is})	Na	A_r	$A_{r[15]}$	P_a	H_o	H_e	uH_e	SE	F_{is}	TPM
1	Old Providence	30	24	0.793	1.924E-08	9.848E-07	58	6.4	4.9	7	0.750	0.709	0.724	0.034	-0.036	0.752
2	San Andrés	30	6	0.172	5.878E-03	1.621E-01	35	3.9	-	0	0.796	0.611	0.667	0.058	-0.194	0.898
3	IC Bolivar	30	17	0.552	1.003E-07	1.016E-05	34	3.8	3.5	1	0.627	0.587	0.605	0.079	-0.037	0.004
4	IC Albuquerque	30	9	0.276	1.414E-03	4.156E-02	30	3.3	-	2	0.679	0.534	0.565	0.049	-0.202	0.633
5	Barú	30	26	0.862	8.543E-09	7.386E-09	55	6.1	4.8	0	0.641	0.664	0.677	0.047	0.054	0.787
6	Isla Palma	30	26	0.862	9.774E-10	2.595E-10	65	7.2	5.0	6	0.735	0.717	0.731	0.032	-0.005	0.715
7	Isla Fuerte	30	29	0.966	2.638E-09	1.281E-08	56	6.2	4.7	3	0.736	0.697	0.710	0.031	-0.037	0.674
8	Isla Tortuguilla	30	24	0.793	1.089E-08	1.049E-06	41	4.6	4.2	1	0.685	0.719	0.734	0.017	0.066	0.001

N: number of ramets (total N=240). *G:* number of genets (Total G=161=total of MLGs). *R*: clonal richness. P_{gen} *max:* highest probability of a clonal identity per population. P_{sex} *max*: probability of obtaining the same genotype by a sexual event. N_a: number of alleles. A_r: allelic richness. P_a: private alleles. H_o: observed heterozygosity. H_e: expected heterozygosity. uH_e: unbiased expected heterozygosity. *SD*: standard deviation. F_{is}: inbreeding coefficient. *TPM*: Two Phase Model Wilcoxon's test for bottleneck.

The population structure analysis using a Bayesian model that tested Δ*K* with two different methods (Puechmaille, 2016; Evanno et al., 2005) (Supplementary material S6) consistently revealed a *K*=5, composed of the following clusters: P (Old Providence), ICS (IC Bolívar and IC Albuquerque), IRSB (San Andrés, Barú and Isla Palma), F (Isla Fuerte), and T (Isla Tortuguilla) **(Figure 2-2a)**. All populations were assigned to a cluster, with an average membership coefficient of 0.805 (minimum coefficient of 0.577 in Isla Palma) **(Figure 2-2b)**. Contrary to expectations, continental islands exhibited significant structuring, showing the same number of clusters as the oceanic islands. This clustering was geographically logical, except for the unexpected grouping of San Andrés with the continental islands of Barú and Isla Palma, rather than with the oceanic islands.

Figure 2-2. Bayesian analysis in STRUCTURE.



a) Clusters at K=5, and b) Map of the populations' proportions of belonging to each cluster when K=5. Cluster P: Old Providence, Cluster ICS: IC Bolívar and IC Albuquerque, Cluster IRSB: San Andrés, Isla Palma and Barú, Cluster F: Isla Fuerte, Cluster T: Isla Tortuguilla. 1. Old Providence; 2. San Andrés; 3. IC Bolívar; 4. IC Albuquerque; 5. Barú; 6. Isla Palma; 7. Isla Fuerte; 8. Isla Tortuguilla.

In addition to the STRUCTURE analysis, the Fixation index (pairwise F_{st}) and the Nei's unbiased genetic distance (*uNei*) **(Table 2-3)**, showed a slight-moderate differentiation between all eight populations (corrected overall F_{st} = 0.095 and *uNei* = 0.425), with a stronger relatedness among the continental islands compared to the oceanic islands (continental F_{st} = 0.050 and oceanic islands F_{st} = 0.125). Albuquerque emerged as the most distanced population overall, with F_{st} > 0.15, significantly related to Bolívar, and both forming the cluster ICS following the STRUCTURE analysis. Regarding the cluster IRSB, results shown in **Table 2-3** suggested that Old Providence and Isla Fuerte are closely related to it despite being located as different clusters since the F_{st} and *uNei* between these populations and Barú and Isla Palma were significantly low. They appeared to be even more related than San Andrés. Old Providence exhibited greater distance from other populations in the Archipelago than from the continental islands, while Isla Tortuguilla, consistent with STRUCTURE results, showed less genetic relatedness to the oceanic populations but maintained a notable genetic distance from nearby meadows.

Table 2-3. Population differentiation of *T. testudinum* based on nine microsatellite loci.

	Old Providence	San Andres	Bolivar	Albuquerque	Baru	Isla Palma	Isla Fuerte	Tortuguilla
Old Providence		0.494	0.495	0.699	0.340	0.191	0.301	0.506
San Andres	0.109		0.320	0.520	0.315	0.283	0.394	0.635
Bolivar	0.115	0.107		0.376	0.218	0.328	0.427	0.548
Albuquerque	0.148	0.153	0.117		0.682	0.809	0.745	0.825
Baru	0.071	0.092	0.069	0.147		0.142	0.171	0.327
Isla Palma	0.041	0.080	0.088	0.154	0.040		0.170	0.357
Isla Fuerte	0.058	0.098	0.101	0.151	0.043	0.039		0.287
Tortuguilla	0.078	0.122	0.113	0.153	0.065	0.062	0.054	

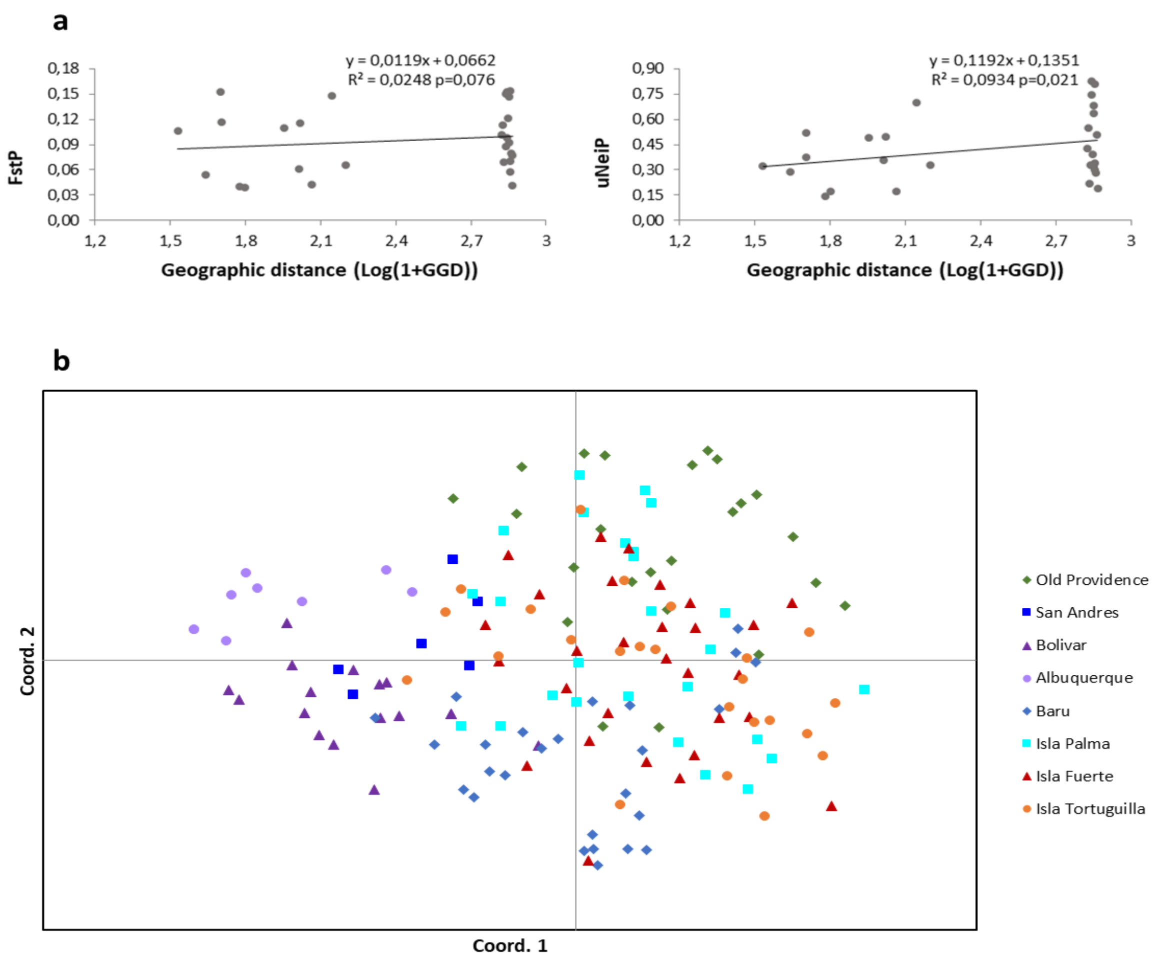
Pairwise F_{st} (below diagonal) and *uNei* genetic distances (above diagonal). Probability, **$p \leq 0{,}001$** for all values. Values range from low genetic distance (green) to high (orange).

The hierarchical AMOVAs were conducted to elucidate the variation among the eight studied populations and the five clusters defined by STRUCTURE. With similar results between populations and clusters, the variation among populations ranged from 10 to 12%, while within populations ranged between 88 and 89% of the total genetic variation **(Table 2-4)**. This indicates significant but not highly pronounced structuring among the populations. Interestingly, despite the genetic relatedness observed among some distant populations, there appeared to be barriers to connectivity between nearby populations. The Mantel test for isolation by distance showed no significant correlation between genetic distance (F_{st} and *uNei*) and geographic distance **(Figure 2-3a** and Supplementary material S7**),** suggesting that geographic proximity did not consistently predict genetic similarity **(Figure 2-3a)**. The Principal Coordinate Analysis (PCoA) showed isolation tendencies mainly in oceanic islands, but in the case of continental islands the differentiation is not clear in the graphic **(Figure 2-3b** and Supplementary material S10-11**).**

Table 2-4. AMOVA results for eight populations and five STRUCTURE-resulting clusters of *T. testudinum* in the Colombian Caribbean.

	8 populations						5 clusters					
Source	**df**	**SS**	**Variance**	**%**	**F_{st}**	**pValue**	**df**	**SS**	**Variance**	**%**	**F_{st}**	**pValue**
Among Populations	7	137.124	0.420	12%	0.119	0.001	4	98.144	0.344	10%	0.097	0.001
Among Individuals	153	470.706	0.000	0%			156	509.685	0.053	1%		
Within populations	161	509.000	3.161	88%			161	509.000	3.161	89%		
Total	321	1116.829	3.581	100%			321	1116.829	3.559	100%		

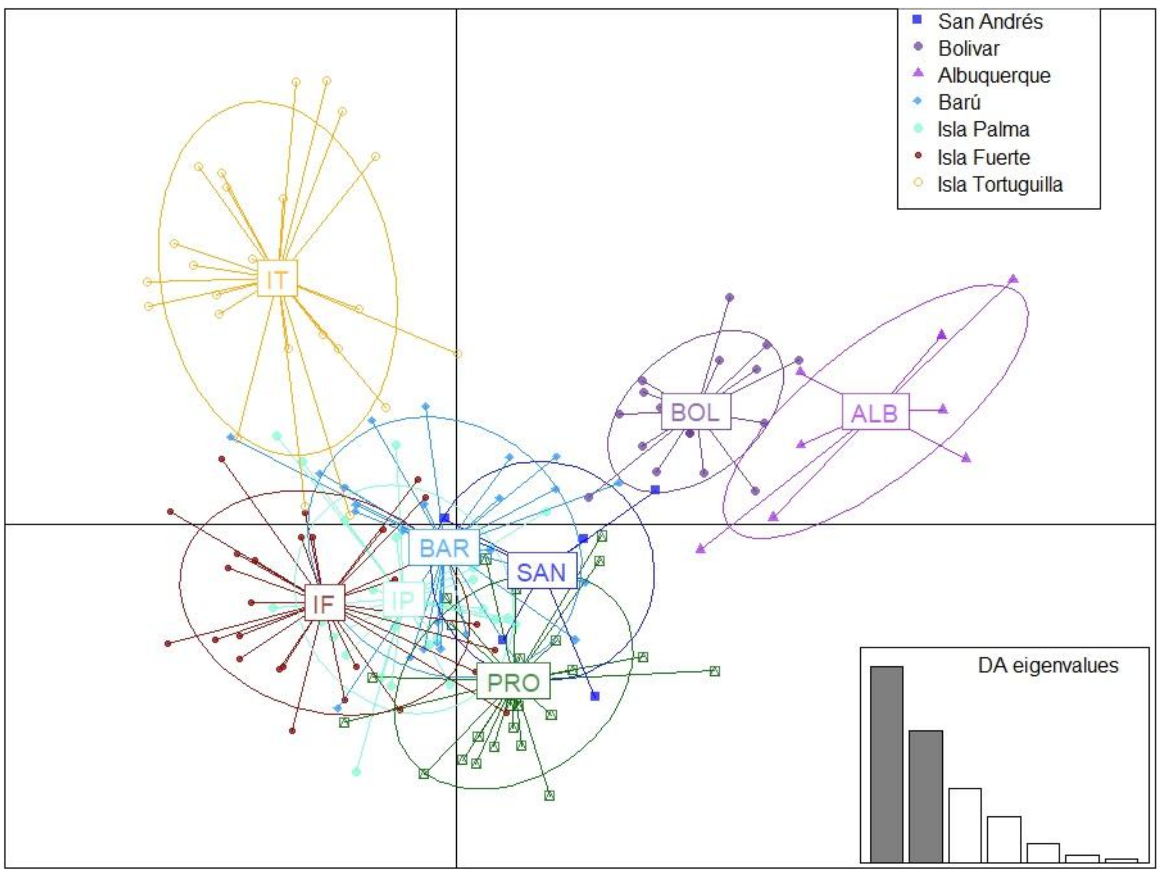
Figure 2-3. a) Mantel test, and b) Principal Coordinate Analysis of the eight geographic *T. testudinum* populations in the Colombian Caribbean.



a) Isolation by Distance for *T. testudinum* calculated through a Mantel test within the Colombian Caribbean, including oceanic and continental populations, and correlating F_{st} and *uNei* with geographical distance. b) Principal Coordinate Analysis (PCoA) employing microsatellite data and F_{st} for the eight populations.

In the DAPC **(Figure 2-4),** populations like Bolivar and Albuquerque remained isolated from the other populations, consistent with the PCoA results, but they appeared more structured in this analysis. Isla Fuerte and Old Providence were positioned close to the populations that comprise the IRSB cluster, despite slight graphical differentiation and higher membership coefficient (Supplementary material S12), indicating a high level of structuring. The analysis also highlighted the notable isolation of Isla Tortuguilla, which aligns with the STRUCTURE results.

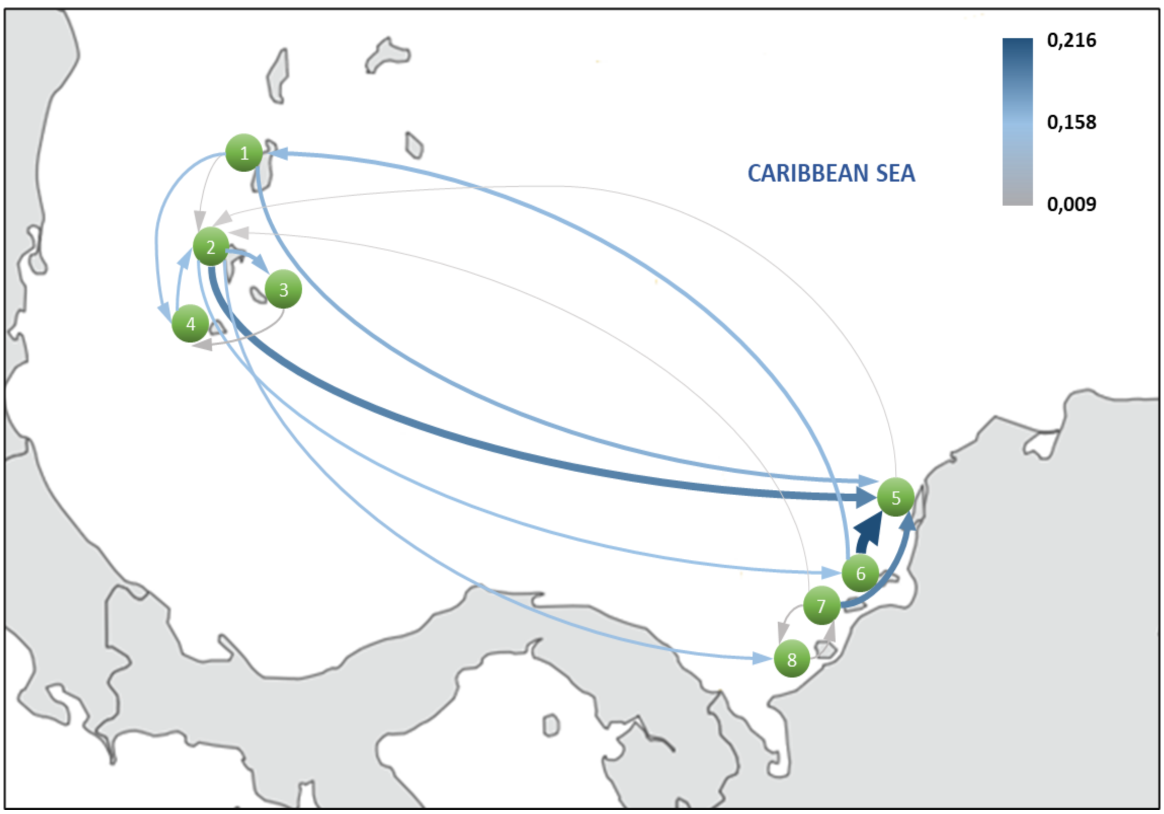
Figure 2-4. DAPC plotted with the first two principal components. Statistical description of the populations' variability with no HWE and LD assumptions.



2.3 Connectivity

Based on the analysis using BAYESASS across the eight original populations, the gene flow evidenced some significant population dynamics that differed somewhat from initial expectations but aligned with the outcomes of the population structure and genetic distance analyses. **Figure 2-5** illustrates both stronger and weaker gene flows among populations. Notably, gene flow analysis revealed asymmetric migration patterns, with stronger migration observed from oceanic to continental islands. Unexpectedly, the primary sources of migration were identified as Isla Palma (0.317) and San Andrés (0.309), while the main recipient of migration was Barú (5.535). Specifically, the highest gene flow was detected from Isla Palma to Barú (0.216), followed by San Andrés to Barú (0.132), Isla Fuerte to Barú (0.071) and Providencia to Barú (0.055). The reduced overall gene flow between the Seaflower Islands was notorious (0.022), contrasting with a slightly higher gene flow observed among the continental islands (0.036). Nevertheless, a significant gene flow was spotted from San Andrés to Bolívar (0.051) (Supplementary material S14).

Figure 2-5. Connectivity and gene flow estimated for each pairwise population of *T. testudinum* in the Colombian Caribbean (continental and oceanic islands), using BAYESASS.

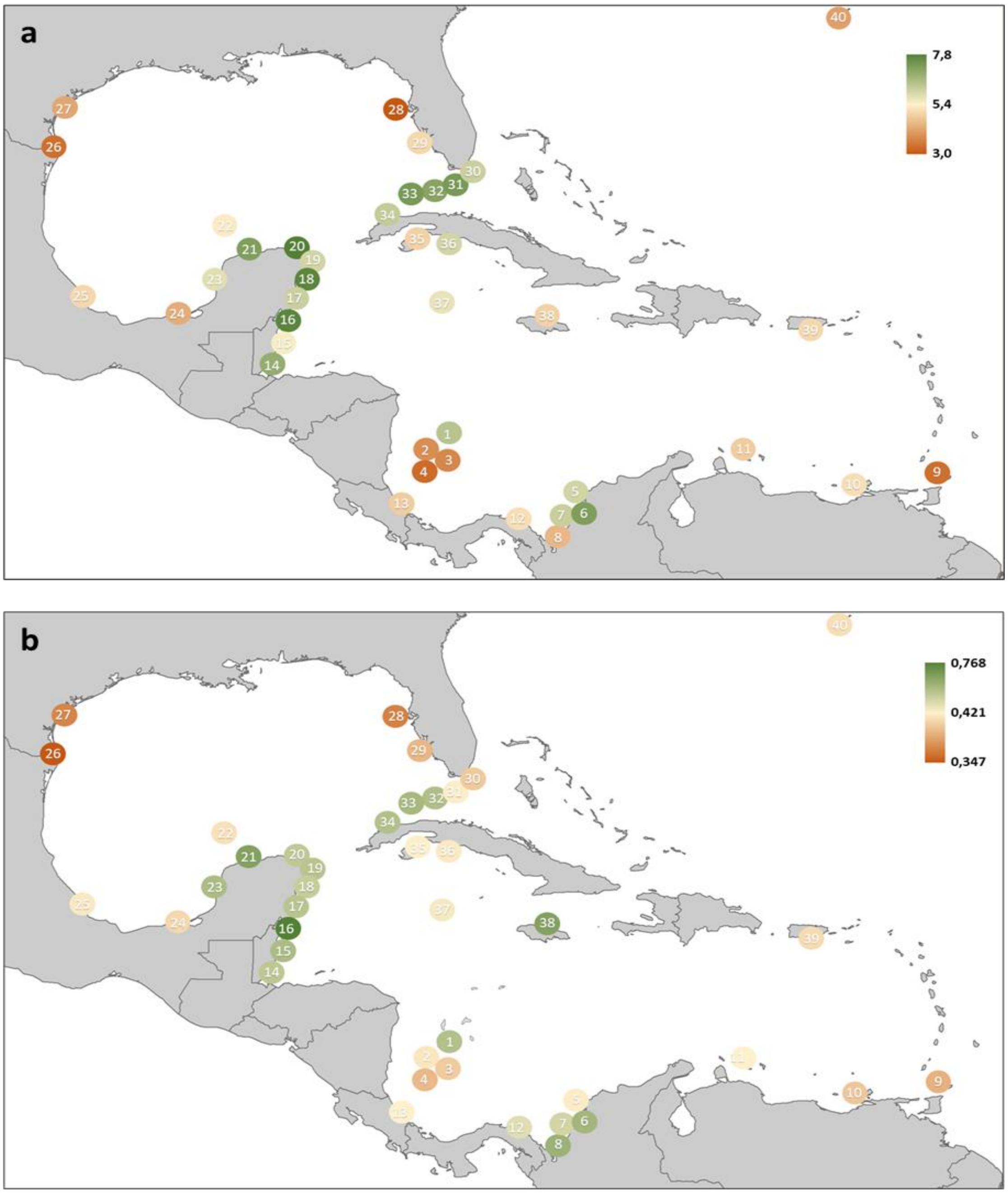


The proportion of gene flow depends on the colour and thickness of the arrows. Between darker blue and thicker is an arrow, the gene flow is higher. Grey-thin arrows represent the lowest gene flows assessed. 1. Old Providence; 2. San Andrés; 3. IC Bolívar; 4. IC Albuquerque; 5. Barú; 6. Isla Palma; 7. Isla Fuerte; 8. Isla Tortuguilla.

2.4 Regional status

The allelic richness (A_r) and the unbiased expected heterozygosity (uH_e) assessed in the national context, correlated with the Great Caribbean information provided by van Dijk et al. (2018) **(Figure 2-6)**, allowed a global vision of the genetic diversity and characteristics of *Thalassia testudinum* in its entire distribution range. The graphical representation clearly supported the hypothesis that populations external to or geographically isolated from central regions tend to exhibit lower allelic richness. Both A_r and uH_e exhibited consistent patterns, highlighting that populations from Southeast Mexico, Belize, South Florida, and Colombia (continental), which are more centralised located and interconnected within the species distribution range, demonstrated higher genetic diversity indicators. Other parameters such as clonal richness (R), observed heterozygosity (H_o), and F_{is} (Supplementary material S15), reinforced these findings. Specifically, F_{is} showed a direct correlation with clonal richness, indicating that highly clonal meadows may have lower inbreeding rates.

Figure 2-6. Regional comparison and recreation of the great Caribbean *T. testudinum* genetic diversity based on microsatellite data, including Allelic richness and Unbiased expected heterozygosity.



a) Allelic richness, b) unbiased expected heterozygosity Modified from van Dijk et al., (2018).. 1. Old Providence; 2. San Andrés; 3. IC Bolívar; 4. IC Albuquerque; 5. Barú; 6. Isla Palma; 7. Isla Fuerte; 8. Isla Tortuguilla; 9. Tobago; 10. Venezuela; 11. Curacao; 12. Panama; 13. Costa Rica; 14. Belize Pl; 15. Belize TC; 16. MX Chinchorro; 17. MX Cozumel; 18. MX Pto Morelos; 19. MX R Nizuc; 20. MX Pta Sam; 21. MX Chabihau; 22. MX Al Isla Perez; 23. MX Cd del Sol; 24. Mx Terminos Lag; 25. MX Veracruz; 26. US L Lag Madre; 27. US Redfish; 28. US Tampa; 29. US Rankin; 30. US Duck; 31. US Arsenicker; 32. US Craig Key; 33. US Fiesta Key; 34. Cuba R Guanabo; 35. Cuba R Luna; 36. Cuba P Ancon; 37. Cayman; 38. Jamaica; 39. Pto Rico; 40 Bermuda.

3. Discussion

3.1 Ecological characteristics

Based on the ecological characteristics described here, it was expected that meadows such as Tortuguilla, Barú, and San Andrés have lower population's "health", and oceanic unpopulated islands presented better conservation conditions. Tortuguilla, for example, showed significant levels of human pressures and physical degradation, which will be specially discussed in genetic terms to widely understand its population dynamics, risks and resilience.

Although visibility, shoot density and coverage are critical factors differentiating the ecological characteristics and anthropogenic pressures on *T. testudinum* meadows between oceanic and continental islands, the species also exhibits multi-temporal trends of shoot density and overall health (Galeano et al., 2016; Gómez-Cubillos et al., 2015; Gómez-López et al., 2020). These cited reports indicate that continental meadows have experienced a significant decline in shoot density from approximately 500 to around 150 since 2013, with a low incidence of infection by the fungus *Labyrinthula* spp. In contrast, meadows in the oceanic islands of the Seaflower Biosphere Reserve have maintained stable shoot density levels since 2014, despite a significant presence of the fungus. However, the impact of the fungus has decreased by half, (100% to ~50%) from 2014 to 2018.

The results of this study suggest that human activities seem to influence the combined effect that natural geographic distance, geological processes (i.e. continental vs oceanic islands) or ecological local aspects could have on the development of seagrass meadows in each island, creating groups depending on the level of human intervention, from unpopulated islands (e.g. Bolivar and Albuquerque) to highly intervened ones (San Andres and Baru). This general ecological view of environmental characteristics, supported by previous monitoring and health assessments of the meadows (Elizabeh Galeano et al., 2016; Gómez-Cubillos et al., 2015; Gómez-López et al., 2020; Gómez-López & Alonso, 2016), evidences the differential features of each population in a specific moment or short timeline. Consequently, the integration of ecological methods with geological understanding and genetic information from this paper, enhances our knowledge and management capabilities for this critical ecosystem, through a genetics-supported marine spatial planning approach (Kershaw et al., 2021; Riginos & Beger, 2022).

3.2 Genetic diversity

The general genetic diversity indices reveal interesting recent and historical processes in *T. testudinum* populations across the Colombian Caribbean, with significant differences between oceanic and continental islands. Of the 79 clones identified in the sample set, 64 were from the oceanic populations and only 15 from the continental populations, indicating that clonality is an effective survival and population establishment strategy, particularly when gene flow is more stochastic. Previous studies have documented extremely large turtle grass organisms, with individuals reaching lengths of 230 meters or more in Mexico (van Dijk & van Tussenbroek, 2010), and a clone extending over 750 meters in Bermuda, the most isolated island within the species' distribution range (van Dijk et al., 2018).

In San Andres Island, we identified 24 ramets of the same multilocus genotype (MLG) along a stretch of approximately 400 ± 30 meters, suggesting that this individual can be even longer and dispersed over a vast area. Using rhizome elongation rates from van Dijk & van Tussenbroek (2010), we estimated the age of this clone to be between 560 and 1040 years. Galeano (2012) suggested that even within a single geographic population, such as that of San Andres Island, turtle grass meadows can exhibit different rates of sexual reproduction depending on environmental conditions and anthropogenic pressures, such as mechanical or chemical stress.

Although *T. testudinum* is a dioecious species with small genetic neighbourhoods, and is subject to various pressures including mechanical disturbances, pollution, and biological threats; it also possesses mechanisms to avoid bi-parental inbreeding. This may be due to selection against homozygotes during sexual reproduction (van Tussenbroek et al., 2016).

In other cases of clonality, such as *Posidonia australis* Hook.f., individuals extend up to 180 km due to polyploidy and vegetative structure dispersion along the same coastline, with no registers of clones between ocean-isolated populations (Edgeloe et al., 2022).

The allelic richness was lower in oceanic islands (overall A_r = 4.4) compared to the continental ones (overall A_r = 6.0), as was the overall uH_e (0.64 vs 0.71). The oceanic populations appear to be genetically expanding, as indicated by an excess of heterozygotes and negative inbreeding coefficients (F_{is}), with significant Chi-squared values for San Andres and Albuquerque (Supplementary material S15). Old Providence Island exhibited similar genetic indices to those of continental populations, likely due to its greater age and the timing of current reef formation. Old Providence, dating back 23-15 million years (Mya) in the early Miocene, is significantly older than the other oceanic islands, which date from the middle Miocene to Pliocene (15-2.5 Mya) (Carvajal-Arenas & Mann, 2018; Geister & Díaz, 2007; Geister, 1972, 1992), and to the continental islands, which date from the early Pliocene (about 5 Mya) (Díaz et al., 2000; López-Victoria et al., 2000). Thus, Old Providence likely hosts a large and highly diverse *T. testudinum* meadow, probably due to colonization before the Last Glacial Period (LGP) and subsequent vertical migration with sea level changes, suggesting that it may be the oldest population in the Colombian Caribbean.

In contrast, Tortuguilla exhibited the lowest allelic richness among the continental islands, with other genetic diversity indices resembling those of the southern oceanic islands of the Seaflower Biosphere Reserve. This suggests that Tortuguilla may be experiencing geographic isolation due to natural barriers or decline in “genetic health” driven by intense anthropogenic pressures, as suggested by the PCA**.** As a result, the relationship between allelic richness (A_r), observed heterozygosity (H_o) and the deficit or excess of heterozygotes is not linearly correlated with the age of the islands or the timing of reef formation. These factors must be analysed in conjunction with oceanographic conditions and potential anthropogenic pressures. Albuquerque and Bolivar, unpopulated and oceanic islands, also showed low genetic diversity indices as they are small and geographically isolated islands. In all these cases, the meadows may expand for several meters, to compete with other organisms for space, but they may lack the genetic diversity to sustain strong disturbances (e.g. hurricanes or diseases).

3.3 Population structure

The genetic structure of the Colombian *T. testudinum* populations is shaped by a complex interplay of historical and recent colonisation events, distinct oceanic and continental conditions of the islands, as well as contemporary anthropic influences. The strong population structuring (*K*=5) identified by both the Puechmaille (2016) and Evanno et al. (2005) methods in STRUCTURE **(Figure 2-2** and Supplementary material S6) provides several key insights: (1) Providence is identified as an ancient, independent population, potentially representing the earliest studied colonization site by the species prior the LGP. (2) The ICS population, comprising the Bolivar and Albuquerque Islands Cays, consists of two young, geographically small (limited coastline expansion), and less genetically diverse oceanic meadows. This population likely consolidated no earlier than 20,000 years ago during the end of the LGP (Geister & Díaz, 2007; Díaz et al., 2000). (3) The IRSB population, surprisingly composed of San Andrés (oceanic), Isla Palma and Barú (continental), reflects different establishment times for turtle grass populations. San Andrés dates back to around 20,000 years ago, while Barú and Isla Palma were established between 5,000 and 3,000 years ago (Cendales et al., 2002; Díaz et al., 2000). (4) Isla Fuerte and Isla Tortuguilla are identified as independent populations with similar ages to those of the other two continental islands, suggesting the presence of significant geographic barriers along the coastline.

In addition to the genetic diversity, population structure also differentiated these environments, with oceanic populations tending to be less diverse and more structured, a pattern observed in other sessile species (Ballesteros-Contreras et al., 2022; Bijak et al., 2018). The average membership coefficient in the oceanic islands is 0.85, compared to 0.76 in the continental islands (less structured), indicating higher connectivity in the latter ones. Excluding Isla Tortuguilla from the continental analysis, due to its evident genetic isolation, the coefficient decreases to 0.72. While Isla Tortuguilla shows a geographically logical connection with Isla Fuerte in *K*=3 (Supplementary material S6), its genetic composition and allele frequencies differ significantly according to STRUCTURE's analysis at K = 5, and it exhibits lower genetic diversity compared to the other continental islands.

Regarding the IRSB population, the close genetic relationship between San Andres and the continent may be explained by natural connectivity driven by ocean currents and biological agents, as turtle grass is known to have high dispersal potential (Kendrick et al., 2012; Ruiz-Montoya et al., 2012; van Dijk et al., 2009). Nevertheless, historical human maritime navigation has also significantly influenced the contemporary population genetics of sessile or sedentary marine organisms, reducing the impact of geographic barriers and altering dispersal potential significantly (Reem et al., 2023; Hudson et al., 2016). Maritime routes between the Archipelago (San Andres and Old Providence islands) and Colombian Continental Caribbean have existed since the XVI century, intensifying during the 1600s due to the British-Spanish conflicts for power, and further increasing in the early 1800s when the Archipelago was claimed by the Spanish crown (James, 2007; Mantilla Valbuena et al., 2016). More recently, the government declaration of the San Andres Department as a freeport in 1953 (James, 2007) led to a significant increase in maritime traffic between San Andres and Cartagena. While the Puechmaille (2016) method effectively determines the number of clusters without bias due to the number of genets per population, further analyses are necessary to corroborate this hypothesis and better understand the specific connectivity between these populations.

The results for genetic distance, F_{st} and Nei's unbiased genetic distance (*uNei)* were consistent with the STRUCTURE outcomes, though some differences were observed. Given the potential underestimation of genetic distance by F_{st} in clonal plants, caused by the longevity and intergenerational overlap of clones, which can persist for decades or even centuries (van Dijk et al., 2018), *uNei* (Nei, 1978; Peakall & Smouse, 2012) was employed to complement the population structuring analyses. This method revealed significantly higher differentiation among populations compared to F_{st}. However, the Mantel test results for Isolation by Distance (IBD) using both indices were nearly identical, showing no significant correlation between genetic and geographic distance among the Colombian populations. This was expected given the population structure described by ΔK in the STRUCTURE test**.** Previous studies on *T. testudinum* have shown a significant increase in genetic differentiation with geographical distance (van Dijk & van Tussenbroek, 2010; van Dijk et al., 2018), particularly when populations were assessed within continuous coastal areas or across the species' entire distribution range.

The main discrepancies between *uNei* - F_{st} and the STRUCTURE clustering involve the closeness of Old Providence, and to some extent Bolivar, to the continent. This suggests that, despite the identified population structuring, the genetic differentiation among the regions might not be as pronounced as expected, indicating significant connectivity within the species (van Dijk & van Tussenbroek, 2010; Bricker et al., 2011; Campanella et al., 2015; van Dijk et al., 2018). Then, the AMOVAs conducted for eight populations and five clusters, revealed low differentiation among populations, with values ≤12%. Interestingly, although Albuquerque and Bolivar are considered a single population at K=3, and Bolivar is the closest population to Albuquerque in the F_{st} – *uNei* analysis, they exhibit higher differentiation values than populations from different clusters, such as Isla Fuerte compared to Barú and Isla Palma, which may reflect the importance of the geologic formations age in the structure understanding.

The Principal Coordinate Analysis (PCoA) based on pairwise F_{st} also indicates that populations and regions are not significantly isolated, though some trends are apparent: i) Bolivar and Albuquerque appear closely related to each other and distinct from the other populations, ii) Tortuguilla is widely scattered on the graph and does not appear isolated, suggesting changes in intra population genetic dynamics or recent isolation, iii) Old Providence shows unique dynamics but is closely related to continental islands, iv) San Andres has few genets but is related to both continental populations and the ICS, and v) Isla Fuerte does not seem to function as an independent population.

The results from DAPC (which does not assume Hardy-Weinberg equilibrium (HWE) or linkage disequilibrium (LD)), PCoA and STRUCTURE (which assumes *HWE* and *LD*) were consistent in describing the population differentiation of *Thalassia testudinum* across Colombia, despite some specific differences. The most notable difference pertained to Isla Tortuguilla, which exhibited significant signs of isolation and population bottleneck, likely due to recent isolation driven by both natural and anthropogenic impacts.

Other seagrass species in the Caribbean, such as *Halodule wrightii* Asch. (Angel, 2002; Tavares et al., 2023), and *Syringodium filiforme* Kütz. (Bijak et al., 2014, 2018) also exhibit asymmetric genetic structure that do not completely align with oceanographic patterns. This suggests that biological factors play a significant role, although anthropic transport was not mentioned in these studies. Research on *T. hemprichii* (Hernawan et al., 2017; Nakajima et al., 2023) has also highlighted the role of stochastic processes in the dispersal potential and species resilience. In contrast, other sessile or movement-restricted species, such as the coral *Madracis auretenra* Locke, Weil & Coates, 2007 (Ballesteros-Contreras et al., 2022) and queen conch *Lobatus gigas* (Linnaeus, 1758) (Truelove et al., 2017), show greater genetic differentiation, IBD and isolation by barriers. These differences may be attributed to their shorter life cycles and generations.

It is accurate to conclude that *T. testudinum* populations across Colombian and the wider Caribbean are not panmictic, despite being highly connected due to long generation times and clonal reproduction. This comprehensive analysis of various studies at different geographic scales not only enhances our understanding of genetic diversity and connectivity across diverse environments but also support the formulation of effective management policies for *T. testudinum* populations and ecosystems. This is increasingly important in the face of environmental changes and anthropogenic pressures.

3.4 Connectivity and gene flow

The results of the gene flow analysis align well with the STRUCTURE outputs, despite the known tendency of BAYESASS to overestimate contemporary gene flow (Samarasin et al., 2017) (**Figure 2-5** and Supplementary material S14). The absence of IBD may be attributed to a combination of oceanographic conditions, biological dispersal mechanisms, recent anthropogenic-driven dispersal, and possibly ancient step-by-step (re)colonization events followed by local recruitment and clonal growth (Arnaud-Haond et al., 2014). This suggests that genetic connectivity between San Andres and the continental Caribbean could result from both natural historical processes and anthropic contemporary activities, such as vessel historical traffic in the region (James, 2007; Mantilla Valbuena et al., 2016), which may enhance the seeds and rhizomes flow. These findings highlight the influence of both microevolutionary and macroevolutionary time scales on the geographic dispersal of *T. testudinum* (Emerson, 2002). Nevertheless, given the clonal richness (*R*) found in the San Andres, and the significant gene flow between this region and the continent, as well as with Old Providence, it is necessary to further investigate the connectivity between both regions and corroborate the hypothesis of recent gene flow (≤ 500 ya) derived from the anthropic activity. This gene flow observed from Old Providence Island should also be considered carefully to avoid population assignment artifacts, which could arise from the small sample size analysed in San Andres. Furthermore, the reduced gene flow between San Andres and Providencia Islands may be because of the CCC (Caribbean Central Current) effect, which acts as a natural barrier between the central and south zones of the Seaflower BR (Ballesteros-Contreras et al., 2022; Benavides, 2020).

Although this gene flow analysis focused only on Colombian seagrass meadows, the genetic diversity characteristics of Costa Rica and Panama populations, as described by van Dijk et al. (2018), suggest a logical oceanic and genetic flow from the Archipelago to the continent, that supports our current analysis. The results indicate that gene flow is stronger from the oceanic islands towards the continent, possibly reflecting a north-south migration of the species that may have reinforced by the final formation of the Isthmus of Panama, 2,8 Mya. This event constricted oceanic barriers, such as seaways, and led to the formation of the Colombia-Panama Gyre (Andrade, 2015; O'Dea et al., 2016) that facilitates the current gene flow towards the continental islands. Furthermore, the contemporary water circulation patterns in the Western Caribbean, characterised by sea currents, cyclonic and anti-cyclonic spins, temperature variations and kinetic energy differences, support the observed coherence between genetic and oceanographic flows (Lopera et al., 2020).

3.5 Bottlenecks

Two populations exhibited noteworthy genetic patterns, suggesting the possibility of bottlenecks. The Bolívar population displayed characteristics such as excess of heterozygotes (H_o>uH_e but not significant Chi^2), low N_a (34 alleles) and A_r (3,8), negative F_{is} (-0,037), and a significant result in the two-phase model (TPM) Wilcoxon's test for bottlenecks (P = 0.007). These indicators suggest a current founder effect, which aligns with the limited geographic extension, the specific ecological features of the meadow, and the potential recent colonization of the species on the island. This premise is based on the concept that the establishment of a new population often leads to a reduction in genetic diversity and a tendency towards excess heterozygosity (Balloux, 2004; Gallego-García et al., 2018). In Bolivar, this is reflected in the low number of individuals but a low level of inbreeding.

The continental islands shared similar genetic features, including clonal (*R* ranging from 0,793 to 0,996), maximum probability of genotypic diversity (P_{gen} max) and clonal diversity (P_{sex} max), and observed heterozygosity ($H_o$ranging from 0,641 to 0,736). However, Isla Tortuguilla exhibited notable lower values for N_a (41 vs. 60 overall) and A_r (4,6 vs 6,5 overall), a significant TPM Wilcoxon's test for bottlenecks (P = 0.002), and the highest F_{is}

(0,066), although this was not statistically significant ($F \leq 0,125$). These findings indicate a potential bottleneck reflecting the consequences of recent isolation.

3.6 Great Caribbean context

The genetic indices of Colombian *Thalassia testudinum* populations fell within the range reported by van Dijk et al. (2018) (**Figure 2-6** and Supplementary material S15). The average unbiased expected heterozygosity (uH_e) for Colombian meadows was 0.68, compared to an average of 0.66 across the entire species distribution range. Although the IBD Mantel test was not significant within the Colombian distribution range, likely due to different biotic and abiotic dynamics, such as sea currents, populations' relatedness, and geographic distances between locations (oceanic and continental), van Dijk et al. (2018) confirmed the central-marginal hypothesis of genetic variation (Eckert et al., 2008a) across the entire *T. testudinum* geographical range. Colombian populations fit this hypothesis coherently when analysed regionally **(Figure 2-6),** based on allelic richness (A_r) and unbiased expected heterozygosity (uH_e). Similarly to its sister species, *T. hemprichii*, the genetic diversity of *T. testudinum*'s tends to decrease in peripheral populations but is not correlated with high latitudes or temperature variability (Hernawan et al., 2017; Nakajima et al., 2023). Populations that are geographically "central" but are isolated, such as those in small or oceanic islands, exhibit low values of these genetic metrics, possibly due to the stochastic effects on gene flow (Emerson, 2002). This pattern is also observed in other seagrass species (Serra et al., 2010; Alberto et al., 2006).

The significant direct correlation observed between clonal richness (R) and inbreeding coefficient (F_{is}) at both the regional (Caribbean) and national (Colombian populations) scales suggest that clonality on its own is not a promoter of inbreeding. This observation is consistent with other studies in the Mediterranean Sea, which also found a positive correlation between clonal indices and F_{is} (Arnaud-Haond et al., 2020). Clonality is an effective strategy for colonising and establishing a population in new environments with few individuals. It supports long-term generations that can exceed 600 years (van Dijk & van Tussenbroek, 2010), facilitating the development of meadows and enabling sexual reproduction with new migrants through stochastic events (Arnaud-Haond et al., 2020; Kendrick et al., 2012).

The analysis of A_r and uH_e**,** revealed several high-diversity core zones within the species' range. These zones, which appear geographically central and possess continuous meadows, support the gene flow across the populations. Notable examples include the Belize and Yucatan Peninsula (pops 14-23), Florida Cays and north Cuba (pops 30-34) and the Colombian continental Caribbean (pops 5-8), including the Guajira population, the largest in the country but not analysed in this study. These areas could be considered potential genetic reservoirs for the species. Additionally, Islands like Old Providence (pop 1) and Jamaica (pop 38), with high values of R, A_r and H_e, but also with some degree of geographic isolation, may also serve as genetic reservoirs based on their historical role as stepping-stone in dispersal (Nakajima et al., 2023). This stepping-stone behaviour of the Colombian oceanic islands has also been identified for other sessile species (Ballesteros-Contreras et al., 2022; Benavides, 2020).

4. Conclusion and recommendations

4.1 Conclusions

The analyses of genetic diversity, structure, and connectivity presented in this study offer crucial insights for the conservation of *T. testudinum* meadows and their associated ecosystems. While previous research identified two primary genetic clusters across the species' range (van Dijk et al., 2018), this finer-scale analyses reveals more specific details relevant to conservation efforts in Colombia, similar to findings in Mexico and Florida (Bijak et al., 2018; Bricker et al., 2011). Colombian populations were clearly structured into five distinct clusters: three for oceanic islands and two for continental islands, with one shared cluster. Genetic distance was lower among continental populations, which exhibited significant isolation by distance following the bidirectional coastal currents. In contrast, connectivity was more restricted in the oceanic islands, where dispersal across open waters and the effect of the Caribbean Central Current (CCC) likely reduces successful settlement, resulting in lower genetic diversity. Notably, San Andrés and Old Providence showed greater connectivity with the continental islands than between them or the closer Albuquerque and Bolívar islands, proving the effects of the currents, and other natural processes, on the gene flow. These nuanced genetic insights can guide targeted management strategies to preserve the resilience of these *T. testudinum* meadows.

The genetic diversity of *T. testudinum* populations is heavily influenced by historical and geological factors, such as other seagrass species like *Posidonia australis* and *Thalassia hemprichii* (Hernawan et al., 2017; Sinclair et al., 2023). For instance, the older oceanic island of Old Providence, with its volcanic fine sediments, exhibits similar genetic diversity indices to those of continental islands. Oceanographic processes such as currents, waves, and winds promote genetic exchange, particularly in continuous coastal areas such as Barú, Isla Palma, and Isla Fuerte. However, coastal barriers, such as river mouths, sedimentation, and discontinuous shorelines, can significantly isolate populations, as observed with Isla Tortuguilla. This study supports the central-marginal hypothesis (Eckert et al., 2008b) discussed in previous studies, by enhancing the population genetics map of *T. testudinum* populations and identifying areas of high allelic richness (A_r) and expected heterozygosity (H_e) across the Caribbean, with the Colombian coast highlighted as a significant hotspot. Additionally, human activities have detrimental effects on population coverage and evolutionary potential, limiting opportunities for genetic exchange through emigration and new propagule settlement. Despite these challenges, maritime transport appears to have facilitated genetic flow, with San Andrés and Providencia playing key roles in contributing migrants to the continental islands. The role of biological vectors, such as animals that inhabit or feed on seagrasses, has been noted but remains unquantified.

Thalassia testudinum exhibits high genetic diversity and connectivity between populations, despite relatively low migration rates. This is attributable to its clonal growth, extremely long lifespans, avoidance of inbreeding, and potential for long-distance dispersal. As a result, the species has persisted in the Caribbean for hundreds of thousands, or even millions, of years, withstanding natural events like the Last Glacial Period and adapting to changes in sea level and temperature. However, the species' long generational times make it vulnerable to accelerated environmental changes caused by human activities. This risk is even higher in oceanic populations, where the evolutionary potential is reduced and recovery from major disturbances is difficult without local recruitment. It is crucial to assess and monitor human impacts on these ecosystems to ensure their protection and sustainability.

4.2 Conservation implications

Mortiz, (1994), distinguishes populations that are demographically independent and exhibit significant divergence in allele frequencies as Independent Management Units (IMUs), which are crucial for short-term conservation efforts. Based on this definition, this study identified five IMUs for *Thalassia testudinum* along the Colombian Caribbean. Although *T. testudinum* is currently categorized as "least concern" by the IUCN (Short et al., 2010), our findings underscore the need for targeted conservation efforts at the national level, encompassing both continental and oceanic environments and including this information in marine spatial planning strategies. National conservation initiatives should be coordinated with ongoing national and regional efforts, such as the bi-annual monitoring of seagrass status conducted by INVEMAR. These findings highlight the importance of protecting not only the core populations with high genetic diversity but also the peripheral and isolated populations that contribute to the species' overall genetic resilience. Special attention should be given to Tortuguilla Islands due to the recent and intense isolation identified, which indicates a significant effect of anthropogenic impacts; and the oceanic islands, where populations are more vulnerable due to their lower genetic diversity and reduced connectivity, particularly considering their importance as stepping-stones in the Caribbean Sea and as a source of gene flow. Effective conservation strategies should aim to preserve the genetic integrity of these populations while facilitating natural processes of gene flow and recolonisation.

Standardised periodical genetic monitoring should be implemented in the Colombian Caribbean, aligned with regional initiatives, to inform and support the conservation and restoration strategies where needed (Konefal et al., 2024; Pazzaglia et al., 2021). Finally, complementary monitoring approaches such as remote sensing (Veettil et al., 2020), seagrass loss impact assessments (Roig-munar et al., 2021), and ecosystem services economic valuation (Serrano et al., 2021), should be continuously conducted and analysed altogether to provide integral management inputs and conservation effectiveness balances.

A. Annex: Supplementary Material

Microsoft Excel document that contains tables, figures and information as follows:

S1. General information related to Table 2-2

S2. Null Alleles

S3. Hardy Weinberg Equilibrium (HWE) and Linkage Disequilibrium (LD) 8 populations

S4. Hardy Weinberg Equilibrium (HWE) and Linkage Disequilibrium (LD) 5 clusters

S5. Rarefaction to 15 samples per population

S6. Population structure table, figures and tests of Evanno and Puechmaile

S7. Mantel test figures and tables based on F_{st} and Nei genetic distance vs geographic distance

S8. Analysis of Molecular Variance (AMOVA) statistical test for 8 populations

S9. Analysis of Molecular Variance (AMOVA) statistical test for 5 clusters

S10. Principal Coordinate Analysis (PCoA) statistical test and figures of the genetic distance of 8 populations

S11. Principal Coordinate Analysis (PCoA) statistical test and figures of the genetic distance of 5 clusters

S12. Discriminant Analysis of Principal Components (DAPC)

S13. Bottleneck test by population

S14. Bayesian analysis of gene flow between the 8 populations

S15. Regional (Great Caribbean) comparisons including Colombian populations and van Dijk et al., 2018 reported populations.

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