Distribution, Relative Abundance, and Intraspecific Genetic Variation of Horseshoe Crabs in the Philippines using environmental DNA

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Purposes

Horseshoe crabs, often called "living fossils," have remained largely unchanged for over 350 million years (Chatterji 1994). Of the four extant species, three inhabit the Indo-Pacific —*Tachypleus gigas*, *Tachypleus tridentatus*, and *Carcinoscorpius rotundicauda*, with *T. tridentatus* and *C. rotundicauda* recorded in Palawan, Philippines (Baylon & Alcantara-Creencia, 2022; Pedrosa-Gerasmio et al., 2025). Historical data also suggest their presence in the Sulu Sea (Laurie et al., 2019).

Horseshoe crabs play vital ecological roles, supporting nutrient cycling (Luo et al., 2020), enhancing biodiversity (Kondo et al., 2018), and providing food for migratory shorebirds (Novcic et al., 2015). However, habitat destruction, overharvesting, and pollution have led to population declines (Wang et al., 2020). *T. tridentatus* has been classified as endangered by the IUCN since 2019 (Laurie et al., 2019), while *C. rotundicauda* remains data deficient, though reports indicate declines in other countries (Shin et al., 2009; Morton & Lee, 2010).

Given the urgency of conservation, understanding their distribution, abundance, and genetic diversity is crucial. Environmental DNA (eDNA) offers a non-invasive, efficient method for assessing genetic variation and abundance (Sigsgaard et al., 2015; Thomsen & Willerslev, 2015). This study proposes using eDNA technology to determine the distribution, relative abundance, and intraspecific genetic variation of horseshoe crabs in the Philippines, particularly in Palawan and historically documented areas.

Methods

Water Sample collection and eDNA extraction

Sampling was conducted in August for the Palawan sites and in September for Tawi-Tawi (Figure 1). At each sampling site, three 1 L seawater replicates were collected using sterile bottles. Each water sample was then filtered onto 47 mm Mixed Cellulose Ester membrane filters with a 0.45µm pore size. DNA bound to the filter membranes was extracted using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's protocol, with some modifications. DNA extraction controls was included for each set of extractions.

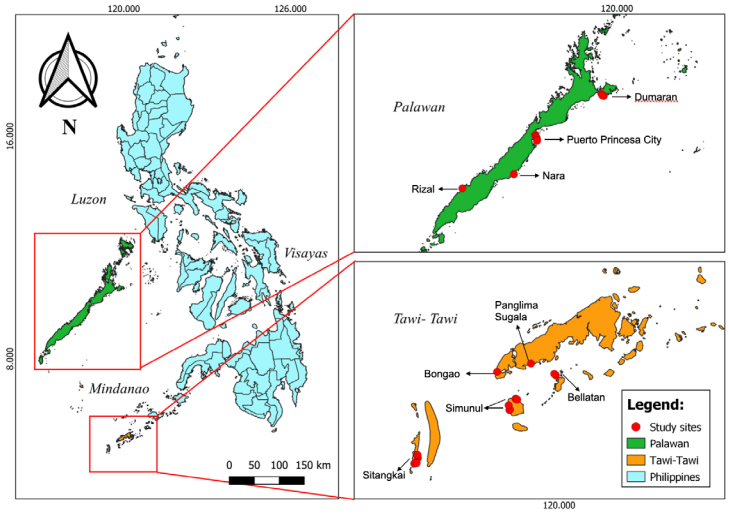


Figure 1. Map of the sampling sites in Palawan Island [Dumaran: Santa Teresita (ST), Bacao (BA), San Juan (SJ); Puerto Princesa City: Santa Lourdes (SL), Tagburos (TAG), San Jose (PSJ) and San Pedro (SP); Rizal : Punta Baja (PB); and, Nara (NA)] and Tawi-Tawi Island [Simunul: Panglima Mastul (PM), Bakong (BAK), Tonggusong (TG); Sitangkai: Panglima Alari (AL), Tumindao (TOM), Poblacion (POB); Bellatan Talinga: Belatan Stations 1, 2 and 3 (BSt1, BSt2 and BSt3, respectively; Panglima Sugala (Pangsu) and Sanga-Sanga, Bongao (Dormitel)] Philippines.

Laboratory analysis

All laboratory works (Figure 2) were performed under the guidance of Dr. Kozo Watanabe at the Molecular Ecology and Health (MECOH) Laboratory, Center for Marine Environmental Studies (CMES), Ehime University, Matsuyama, Japan. PhD student Dan Joseph Logronio supervised laboratory protocol implementation at MECOH Laboratory. Travel funds and laboratory supplies were provided by LaMer and MECOH Laboratory.

Figure 2. Images from the MECOH Laboratory during laboratory analysis and eDNA library preparation (a–d) and (e) after a research consultation with Dr. Kozo Watanabe.

End-point and Quantitative PCR

End-point PCR was initially performed for the positive control samples to assess the three qPCR primer pairs used for detecting the presence of horseshoe crabs at the study sites. These primers targeted the following mitochondrial DNA regions: 16S, COI, and 12S (Table 1). The 16S primer pair was then used to check amplification of all the samples using end-point PCR before performing quantitative real-time PCR (qPCR).

Table 1. *Primers used in this study.*

Primer Name	Primer Sequence (5'-3')	Application	Reference
16S_HsIPG-F	GAGGTCGCAAACTTCTTCGC	qPCR	This study
16S_HsIPG-R	TGGGGCGGTAGAGAAAGAGA	qPCR	This study
Ttcox1_F	TAACTGACTAACCCCATTAATAT	qPCR	Koyama et al., 2022
Ttcox1_R	CTAAAGGAGGATAAACTGTTCATC	qPCR	Koyama et al., 2022
12S_HsIPG-F	TGTGTACATGCTTTAGAGCCACA	qPCR	This study
12S_HsIPG-R	TGATGTCAGGTCAAGGTGCAG	qPCR	This study
eDNA16S_HsIPG-F	ACACTCTTTCCCTACACGACGCTCTTCCG ATCTGAGGTCGCAAACTTCTTCGC	eDNA library preparation	This study
eDNA16S_HsIPG-R	GTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTTGGGGCGGTAGAGAAAGAGA	eDNA library preparation	This study

For qPCR, the standard curve was generated using a serial dilution of gDNA extracted from the spine of *C. rotundicauda* (10^{0}-10^{-5}). All standards and samples were run in duplicate. Together with the standards and samples, negative (blank) and positive controls (eDNA from water containing horseshoe crabs) were included. Amplification was performed with BioRad CFX96 real-time PCR system using the following conditions: 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 65°C for 5 s and 72 for 5s.

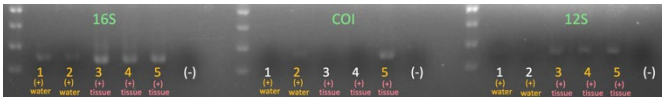
eDNA Library Preparation, Sequencing and Analysis

For eDNA library preparation, the 16S region was selected as the target region. Only the positive control libraries (HS2 – water from a pail with two horseshoe crabs (HS) and HS6 – with 6 HS) were successfully amplified, so we proceeded with NGS alongside fish and decapod samples. Bioinformatics analysis was conducted using R and RStudio.

Results

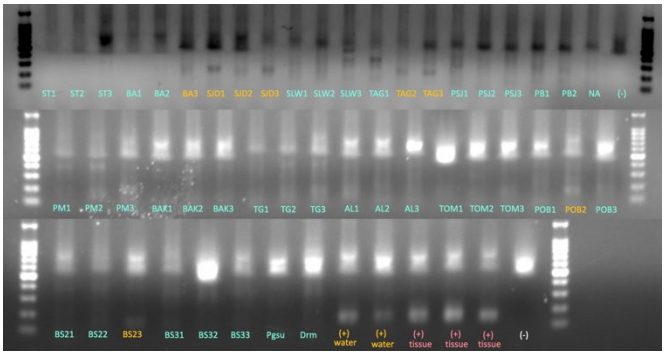
Among the primers used in this study, the 16S primer pair consistently produced positive bands across all tested horseshoe crab samples (Figure 3) and was subsequently used to screen the remaining samples (Figure 4).

Figure 3. Agarose gel electrophoresis results of end-point PCR for three markers.



Of the tested samples, only those labeled in yellow (Figure 4) showed potential positive results. All positive controls displayed clear bands.

Figure 4. Agarose gel electrophoresis results of end-point PCR of samples from Palawan and Tawi-Tawi Islands. Kindle refer to Figure 1 for the labels.



In the qPCR analysis using the 16S primer, no target DNA was detected in any samples (0 ng/µl), except for the positive control (HS6) at 5.82 ng/µl, suggesting a need to refine the sampling protocol due to the rarity of horseshoe crabs. We confirm the presence of *Carcinoscorpius rotundicauda* in Tawi-Tawi, as wild individuals were briefly housed in a tank by local collaborators. These specimens served as positive controls using (1) spine tissue and (2) eDNA water samples from pails containing two (HS2) and six (HS6) individuals. All pail water samples showed detectable DNA, likely due to higher genetic material concentration in a confined volume.

For the eDNA analysis, we identified the amplicon sequence variants (ASVs) obtained from samples HS2 and HS6. A total of 12 ASVs were detected, six of which closely matched horseshoe crabs. Among the non-horseshoe crab ASVs, two were identified as bacteria, two were unknown, one was a virus, and one was a nematode. However, the read counts for these non-horseshoe crab ASVs were all below 30, having minimal impact on the total read count. Five ASVs showed significant similarity to a known sequence (JX437074.1) in the NCBI database, with sequence identity ranging from 98.14% to 99.32%. One ASV matched *Tachypleus gigas*, a species not found in the Philippines, but with a lower sequence identity of 88.89% and fewer than 30 reads.

The results also serve as a proof of concept for using eDNA to detect horseshoe crabs, demonstrating that higher organism density in the water leads to increased DNA detection. HS6 had a higher read count than HS2 (Table 2), supporting this idea, as HS6 contained six individuals in the pail during sampling, while HS2 had only two.

Table 2. *The read counts for the ASVs detected for from HS2 and HS6 eDNA samples.*

ASV	HS2	HS6	Identification	Closest Match	% Identity	Length (bp)
1	32391	89286	*C. rotundicauda*	JX437074.1	99.32%	147
2	1166	11204	*C. rotundicauda*	JX437074.1	98.64%	147
3	116	287	*C. rotundicauda*	JX437074.1	98.14%	161
4	71	175	*C. rotundicauda*	JX437074.1	98.65%	147
5	30	106	*C. rotundicauda*	JX437074.1	98.65%	147
6	0	26	*T. gigas*	MK595787.1	88.89%	164
Total	**33774**	**101084**				

Future challenges

This study confirms the presence of horseshoe crabs in Tawi-Tawi, expanding their known range beyond Palawan, Philippines. While eDNA from wild populations was undetected, likely due to low density, our findings highlight the need for improved sampling methods. We recommend collecting water samples only when live individuals are observed and enhancing DNA capture by increasing water volume or refining filtration. While this approach relies on the presence of horseshoe crabs at the time of sampling, it remains a non-invasive strategy that helps protect these rare and endangered species. Furthermore, exploring the use of different primer lengths could help determine the optimal size for eDNA detection, potentially increasing sequence data availability for studies requiring more comprehensive analysis.