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Revealing the Genetic Landscape of *Portunus segnis* and *Portunus reticulatus* in Indo-West Pacific Continuum

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Abstract

Crab identification based on morphology is not precise and requires high taxonomic expertise; therefore, molecular identification is used as an alternative and accurate technique for identification. This study investigates the population dynamics and genetic diversity of the *P. pelagicus* species complex. Two mt-DNA genes, 16S rRNA and Cytochrome C Oxidase Subunit I (COI), were employed for analysis. The study identified significant genetic diversity within the two new records from Pakistan, with 19 haplotypes in *P. reticulatus* and 9 in *P. segnis*. Neutrality tests and mismatch distributions indicated population expansion, with significant negative values. AMOVA results highlighted genetic differentiation and population structuring in *P. segnis*, necessitating urgent local conservation efforts to protect the unique genetic compositions of each population. Simultaneously, the genetic connectivity observed in *P. reticulatus* suggests that conservation strategies should prioritise maintaining and enhancing gene flow among populations. The median-joining network clarified phylogenetic relationships, revealing shared and rare haplotypes. Haplotype network analysis uncovers the central role of specific haplotypes in maintaining genetic connectivity, offering insights into past population dynamics. This study highlights the importance of species-specific conservation approaches in preserving the genetic diversity and evolutionary potential of species in the Indo-West Pacific region, with practical implications for conservation efforts.

Introduction

Crabs belong to the order Decapoda, Crustacea, with almost 10,000 species described (Brown and Abel 1982; Kazmi et al. 2003). Decapod crustaceans play a crucial role in commercial fisheries (Ozcan 2012; Safaie et al. 2013). The crab fishery predominantly relies on members of a single family, Portunidae: mud crabs (genus *Scylla* De Haan 1833), blue crabs (*Portunus pelagicus*, Linnaeus 1758), three-spotted crabs (*Portunus sanguinolentus*, Herbst 1783), and gazami crabs (*Portunus trituberculatus*, Miers 1876). These crabs are caught in several countries, including Pakistan, India, Sri Lanka, Thailand, Indonesia, the Philippines, and Australia. The market demand for wild and aquaculture products remains significant, with over 1.5 million tonnes landed per annum (Otto et al. 2001). Portunid crabs serve as a food source in coastal areas and represent an important resource for local fisheries in Pakistan (Rasheed and Mustaquim, 2010), constituting about 63% of the total catch of the fishery (Tirmizi and Kazmi 1996; Takween and Qureshi 2001). The annual landings of Portunid crabs were 9,321 m. tonnes in 2013 and 9,000 m. tonnes in 2015; the annual export of live crab remained high (around 6,000 m. tonnes) between 2008 and 2015, whereas a decreasing trend was observed in 2016 at 5,436 m. tonnes. However, an increasing trend was again observed in 2022 when it

reached a level of about 6,941 m. tonnes. Presently, around 3,000 m. tonnes of live mud crabs are annually exported from Pakistan (Moazzam and Osmany 2024). Swimming crabs (Portunidae) distributed throughout the Indo-West Pacific have a planktonic larval duration (PLD) of approximately two weeks, which is considered relatively short compared to other marine species. While this PLD allows for some dispersal via ocean currents, their larvae are often retained in estuarine and coastal areas due to behavioural adaptations such as vertical migration and selective tidal-stream transport. This retention strategy, along with a preference for intertidal and estuarine nursery habitats, can limit long-distance larval dispersal and reduce gene flow across broader geographic scales. Despite these limitations, seasonal migrations by adult crabs, particularly from estuaries to offshore waters during the rainy season to escape low salinity conditions, may facilitate some regional dispersal. As a result, moderate gene flow may occur between conspecific populations over mesoscale distances (tens to perhaps low hundreds of kilometers), especially along continuous coastal habitats (Klingbunga et al. 1990; Vartak et al. 2008). In terms of morphology, species of swimming crabs have traditionally been identified based on external morphological traits. However, significant morphological variation (i.e., different morphs) has been observed within what were previously considered single "biological" species, raising the possibility of cryptic species or locally adapted populations (Keenan and Shaklee, 1995).

In recent years, genetic analyses combined with morphological studies have shown that the species reported for long as a single species is in fact a complex of more than one species or subspecies. Molecular data demonstrated that mud crab *S. serrata* (Forskål 1775) is a complex of four similar species (Keenan et al. 1998). Likewise, *Plegicus* sensu lato in the Indo-Pacific region is known by seven other synonym names: *Cancer pelagicus* (Linnaeus 1758), *C. segnis* (Forskal 1775), *C. cedonulli* (Herbst 1794), *C. reticulatus* (Herbst, 1799), *P. denticulatus* (De Procé 1822), *P. armatus* (A. Milne-Edwards 1861), *P. pelagicus* var. *sinensis* (Shen 1932), and *P. mauritianus* (Ward 1942). According to Lai et al. (2010), the eastern Indian Ocean and the Bay of Bengal may represent a hybridisation zone between *Portunus pelagicus* and *Portunus reticulatus*, whereas *Portunus segnis* exhibits a distribution restricted to the western Indian Ocean, extending from the Pakistani coastline to South Africa. The Erythrean population of this species entered the Mediterranean from the Red Sea through the Suez Canal (Castriota et al. 2022). Bagheri (2020) examined the genetic diversity and population structure of *P. pelagicus* along the Iranian coast of the Persian Gulf and Oman Sea using mitochondrial markers and revealed significant genetic differentiation among populations, indicating restricted gene flow and suggesting localised adaptation or limited larval dispersal. Lovrenčić et al. (2020) analyzed *P. segnis* populations in the Mediterranean following its Lessepsian migration, highlighted founder effects and reduced genetic variability, contributing to our understanding of how species respond genetically to range expansion and environmental change. Castriota et al. (2022) further investigated *P. segnis* in Mediterranean waters, supporting earlier findings of reduced genetic diversity and offering implications for monitoring invasive population dynamics. Although these studies are region-specific, they underscore critical genetic principles such as population structure, gene flow, and evolutionary potential that are universally applicable across the species complex. Understanding these dynamics in adjacent or ecologically similar regions provides comparative context and helps infer patterns likely occurring in the Indo-Pacific. Moreover, such studies offer methodological frameworks and baseline expectations for evaluating population connectivity, adaptive capacity, and conservation needs of *P. pelagicus* and related taxa.

The DNA analysis technology is beneficial for the molecular identification of species, molecular characterization to construct phylogenetic trees and find its divergence and lineage. Some mitochondrial markers such as 16S rRNA and *cytochrome c oxidase subunit 1* are used in studies of species identification, genetic diversity, molecular evolution of the species (Yu et al. 2012; Sahoo et al. 2015; Korkmaz et al. 2016) and connectivity and describe the evolutionary history and phylogeography of a species (Cowen et al. 2006). The current investigation was designed to explore the genetic diversity, population structure, and connectivity and cryptic species of the *Portunus pelagicus* species complex in the coastal waters of Pakistan, with particular attention to the Indo-Pacific region and Mediterranean (Red Sea) by analyzing mitochondrial DNA (mtDNA) marker 16S rRNA and Cytochrome C Oxidase Subunit I (COI).

Materials and Methods

Specimen Collection and Amplification

Samples were collected (n=20 *P. segnis* and n=9 *P. reticulatus*) from freshly caught specimens provided by local fishermen, companies, and fish harbors along the coastal waters of Pakistan (Figure 1). Specimens immediately stored in an icebox killed by freezing, transferred to the laboratory and identified according to their morphological characteristics (Lia et al. 2010) (Figure 2). Extracted DNA from >25 mg of muscle tissue of chelipeds by using Qiagen's DNeasy Blood and Tissue Kit (Cat. No. 69504), ensuring the instruction with little modifications of the original procedure to improve the yield of the DNA extract. Quantifying the extracted template DNA using a Beckman Coulter DU 730 spectrophotometer and agarose gel electrophoresis to determine the concentration and purity of the DNA. Amplify the region of the 16S rRNA gene and COI coding

sequence in the mt-DNA genome. Primers used in the 16S fragment and COI were 16Sar (F) (5′-CGC CTG TTT ATC AAA AAC AT-3′)**;** 16Sbr (R) (5′-CCG GTC TGA ACT CAG ATC ACG T-3′) (Schubart et al. 2000b). COI a (F) (5′-AGT ATA AGC GTC TGG GTA GTC -3′); COIf-L (R) (5′-CCT GCA GGA GGAGGAGGA GAY CC -3′) (Palumbi et al. 1991). The polymerase chain reactions (PCR) technique used according to Mantelatto et al*,* (2009). 50µl volumes containing 15 µl of DNA template (150 ng), 25 µl Go Taq Green Master Mix 2X (Promega, USA), and 5 µl forward and reverse primers (10 pmol/ µl). Amplification was performed by Applied Biosystems 2720 thermal cycler, 16S rRNA, the process included denaturation for 10 minutes at 95°C, 40 cycles of 1 minute at 95°C, 1 minute at 46°C, and at 72°C for 2 minutes, at 72°C for 10 minutes' final extension. COI amplification, the protocol involved denaturation for 2 minutes at 94°C, 1 minute for 40 cycles at 94°C, at 50°C for 1 minute and 1.5 minutes at 72°C, and a final 10 minutes for an extension at 72°C.

Sequencing of 16S rRNA and COI mtDNA

Envision the PCR product through the agarose gel system. 5µL of PCR products were checked through 1% of agarose gel by horizontal gel electrophoresis in 80 volts of electric current and also compared with a Gene Ruler 100 bp Plus DNA ladder (Promega, USA). 0.5µg/ml

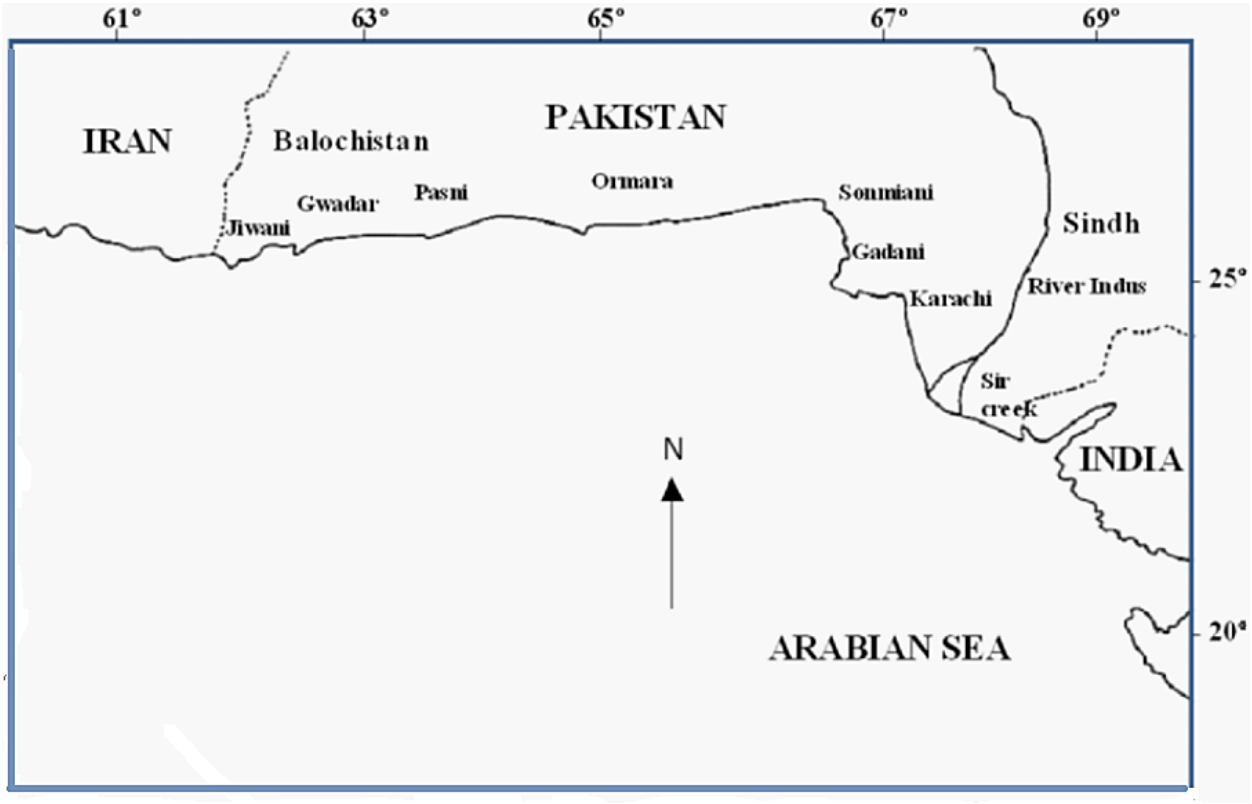

Figure 1. Map of study area coastal waters of Pakistan.

Figure 2. *Portunus segnis* (A), *Portunus reticulatus* (B).

ethidium bromide was used for staining, visualized by UV, and sent to Macrogen, Korea, for purification and sequencing. Sequences were analyzed through Applied Biosystem Sequence Scanner v1.0 software.

DNA Sequence Alignment and Species Identification

DNA sequences were carefully searched and compared for similarity using the NCBI BLAST web server (https://www.ncbi.nlm.nih.gov/) with BLASTn 2.2.26+ (Zhang et al., 2000). Up to 100 hits showing at least 97–100% identity were retrieved for each query sequence, and the sequences were assigned to the most similar species based on the best hit (highest bit score). The new nucleotide sequences of the 16S rRNA gene were submitted to the NCBI nucleotide-sequence databases through the BankIt submission tool (BankIt is a recognized NCBI web-based tool used for submitting nucleotide sequences to GenBank.

Data Analysis

The procured DNA sequence of 16S rRNA and COI was utilized to calculate and estimate various interspecific and intraspecific genetic variability and relationships in the identified species of *Portunus plegicus* species complex (*P. segnis* and *P. reticulatus*). Initially, the 16S rRNA and COI sequences were first analyzed with the software MEGA 11. All sequences were ATG transformed and aligned in CLUSTAL W (Thompson et al. 1997) using the default parameters to evaluate the evolution model (Maximum Likelihood fits of 24 different nucleotide and amino acid substitution models) that best fit the data. The selected models, the lowest Bayesian Information Criterion scores, AICc value (Akaike Information Criterion), and the correct ones are considered.

Evolutionary and Phylogenetic Analysis

Procured mitochondrial 16S rRNA was used for species identification and haplotype diversity whereas the Cytochrome Oxidase subunit I gene sequences from Pakistan (Table 1) and other sequences in molecular databases described by Lai et al. (2010) was included in this study to evaluate evolutionary and phylogenetic relationships. In total, 29 sequences of *P. segnis* population from Indo-Mediterranean Region: Mozambique: Maputo Bay, Madagascar: Tulear (Population 1), Pakistan (Population 2) (current study) Israel: off Ashjod (Population 3), United Arab Emirates: Abu Dhabi (Population 4), and 30 sequences of *P. reticulatus* population from Pakistan (Population 1) (Current study), India: Chennai (Population 2), Sri Lanka (Population 3) and Thailand: Phuket (Population 4) (Table 2). The evolutionary divergence analyses were estimated based on the selected evolutionary model, which included average divergence between and within overall sequence pairs. For the population analysis, DnaSP software version 5.10, Librado and Rozas (2009) were used to calculate haplotype diversity (hd) and nucleotide diversity (π) as described by Nei, (1987). The haplotype diversity (hd) indicates the probability that two randomly chosen haplotypes are different within a single population, while nucleotide diversity (π) represents the average number of differences between all pairs of haplotypes in a single population. Neutrality tests, including Ewens-Watterson (Ewens 1972; Watterson 1978), Chakraborty (Chakraborty 1990), Tajima's D (Tajima 1989), and Fu's Fs (Fu 1997), were also conducted. A median-joining (MJ) network (Bandelt et al. 1999) of phylogenetic relationships among haplotypes was used to clarify the evolutionary relationship constructed by using software Network version 4.51 (version 4.5.1.0; Fluxus engineering 2008) (Polzin and Daneshmand 2003).

The study utilized a comprehensive approach, incorporating all currently available molecular data to infer the genetic diversity, genetic relatedness, and evolutionary history of *P. reticulatus* Indo-Mediterranean and *P. segnis* in the Indo-West Pacific region. This was achieved through various methods, including nucleotide diversity tests, network analysis, mismatch analysis, Molecular variance analysis (AMOVA), and statistical tests for differentiation among multiple samples, using a Markov Chain Monte Carlo (MCMC) method (significance level= 0.0500) by Arlequin ver 3.5.2.2. Constructing a network diagram or a mesh representation provided insights into the complexity and interconnectedness of evolutionary processes using Spilits Tree: Version 4.14.2 (Huson and Bryant 2006).

Table 1. GenBank accession numbers for 16S and COI sequence of *P. segnis* and *P. reticulatus* from the coastal waters of Pakistan.

S. No	Portunid Crabs	16S rRNA GenBank accession numbers	No	COI GenBank accession numbers	No
1	*P. segnis*	KU 296938.1	1	KY290382.1, KY290383.1, KY290384.1, KY290385.1, KY290386.1, KY290387.1, KY428867.1, KY587761.1, KY587764.1, KY587776.1, KY587777.1, KY587780.1, KY618712.1, KY618713.1, KY618711.1, KY623445.1, KY623448.1, KY695090.1, KY695091.1	19
2	*P. reticulatus*	KU296927.1, KU296928.1, KU 296940.1, KU296941.1	4	KY428868.1, KY623446.1, KY587765.1, KY587775.1, KY695089.1, KY623447.1, KY587762.1, KY587763.1	8

Results

Mitochondrial DNA Analysis

In the field, species identification was conducted based on established morphological keys and diagnostic characteristics specific to the *P. pelagicus* species complex. These included features such as carapace shape and color, the number and arrangement of lateral spines on the carapace, and distinctive patterns on the chelae and legs. Identification followed the taxonomic criteria of Stephenson (1962) and Lai et al. (2010), which are widely used for distinguishing cryptic and closely related *P. plegicus* species in the Indo-Pacific region. The amplified product of 16S rRNA and COI gene was displayed in the form of a glowing band when exposed to UV light for visual analysis, and the number of base pairs was also estimated through the amplified product by comparing with the known base pair (100 bp plus DNA ladder) Gene Ruler. Band size for amplified 16S rRNA gene ~1,450–1,550 bp and COI (Cytochrome oxidase I) ~650 bp. The procured DNA sequences were initially searched for sequence similarity by using BLAST (Basic Local Alignment Search Tool) (Zhang et al. 2000), and the initial species identification and confirmation were based on at least 97%-100% identity for each query sequence. Each procured and analyzed sequence was submitted to GenBank, and a GenBank accession number was received for each isolate sequence. The BLAST search showed high sequence similarity (97-100%) to their respective species sequences in GenBank (Table 1).

An Evolutionary Model for Evolutionary Divergence and Phylogenetic Inference and Neutrality Tests for Cytochrome Oxidase I (COI) from Pakistan.

Multiple sequence alignments of the 16S rRNA and COI genes yielded 580 bp and 697 bp, respectively, following the exclusion of hypervariable regions. Subsequently, 517 bp of the 16S alignment and 327 bp of the COI alignment were retained for downstream phylogenetic inference. Likelihood ratio test revealed the selected optimum model (The T92+G Tamura 3- 3-parameter+Gamma distribution) under the Akaike information criterion (AIC). However, the models with the lowest BIC scores were considered to best describe the DNA substitution pattern as implemented in MEGA 11 (Kumar et al. 2016). The number of haplotypes and their diversity for the 16S and COI genes were estimated to assess genetic variation and differentiation in *P. segnis* and *P. reticulatus*. For the 16S rRNA gene, three haplotypes were identified in *P. reticulatus*, with a haplotype diversity (hd) of 0.833 (P≤0.04948) and a nucleotide diversity of 0.01518 (P≤0.0001). In contrast, only a single sequence was available for *P. segnis*,

Table 2. GenBank accession numbers for COI sequence of *P. segnis* and *P. reticulatus* from the different populations: Pakistan (Pk), India (Ind), Thailand (Thi), Abu Dubai (AbD), Srilanka (Sri), Israel (Isl)

Haplotype N	Population	Hn	*P. reticulatus*	Haplotype N	Population	Hn	*P. segnis*
1	Pk, Ind Sri	2	EF661959.1, MT831513.1	1	Isl	1	EF661958.1
2	Ind Sri	5	EF661960.1, EF661962.1, KY587762.1, KY587763.1, MH395331.1,	2	Pk	19	EF661949.1, KY290382.1, KY290383.1, KY290384.1, KY290385.1, KY290386.1, KY290387.1, KY428867.1, KY587761.1, KY587764.1, KY587776.1, KY587777.1, KY587780.1, KY618712.1, KY618711.1, KY623445.1, KY623448.1, KY695090.1, KY695091.1,
3	Sri	1	EF661961.1	3	Isl	1	EF661950.1
4	Ind	1	EF661963.1	4	Isl	1	EF661951.1
5	Ind	1	EF661964.1	5	Thi	3	EF661952.1, EF661953.1, EF661954.1
6	Ind	1	EF661965.1	6	AbD	1	EF661955.1
7	Thi	2	EF661966.1, EF661968.1	7	Isl	1	EF661956.1
8	Thi	1	EF661967.1	8	Isl	1	EF661957.1
9	Ind Thi	2	EF661969.1, MH395333.1	9	Pk	1	GQ272556.1
10	Thi	1	EF661970.1				
11	Ind Thi	2	EF661971.1, MH395332.1				
12	Thi	1	EF661972.1				
13	Thi	2	EF661973.1, EF661975.1				
14	Thi	1	EF661974.1				
15	Thi	1	EF661976.1				
16	Pk	2	KY428868.1, KY623446.1				
17	Pk	1	|KY587765.1				
18	Pk	2	KY587775.1, KY695089.1				
19	Pk	1	KY623447.1				

preventing diversity estimation. For the COI gene, three haplotypes were identified from 19 sequences of *P. reticulatus*, with a haplotype diversity of 0.582 (P≤0.001) and a nucleotide diversity of 0.00128 (P≤0.0001). In total, across all samples, 19 haplotypes were detected, with an overall haplotype diversity of 0.961 (P≤0.00042), whereas the nucleotide diversity was (0.01156; P<=0.000) in *P. segnis*. The maximum number of haplotype descriptions likely due to the selection of morphological differences in individuals (morphotype) from each population (Table 3). Neutrality and Mismatch Analysis Tajima's D (Neutrality test) was estimated for 16S rRNA and COI in *P. reticulatus* D= 0.330 (P>0.10), whereas Fu's Fs 0.84371 in *P. segnis* (P>0.10) and Fu's Lis D 0.884; P>0.10). Neutrality and Mismatch analysis Tajima's D (Neutrality test) was estimated for COI in *P. reticulatus* D= -2.182 (**P<0.01), whereas Fu's Fs -2.64007 (*P<0.05) and Fu's Lis D -2.64007, *, P<0.05 (Table 4).

An Evolutionary Model for Evolutionary Divergence and Phylogenetic Inference and Neutrality Tests for Cytochrome Oxidase I (COI) from the Indo-west Pacific and Mediterranean Region.

The analysis involved 30 sequences of *P. reticulatus* (729 bp) and 29 sequences of *P. segnis* (714 bp) nucleotide sequences. Hypervariable regions that could not be aligned and excluded from the sequences and the remaining (327 and 462 bp COI) of *P. reticulatus* and *P. segnis,* respectively, were used for the phylogenetic analysis. Likelihood ratio tests revealed that the selected optimum model for the data set, selected under the Akaike information criterion (AIC), models with the minimum BIC scores according to (Bayesian Information Criterion) were considered to explain the DNA substitution pattern the best as implemented in MEGA 11 (Kumar et al. 2016). COI was the T92+1 (Tamura 3-parameter+Gamma distribution). The T92+1 (Tamura 3-parameter+Gamma distribution) was a simplified model in which the frequencies of the two types of transitions (A=>C and G=>T) were equal for the neighbour-joining and Maximum likelihood methods.

Evolutionary Divergence (COI gene)

The number of base substitutions per site from an average of overall sequence pairs within each species and among species was estimated. The evolutionary divergence in the overall mean sequence pairs within species of *P. reticulatus* and *P. segnis* was 0.02, indicating low intraspecific variation and a relatively homogeneous genetic makeup within each species. The mean genetic distance between the two species was 0.0927, which is substantially higher than the within-species value, suggesting clear interspecific differentiation. The net mean species difference was 0.0759, further supporting that *P. reticulatus* and *P. segnis* are genetically distinct taxa within the *P. pelagicus* species complex.

Haplotype and Nucleotide Diversity

The percentage of haplotypes for *P. segnis* and *P. reticulatus* was estimated (Figure 3). A total of 19 haplotypes were identified out of 30 individuals of *P. reticulatus*, whereas 9 haplotypes were detected among 29 individuals of *P. segnis*. Haplotype diversity was high in *P. reticulatus* (hd= 0.961) and moderate in *P. segnis* (hd= 0.5714). The markedly higher number of haplotypes and the elevated haplotype and nucleotide diversity in *P. reticulatus* indicate a genetically more diverse population compared to *P. segnis*. Nucleotide diversity (π) also showed clear differences between the species, with *P. reticulatus* exhibiting higher variability (π= 0.01156) compared to the very low nucleotide diversity in *P. segnis* (π= 0.000261). Both haplotype diversity and nucleotide diversity values were highly significant (P≤0.01 and P≤0.000, respectively) (Table 5).

Table 3. Summary statistics of 16S rRNA and COI sequences, species, no of sequences used, no of sequence analyzed, no of sites, total no of sites and variable sites, Haplotypes (P) and Haplotype diversity (Hd) at significance level (P<=0.000); nucleotide diversity (π) at significance level Theta per site (P<=0.000) by using Dna SP V5 from the coastal waters of Pakistan

S.No	Gene	Portunid Crabs	Sequence	Site	Haplotype	Hd	hd (P <=0.000)	Π	Pi (P<=0.000)
1	16S rRNA	*P. segnis*	1	-	-	-	-	-	-
		P. reticulatus	4	524	3	0.833	0.04948	0.01518	0.0001
2	COI	*P. segnis*	18	517	3	0.582	0.00128	0.00128	0.000
		P. reticulatus	30	327	19	0.961	0.00042	0.01156	0.000

Table 4 Tajima's *D* and Fu's *F* Test for COI mitochondrial DNA from the coastal waters of Pakistan

Neutrality test	16S rRNA			COI		
Species	Tajima's D	Fu's Lis D	Fu's Lis F	Tajima's D	Fu's Lis D	Fu's Lis F
P.s egnis	-	-	-	0.330 P > 0.10	0.884 P > 0.10	0.84371 P > 0.10
P. reticulatus	0.847 P > 0.10	-0.847 P > 0.10	-0.866 P > 0.10	-2.182 **, P < 0.01	-2.265 P > 0.10	-2.64007 *, P < 0.05

Neutrality Test

Neutrality test: Tajima's D was estimated for COI in *P. segnis.* Tajima's D: -2.11587 (Significant) indicates a significant excess of low-frequency polymorphisms. This suggests a recent population expansion, or purifying selection removing deleterious mutations. Fu and Li's D* and F* (both biallelic and full dataset): Strongly negative values (all below -2.5) and statistically significant, indicate a significant deviation from neutrality, Strong support for population growth, where new mutations are still rare or selection, especially purifying selection that removes harmful variants. Neutrality test: Tajima's D was estimated for COI in *P. reticulatus.* Tajima's D = -2.18254 (P<0.01) a significant excess of rare (low-frequency) alleles, indicating population expansion (new mutations still rare), or Purifying selection (removal of deleterious mutations). Fu and Li's D* and F* (biallelic), Negative and close to or below -2, which is generally considered statistically significant**,** support the same conclusion as Tajima's D. Strong indication of demographic expansion**,** or selection pressures, especially purifying selection (Table 6). The consistently strong negative values in both Tajima's and Fu & Li's tests imply that similar evolutionary forces may be acting on both species, although the magnitude of values suggests *P. segnis* may be experiencing slightly stronger demographic or selective effects.

Markov Chain Monte Carlo (MCMC) Test of Differentiation among Populations

The global test suggests no significant overall differentiation among all populations of *P. reticulatus*. Pairwise tests show significant differentiation between populations one and four, but not among other populations. For pairs (P1, P2), (P1, P3), (P2, P4) and (P3, P4), the P values are above 0.05, indicating non-differentiation (i.e., samples are not significantly different). The P value of the population (1, 4) is 0.02768, below 0.05, indicating significant differentiation. There is a significant overall differentiation among the populations of *P. segnis* (P value= 0.00000). Significant differentiation exists

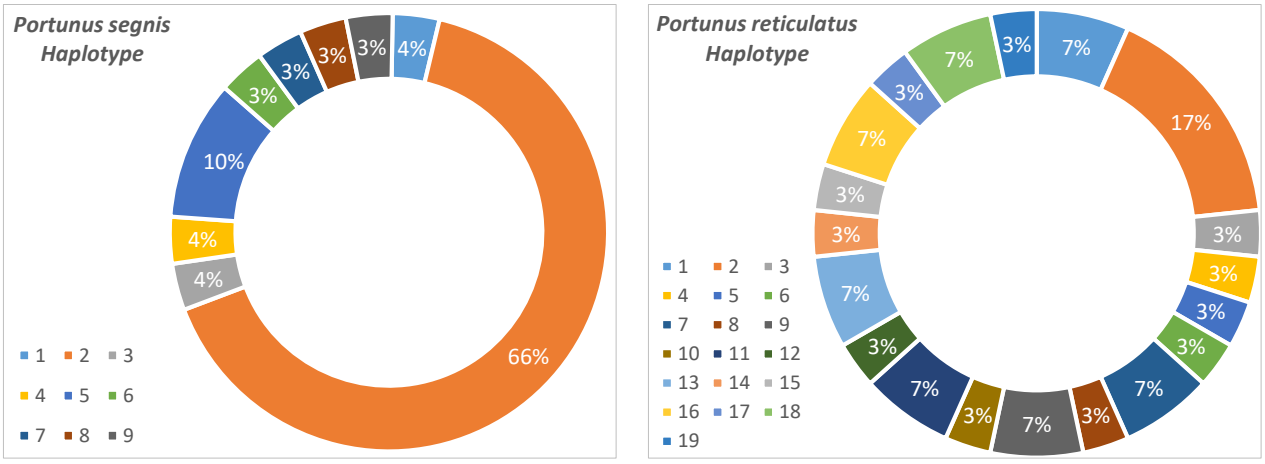

Figure 3. Percentage of haplotype *P. segnis* and *P. reticulatus* from the coast of Pakistan.

Table 5. Summary statistics of COI sequences, species, no of sequences used, no of sequence analyzed, no of sites, total no of sites and variable sites sites, Haplotypes (P) and Haplotype diversity (Hd) at significance level (P<=0.000); nucleotide diversity (π) at significance level Theta per site (P<=0.000) by using Dna SP V5 different populations.

Species	Gene	No of Sequence	No Sites	Total no of sites used	Variable Site	Haplotype	hd	hd (P<=0.000)	π	Pi (P<=0.000)
P. reticulatus	COI	30		327	34	19	0.961	0.00042	0.01156	0.0000***
P. segnis		29	714	462	12	09	0.5714	0.01147	0.00261	0.000***

Table 6. Tajima's *D* and Fu's *F* Test for COI mitochondrial DNA from the different populations.

Neutrality test	COI		
	Tajima's D	Fu's Lis D	Fu's Lis F
P. reticulatus	-2.18254	-2.26578	-2.64007
P. segnis	-2.11587	-2.52963	-3.08440

between populations: populations one and two (P= 0.00028) and populations two and three (P= 0.00123). In contrast, no significant differentiation exists between other pairs of populations. (Table 7 and 8).

Mismatch Distribution

Mismatch distribution describes the distribution of pairwise differences among populations, *P. segnis* and *P. reticulatus* showed a unimodal pattern, the minor fluctuations in the tail do not constitute a second mode. It does support the hypothesis of a sudden population expansion the overall shape closely follows the smooth, unimodal expected (blue) curve predicted by the expansion model (Figure 4A). *P. reticulatus* also showed unimodal, the slight bump in the tail is likely due to sampling variance or low-frequency divergent haplotypes. The pattern still broadly follows the expected sudden expansion curve, with some noise in the data. This type of unimodal mismatch distribution is

Table 7. Statistical test for differentiation among population of and *P. reticulatus*, using a Markov Chain Monte Carlo (MCMC) method (significance level=0.0500) P= Population)

	Pakistan (P1)	India (P2)	Sri lanka (P3)	Thailand (P4)
Pakistan				
India	0.27951±0.0069 (-)			
Sri lanka	0.47278±0.0048 (-)	1.00000±0.0000 (-)		
Thailand	0.02768±0.0021 (+)	0.47771±0.0050 (-)	0.39394±0.0068 (-)	

Table 8. Statistical test for differentiation among population of and *P. segnis*, using a Markov Chain Monte Carlo (MCMC) method (significance level=0.0500)

	Thailand (P1)	Pakistan (P2)	Israel (P3)	Abu Dubai (P4)
Thailand				
Pakistan	0.00056+-0.0001 (+)			
Israel	0.24337+-0.0039 (-)	0.00079+-0.0003 (+)		
Abu Dubai	0.24918+-0.0018 (-)	0.09807+-0.0031 (-)	1.00000+-0.0000 (-)	

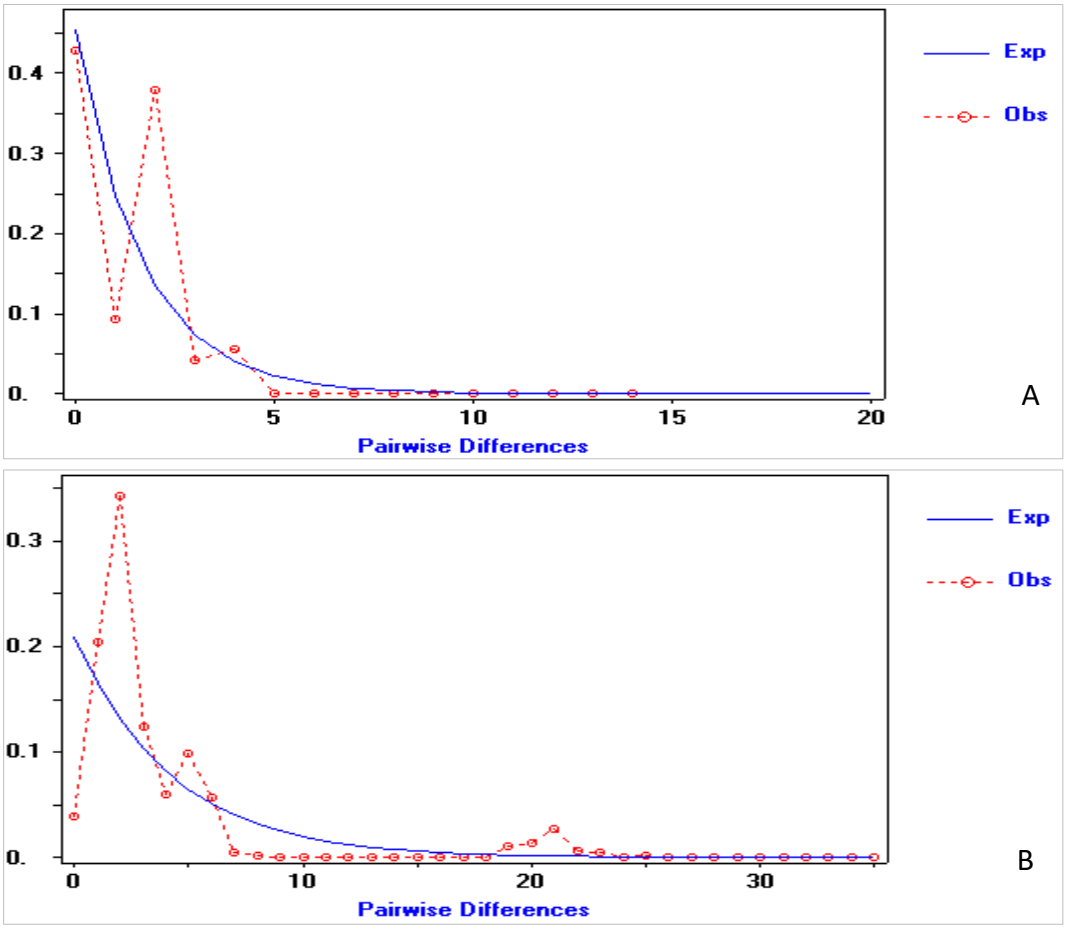

Figure 4. unimodal pattern of mismatch analysis of *P. segnis* and *P. reticulatus* in different populations.

very common in marine invertebrates, where populations may have undergone historical expansions after bottlenecks or glacial retreats (Figure. 4B).

Molecular Variance Analysis (AMOVA)

The Analysis of Molecular Variance (AMOVA) was conducted to evaluate the genetic structuring among four geographically distinct subpopulations of *P. segnis* and *P. reticulatus*. The subpopulations were grouped based on their respective collection locations (*P. segnis* population from Indo-Mediterranean Region: Mozambique: Maputo Bay, Madagascar: Tulear (P1), Pakistan (P2) (current study) Israel: off Ashjod (P3), United Arab Emirates: Abu Dhabi (P4), and 30 sequences of *P. reticulatus* population from Pakistan (P1) (Current study), India: Chennai (P2), Sri Lanka (P3) and Thailand: Phuket). The AMOVA analysis, based on the Tamura and Nei distance method, was conducted among four subpopulations of *P. segnis*. The molecular variance analysis (AMOVA) indicated genetic structuring within and among four populations of *P. segnis*, with 63.42% of the total variation occurring among populations and 36.58% within populations. The FST value of 0.63417 indicates a high level of genetic differentiation among the populations. The P-value= (0.00000) indicates that the observed genetic differentiation (Va and FST) is highly significant, meaning the differentiation among populations (Table 9). The AMOVA analysis, based on the Tamura and Nei distance method, was conducted among four subpopulations of *P. reticulatus* population also indicated the genetic structuring within and among populations. The results revealed significant genetic differentiation among populations, with 21.92% of the total variation occurring among populations and 78.08% within populations. The FST value of 0.21918 indicates moderate genetic differentiation. The P-value (0.00684) indicates that the observed genetic differentiation (Va and FST) is highly significant, meaning that the differentiation among populations is not due to random chance (Table 10).

Network Analysis

A median-joining (MJ) network (Bandelt et al., 1999) of phylogenetic relationships among haplotypes was used to clarify the evolutionary relationship among haplotypes within *P. segnis* and *P. reticulatus* populations and highlighting both common and rare haplotypes and the genetic distances between them. In the *P. segnis* population H2 is the central and most frequent haplotype, connected to H1, H3, H4, H5, H6, H7, H8, H9, and mv1 through various mutational steps. Haplotypes H1, H4, and H6 have a single or few mutational steps (542, 497) from H2, indicating close genetic relationships. Haplotypes H3 and H5 are more distantly related with more mutational steps (265, 381). The presence of an inferred median vector (mv1) indicates that there might be an un sampled or extinct intermediate haplotype. In *P. reticulatus*, H9 is a central and diversely shared haplotype among the populations. In contrast, peripheral haplotypes like H8, H10, H11, and H12 are less common and show varying degrees of differentiation from H9, and Median vectors illustrate the most likely genetic pathways and connections among the observed haplotypes (Figure 5). The numbers on the connecting lines indicate the number of mutational steps between haplotypes. The small red circle (mv1) represents a median vector, which denotes a hypothetical ancestral or unsampled haplotype.

Splits Tree Phylogenetic

Splits Tree phylogenetic network (Dress and Huson 2004) was reconstructed using the inclusive sequence dataset of mtDNA Cytochrome Oxidase I gene. The Splits Tree network represents evolutionary relationships with more complexity than a traditional phylogenetic tree. It can show reticulate events such as hybridization or recombination. The lengths of the branches and the positions of the clusters indicate genetic distances. Longer branches signify greater genetic divergence. The clear genetic differentiation between *P. reticulatus* and

Table 9. AMOVA using the Tamura and Nei distance method from five subpopulations for *P. segnis*

Source of Variation	df	Sum of squares	Variance components	Percentage of variance
Among populations	3	10.490	0.62831Va	63.42
Within population	25	9.061	0.36246Vb	36.58
Total	28	19.552	12.56384	
Fixation Index (FST)		0.63417		

Significance tests (1023 permutations), Va and FST: P(rand. value>obs. value)= 0.00000, P(rand. value= obs. value)= 0.00000, P-value= 0.00000

Table 10. AMOVA using the Tamura and Nei distance method from five subpopulations for *P. reticulatus*

Source of Variation	df	Sum of squares	Variance components	Percentage of variance
Among populations	3	185.184	5.77128Va	21.92
Within population	26	534.549	20.55959Vb	78.08
Total	29	719.733	26.33086	
Fixation Index (FST)		0.21918		

Significance tests (1023 permutations), Va and FST: P(rand. value>obs. value)= 0.00684, P(rand. value= obs. value)= 0.00000, P-value= 0.00684+-0.00231

P. segnis implies that these species have distinct evolutionary histories and population structures. Conservation efforts should focus on maintaining genetic connectivity within *P. reticulatus* to preserve its genetic diversity, whereas the conservation strategies should aim to protect the unique genetic characteristics of each population of *P. segnis*, potentially through localized efforts (Figure 6).

Discussion

In the current study, the genetic divergence and population structure of *P. reticulatus* and *P. segnis* were estimated; the evolutionary divergence between *P. reticulatus* and *P. segnis* was 0.014±0.042, and the haplotype diversity was 0.961 and 0.5714, whereas the nucleotide diversity was 0.01156 and 0.000261

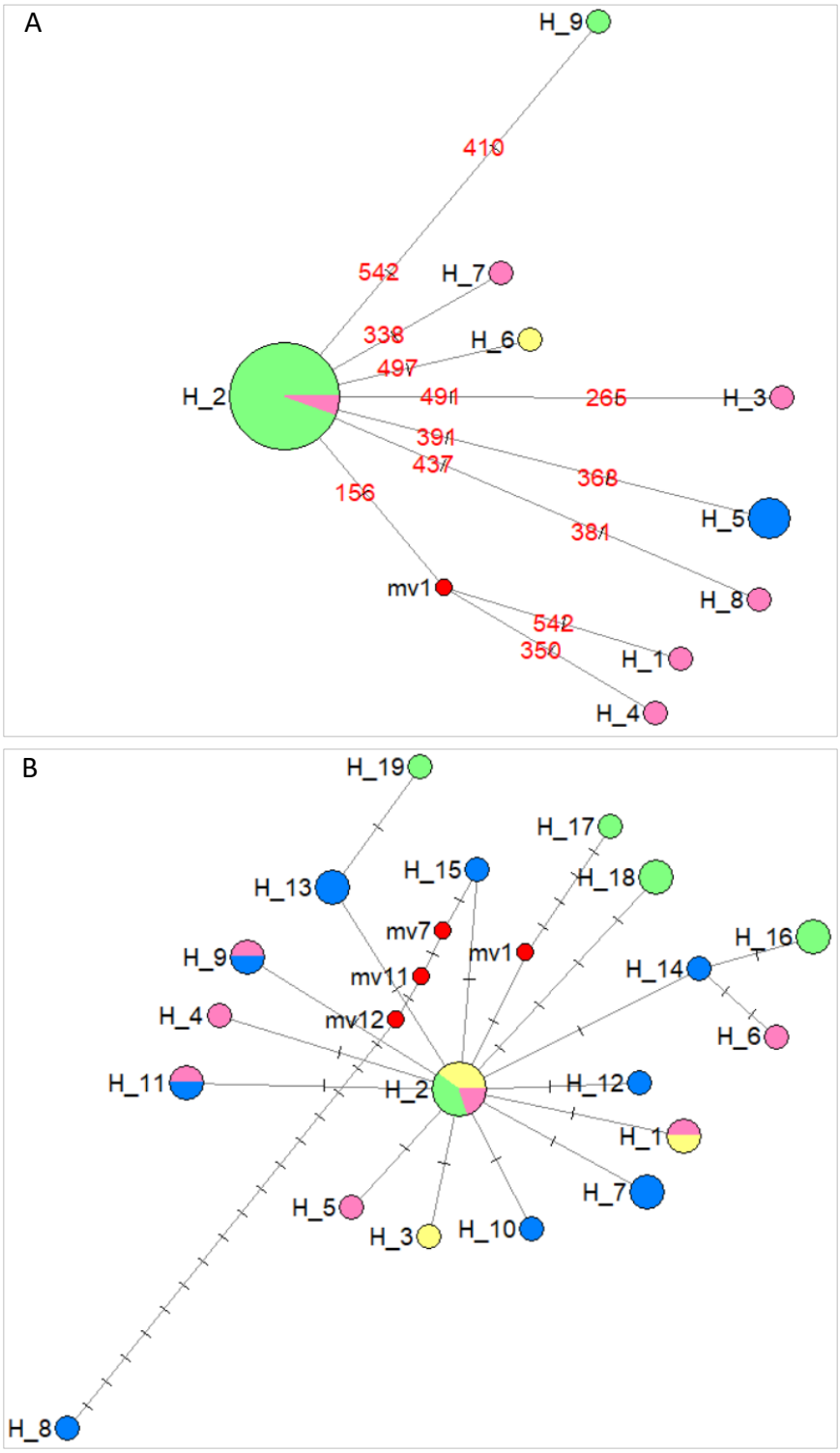

Figure 5. Haplotype network of *P. segnis* based (A), and *P. reticulatus* (B) on mitochondrial DNA sequences. Each circle represents a unique haplotype (H1–H9), with the size of the circle proportional to the haplotype's frequency. Colors within the circles represent different sampling locations. Green (Pakistan), Pink (India), Blue (Thailand) and Yellow (Silanka).

respectively. Similarly, Lu et al. (2022) found two distinct lineages of *P. sanguinolentus* lineage A was primarily found in the South China Sea, whereas lineage B was dominant in the East China Sea and observed the lineage haplotype diversity h= 0.987 (π= 0.008), nucleotide diversity (π= 0.013; h= 1.000) in lineage B was significantly higher than the haplotype diversity (h= 0.987, π= 0.008) that indicate that lineage B is older than lineage A, and inferred that populations containing ancestral genotypes tendency to retain a higher level of haplotype and nucleotide diversity over time (Wang et al. 2000 and Li et al. 2000). The neutrality tests conducted for *P. reticulatus* and *P. segnis* provide insights into these species' evolutionary dynamics and demographic history. Tajima's D indicated a negative value that suggests an excess of low-frequency polymorphisms relative to expectation, which can indicate population expansion or purifying selection. Similarly, Fu and Li's F* test statistic shows a significant deviation from neutrality and supporting the hypothesis of population expansion. However, Fu and Li's D* test statistic was not significant, suggesting that the results for this test might be less conclusive for *P. reticulatus.* For *P. segnis*, Tajima's D also indicated a potential population expansion or purifying selection. The significant negative values in Fu and Li's F and Fu and Li's D further support this interpretation, indicating a deviation from neutrality likely due to a recent population expansion. These findings align with broader observations in marine species where demographic expansions are often detected through negative values in neutrality tests, as Avise (2000) and Grant and Bowen (1998) reported. Such expansions may be attributed to historical events like post-glacial colonization or ecological opportunities in newly available habitats. Overall, the significant negative values of these neutrality tests in *P. reticulatus* and *P. segnis* suggest that both species have likely experienced recent population expansions, which could have important implications for their genetic diversity and evolutionary trajectories.

The results of the global and pairwise differentiation tests are as follows: *P. reticulatus* and *P. segnis* provide valuable insights into the population structure and genetic diversity of these species. For *P. reticulatus*, the global test indicates no significant overall differentiation among all populations, suggesting a relatively homogenous genetic structure across the sampled populations. However, pairwise tests reveal significant differentiation between populations 1 and 4. In contrast, no significant differentiation was observed between the other pairs, indicating that while most populations are genetically similar, population 4 differs from population 1. Such localized differentiation could be due to restricted gene flow, possibly as a consequence of geographic barriers, and long-term separation leading to genetic divergence in these specific populations (Wright 1943; Slatkin 1987).

In contrast, *P. segnis* shows significant overall differentiation among all populations, indicating a more pronounced population structure and greater genetic variability across the sampled populations. Significant pairwise differentiation is observed between populations 1 and 2 and between populations 2 and 3, suggesting that these populations are genetically distinct. The lack of significant differentiation among other population pairs implies that while some populations are well-differentiated, others may still share substantial genetic similarities (Hutchison and Templeton 1999). The observed significant differentiation between specific pairs of populations in *P. segnis*, but not in *P. reticulatus*, could be influenced by several factors, including different historical biogeographical events, varying levels of gene flow, and distinct ecological pressures. For instance, marine species with broad distributions often exhibit complex

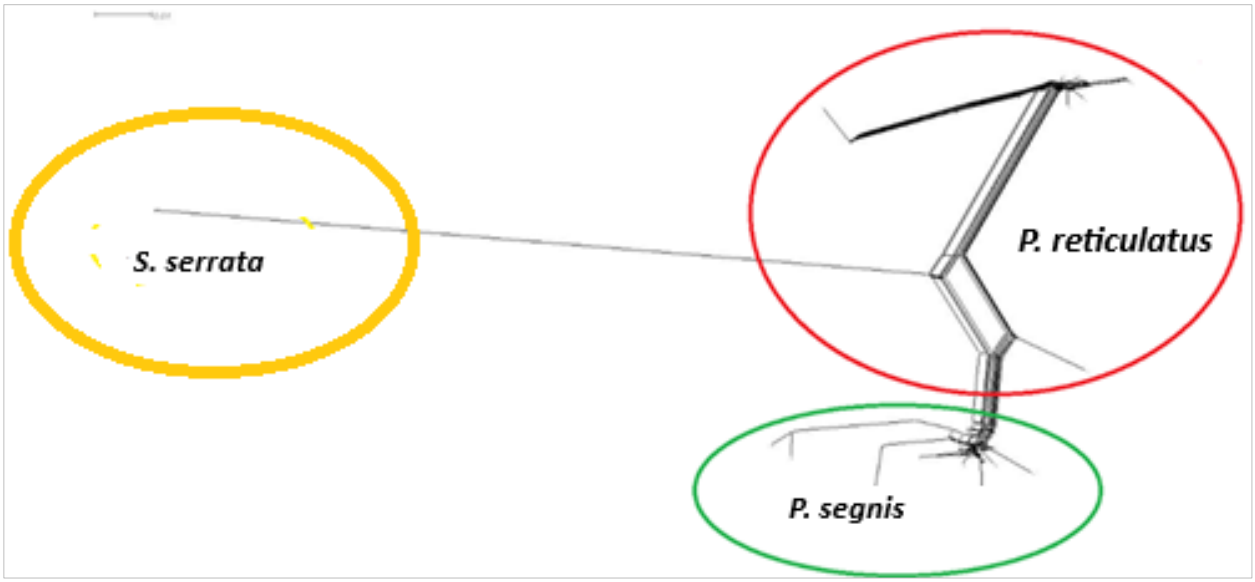

Figure 6. Splits Tree phylogenetic network (Dress and Huson 2004) reconstructed using the inclusive sequence dataset of mt-DNA Cytochrome Oxidase I gene.

population structures due to past climatic changes, ocean currents, and habitat discontinuities, which influence their genetic diversity and differentiation patterns (Palumbi 1994; Hellberg 2009). These conclusions underscore the significance of population-specific dynamics in managing and conserving marine species, a task that is integral to the field of marine biology and genetics. For *P. reticulatus*, conservation efforts might focus on maintaining gene flow and connectivity between populations to prevent further differentiation. The distinct population structure highlights the need for targeted management strategies to preserve the genetic integrity of significantly differentiated populations, ensuring their long-term viability and adaptability (Allendorf et al. 2010). The unimodal mismatch distribution observed for *P. segnis* and *P. reticulatus* aligns closely with the expected distribution under a model of sudden demographic expansion. This pattern suggests that both species have likely undergone recent population growth, possibly due to colonization of new habitats or expansion into new ecological niches. The close fit between observed and expected distributions, along with the lack of high pairwise differences, supports a recent event, such as post-glacial recolonization or anthropogenic dispersal. Further analyses, such as neutrality tests and phylogeographic studies, are recommended to confirm these inferences and understand the potential ecological impacts of these expanding populations. Recent studies on *Portunus* species have provided valuable insights into their population genetics and demographic history. Hui et al. (2019) analyzed the genetic structure of *P. trituberculatus* across the China seas and Japan using mitochondrial DNA control region sequences. Their findings revealed multimodal mismatch distributions in certain populations, suggesting a complex demographic history shaped by factors such as historical fragmentation and secondary contact. Similarly, Guo et al. (2011) studied the genetic variation of *P. trituberculatus* along the coast of China. Their mismatch distribution analyses indicated that specific populations had undergone sudden demographic or spatial expansions, likely influenced by historical events like Pleistocene glaciations. Lu et al. (2022) focused on *P. sanguinolentus* in East Asia, employing mitochondrial DNA analyses to examine genetic diversity and population structure. Their mismatch distribution results suggested historical demographic expansions, underscoring the role of past climatic events in shaping current genetic patterns. Collectively, these studies highlight the importance of considering historical events, population connectivity, and complex demographic processes in the conservation and management of swimming crabs. Such complex genetic structures necessitate not just any conservation approaches, but tailored ones that consider these populations' historical and demographic contexts. The AMOVA results for *P. segnis* indicate substantial genetic structuring, and the high FST value signifies strong genetic differentiation among populations, and the P-value confirms that this differentiation is highly significant. This significant genetic differentiation could be attributed to limited gene flow among populations, potentially due to geographical barriers, ecological specializations, or historical isolation events (Slatkin, 1987; Waples and Gaggiotti 2006). Recent studies have shown that marine species with high levels of population structure, like *P. segnis*, often exhibit local adaptations and distinct genetic lineages, which are critical for their long-term survival and adaptability (Bernatchez 2016; Nielsen *et al*. 2009). Therefore, the urgency of conservation efforts for *P. segnis* is underscored, as they should not just focus on maintaining the genetic integrity of each population, but also on addressing local threats, and preserving habitat connectivity to facilitate potential gene flow where possible. For *P. reticulatu*s, the AMOVA results reveal that the moderate FST value suggests significant but less pronounced genetic differentiation compared to *P. segnis*, with a P-value indicating statistical significance. This pattern suggests more frequent gene flow among populations of *P. reticulatus*, maintaining higher levels of genetic connectivity (Palumbi 1994; Cowen and Sponaugle 2009). In marine species, moderate genetic differentiation coupled with significant within-population variation often reflects a balance between local adaptation and gene flow, enhancing populations' overall genetic diversity and resilience (Hellberg 2009; Gagnaire et al. 2015). For *P. reticulatus*, conservation strategies should not just aim to preserve this connectivity, but also to mitigate habitat fragmentation and ensure the maintenance of corridors that facilitate dispersal and gene flow.

Network Analysis

In *P. segnis*, the MJ network shows that H2 is the central and most frequent haplotype, indicating it might be an ancestral or more recent, widespread haplotype within this population. As indicated by a single or few mutational steps, the close genetic relationships between H2 and haplotypes H1, H4, and H6 this situation suggest recent divergence and ongoing gene flow among these haplotypes (Bandelt et al. 1999). Conversely, the more distantly related haplotypes H3 and H5, connected through numerous mutational steps, indicate historical separations and potentially isolated evolutionary pathways. The presence of an inferred median vector (mv1) highlights possible unsampled or extinct intermediate haplotypes, suggesting historical demographic events like population bottlenecks or expansions, which may have shaped the current genetic landscape (Excoffier et al. 2009). The network structure, with a central haplotype and multiple peripheral haplotypes, reflects a star-like pattern often associated with population expansions from a common ancestor (Slatkin and Hudson 1991). This pattern suggests that the central haplotype, H2, may have played a crucial role

in the population's expansion and maintenance of genetic diversity.

In contrast, the peripheral haplotypes H1, H4, H6, H3, and H5 may represent localized adaptations or genetic drift in smaller, isolated subpopulations. For *P. reticulatus*, H9 emerges as the central and most diversely shared haplotype, suggesting it plays a similarly central role within this species' population structure. The peripheral haplotypes (H8, H10, H11, H12) are less common and show varying degrees of genetic differentiation from H9. This pattern indicates that while H9 remains prevalent across populations, the peripheral haplotypes may represent localized adaptations or genetic drift in smaller, isolated subpopulations. The presence of median vectors in *P. reticulatus* further illustrates potential genetic pathways and historical connections among haplotypes, providing insights into past population dynamics and movements. Such patterns may result from historical fragmentation followed by secondary contact or continuous but limited gene flow between populations (Hey 2005). The MJ network analyses for both *P. segnis* and *P. reticulatus* underscore the importance of central haplotypes (H2 in *P. segnis* and H9 in *P. reticulatus*) in maintaining genetic connectivity across populations. These central haplotypes likely represent crucial genetic reservoirs that contribute to the resilience and adaptability of these species in their respective environments (Templeton et al. 1995). The identification of peripheral and more differentiated haplotypes points to areas where genetic diversity is remarkably high, a factor that could be of paramount importance for conservation efforts. The preservation of such diversity is not just a goal; it is a necessity for the species to effectively cope with environmental changes and other stressors (Allendorf et al. 2010).

Effective conservation strategies should aim to preserve the central haplotypes that ensure genetic connectivity and the unique peripheral haplotypes that contribute to overall genetic diversity. Management practices should protect critical habitats, promote gene flow, and prevent habitat fragmentation to sustain these species' genetic health and evolutionary potential (Frankham et al. 2010).

Conclusion

In conclusion, the comprehensive analysis of *Neptunus* (*Portunus*) *pelagicus* and related species, including *P. segnis* and *P. reticulatus*, sheds light on their intricate population structures, evolutionary histories, and genetic dynamics. The delineation of distinct genetic lineages within these species highlights the necessity for tailored conservation strategies.

Detecting significant genetic differentiation and population structuring within *P. segnis* and *P. reticulatus* emphasizes the importance of implementing species-specific conservation measures. For *P. segnis*, characterized by high genetic differentiation among populations, localized conservation efforts targeting the unique genetic makeup of each population are imperative. In contrast, for *P. reticulatus*, which exhibits more genetic connectivity, conservation strategies should prioritize maintaining and enhancing gene flow among populations to preserve overall genetic diversity.

The neutrality tests conducted provide insights into these species' evolutionary dynamics and demographic history. The significant negative values observed in the neutrality tests for both species suggest recent population expansions, highlighting the significance of historical events in shaping their genetic diversity and population structures.

The results of the global and pairwise differentiation tests further underline the need to consider population-specific dynamics in managing and conserving marine species. Conservation strategies should focus on maintaining genetic connectivity, preserving habitat integrity, and promoting gene flow to ensure these species' long-term viability and adaptability.

Moreover, the network analysis of haplotype relationships within *P. segni* and *P. reticulatus* illuminates the central role of specific haplotypes in maintaining genetic connectivity across populations. Understanding the genetic pathways and historical connections among haplotypes provides valuable insights into past population dynamics and movements, aiding in developing effective conservation strategies.

In conclusion, the comprehensive genetic analyses presented in this study underscore the importance of adopting species-specific conservation approaches tailored to the unique genetic characteristics and population structures of Neptunus (*Portunus*) *pelagicus, P. segnis*, and *P. reticulatus*. These findings provide a foundation for informed conservation efforts to preserve these species' genetic diversity and evolutionary potential in the Indo-West Pacific region and beyond.

Ethical Statement

Not Applicable.

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Author Contribution

Author 1 conceived and designed the study, performed all experiments, analyzed the data, and drafted the manuscript.

Author 2 and Author 3 provided supervision, critical guidance, and valuable insights throughout the research process. All authors have read and approved the final version of the manuscript.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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