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Article

Integrated Genetic and Statolith Shape Analysis Reveals the Population Structure of *Todarodes pacificus* in the Coastal Waters of China

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Abstract

Understanding stock structure is a priority for evaluating population dynamics of commercially fished cephalopods under fishing pressure and environmental changes. This study employed a multidisciplinary approach to clarify the stock structure of *Todarodes pacificus*, a highly migratory squid species exploited by Asia-Pacific countries. Based on the analysis of statolith increments, summer and autumn cohorts were identified. Low haplotype diversity was revealed by two mitochondrial DNA markers (COI, 16S) and one nuclear DNA (nuDNA) marker (ODH). There was no significant genetic differentiation through phylogenetic trees and haplotype networks. The overall shapes between cohorts were significantly different, along with high classification success, could be used as a classification marker. This result offers new insights into the influence of genetic and environmental factors on statolith shape. The integrated results provide a comprehensive understanding of the stock structure of *T. pacificus*, laying the foundation for resource development and the conservation of the species.

Keywords: *Todarodes pacificus*; population structure; genetic diversity; cohort; statolith morphology

1. Introduction

In recent decades, cephalopod fisheries globally expand and are viewed and exploited as alternatives to many depleted conventional finfish fisheries[1]. The marked rise in cephalopod landings has contributed to the relative stability and high level of global marine fisheries. Correspondingly, fishing pressure on cephalopod populations has increased, and catches have leveled off to 3.6 million tones in 2018 from a peak of 4.9 million tones in 2014 [2]. This makes identifying the population structure of the commercial species crucial, since population is the basic biological unit considered when evaluating ecosystem conservation and managing fishery resource [3]. However, cephalopod has unique life history characteristics, such as short lifespan, high natural mortality and turnover rate, and diversified habitat [4]. These characteristics make them can quickly adapt to environmental changes, thereby gaining a competitive advantage and filling the niche of other biological populations, and also resulting in relatively complex populations [5]. Consequently, it becomes imperative to integrate diverse stock identification techniques. Moreover, the extensive oceanic migrations of nektonic squids, which traverse multiple oceanic ecosystems during their movements, pose a further complication in harmonizing operational and biological management approaches for effective population governance.

Todarodes pacificus (Cephalopoda: Ommastrephidae), is distributed in the northwest Pacific between 20 and 60°N. It is one of the earliest developed and utilized species worldwide, and largely

exploited by Asia-Pacific countries including China, Japan, and Korea [6]. At present, the research on *T. pacificus* mainly focuses on resource abundance and its assessment, basic biology, genetic diversity of prefectural-level populations, and the spatio-temporal distribution of fish farms [7]. The species has three cohorts with different peak spawning seasons: summer, autumn and winter. The three cohorts have different distribution, migration route, spawning areas and resource dynamic. Long-term abundance variability and its relationship with environmental changes has been evaluated, which contribute to better management [8]. However, due to the complex migration route, different cohorts of *T. pacificus* mix in some fishing ground, and then stock composition need be evaluated, namely cohort discrimination method needed. Furthermore, as an important part of population, biological knowledge on the stock in the East China Sea and Yellow Sea is quite lacking, and cohort composition is urgently required to provide comprehensive knowledge on the population structure of *T. pacificus*.

Because of challenging in identifying squid stocks, wider range of tools to investigate stock structure become available and a multidisciplinary approach combining several tools can be helpful. Molecular genetic markers are versatile tools for researching the genetic diversity of marine organisms and uncovering phylogenetic relationships [9]. At present, mitochondrial DNA (mtDNA) and nuclear DNA (nuDNA) markers, two common molecular markers, play important roles in revealing the genetic structure and dynamics of a population [10]. Mitochondrial DNA markers are widely used in population genetics studies of cephalopods [11]. Nuclear genes can provide more genetic information about organisms and are equally effective in detecting a populations genetic structure [12]. In addition, statolith is also an effective tool for studying population structure, because it has a stable shape and contains important biological and ecological information of cephalopods [13]. Because its outline varies predictably with developmental temperature, diet and depth, geometric analysis of statolith outlines discriminates among conspecifics originating from disparate habitats and thereby illuminates both ecological behaviour and demographic subdivision in oceanic squid [9].

To obtain deeper insights into population diversity and genetic structure, jointly analysing molecular markers alongside statolith data analysis offers a more comprehensive perspective unattainable through either dataset alone [14]. In this study, the genetic diversity of *T. pacificus* and the statolith shape was analyzed to identify the stock structure, the aim was to: 1) better understand the migration patterns and gene flow of *T. pacificus*; 2) examine the morphological differences in statoliths caused by different spawning seasons (summer and autumn), providing better classification criteria and better understanding of the dynamics of cephalopod resources in China’s seas.

2. Materials and Methods

2.1. Sample Collection and Processing

Between 2019 and 2024, *T. pacificus* specimens were acquired across three sectors of the China Seas: the northern Yellow Sea, the northern East China Sea, and the southern East China Sea (Table 1; Figure 1). The samples were obtained from local fishermen and were immediately placed on ice and transferred to the laboratory.

Table 1. Statolith and genetic samples data for *T. pacificus* in the China Seas.

Region	Number of analyzed individuals	Cohort composition (Summer:Autumn)	Sampling time	Mantle length range/mm
NYS	48	48:0	2019.11	162~204
	26	26:0	2023.11	235~305
	68	68:0	2024.11	160~320

Statolith samples		51	0:51	2024.12	150~317
	NECS	24	0:24	2024.3	205~280
		49	49:0	2024.11	215~296
	SECS	60	0:60	2024.3	165~283
	NYS	20	20:0	2023.11	235~305
		20	20:0	2024.11	160~320
		20	0:22	2024.12	150~317
Genetic samples	NECS	20	0:20	2024.3	216~296
		20	20:0	2024.11	215~296
	SECS	20	0:20	2024.3	165~284

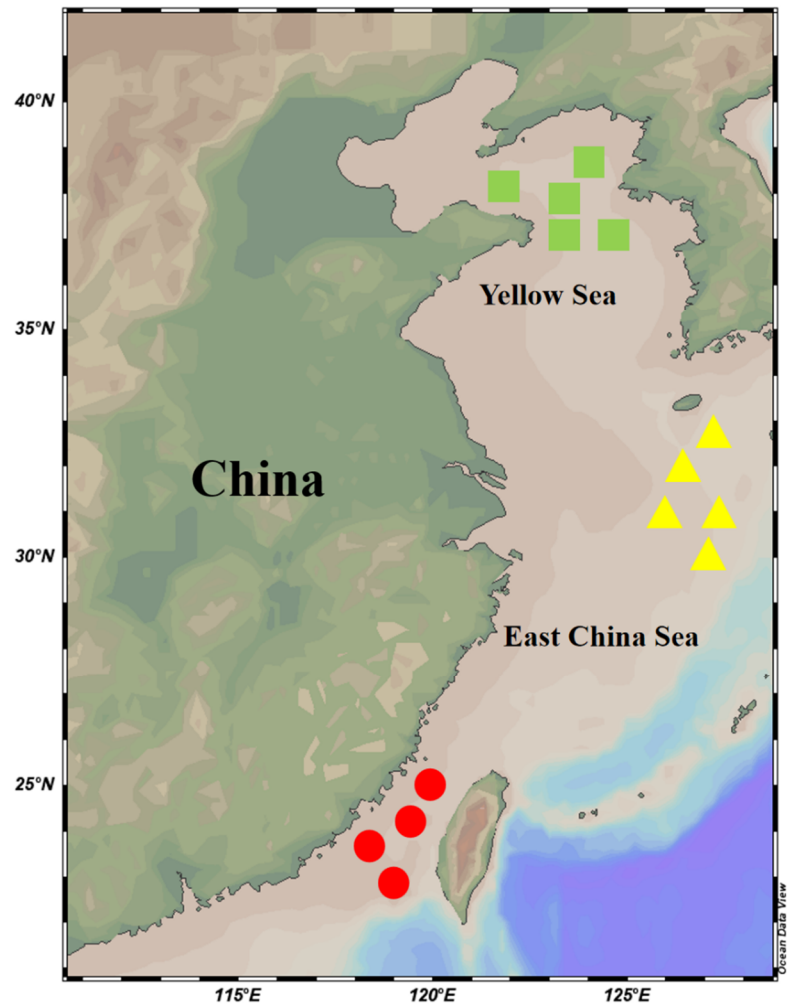


Figure 1. The sampling map for *T. pacificus* from different locations. The green rectangles represent the northern part of the Yellow Sea, the yellow triangles represent the eastern part of the East China Sea, and the red circles represent the southern part of the East China Sea.

Mantle muscle specimens were ethanol-preserved (100%) and maintained at -30 °C for genetic analysis. For each squid, the dorsal mantle length (DML, mm) and body weight (BW, 0.1g) were measured, and gonad development stages were visually assessed [9]. Squid statoliths were extracted, cleaned with an ultrasonic cleaner, dried, and stored in centrifuge tubes for further analysis.

2.2. Analysis of Otolith Age Identification

Statolith was embedded in epoxy resin (Buehler, Epothin) on a glass slide with posterior side up. After hardening, the posterior side was hand ground with alternating fine waterproof sandpapers (Buehler, 600 to 4000 grits) until fully exposure of nucleus, and then polished with 0.3µm aluminium powder [15]. For larger statolith, the grinding direction should be adjusted during grinding process to avoid over ground in the dorsal dome.

The polished statoliths were photographed under an optical microscope (×200 magnification) using a microscope system (OLYMPUS BX53 and DP74) (Figure S1). For each statolith the total number of increments was counted from the hatch ring to the margin of dorsal dome. The age of specimens was determined by counting all dark rings. An experienced reader counted the increments three times, with a one - month gap between each count. When the differences in increment counts were under 10%, the average of the three counts was used to estimate daily age. The hatch time for each individual was found by subtracting the daily age from the capture date, and specimens were then assigned to a cohort.

2.3. DNA Extraction and PCR Amplification

Total genomic DNA was extracted using following the CTAB protocol refined by Winnepenninckx and colleagues [16]. The target genes were amplified by PCR using primers, whose sequences are catalogued in Appendix 1 (Table S1). The amplification of target genes was reacted in a volume of 25 µL: 1 µL of 100 ng/µL DNA template, 1 µL of each 10 µM/µL primer, 12.5 µL of 2 × Taq Master Mix, and 9.5 µL water. The PCR cycle was 95°C for 5 min, followed by 35 cycles of 95°C for 10 s, 55°C for 20 s, 72°C for 30 s, and a final 72°C for 5 min extension. The products were detected by 1.5% agarose gel electrophoresis and sent to Sangon Biotech (Shanghai, China) for sequencing.

2.4. Sequencing Analysis

2.4.1. Population Genetics

For sequence assembly and alignment of *Todarodes pacificus* genetic data, SeqMan v.7.2 [17] from the DNASTAR package and MEGA v.11 [18] were used. MEGA v.11 [18] also calculated the average base composition, variable site count, and genetic distances for all sequences. The DnaSP v 6.1 [19] software computed genetic diversity indices and inferred population demographic history via mismatch distribution plots for all *T. pacificus* populations and lineages. The haplotype network maps were constructed with Popart v.1.7 [20] and then manually adjusted. The genetic differentiation indices (Fst) can explore the level of genetic differentiation among populations. An Analysis of Molecular Variance (AMOVA) [21] with the Kimura-2-parameters model, and neutrality tests, Tajima's D [22] and Fu's Fs tests [23], were determined with the Arlequin 3.5 [24].

2.4.2. Phylogenetic Relationship

Phylogenetic inference relied on four datasets: the first three datasets consisted of haplotype sequences of COI, 16S and ODH genes, and a fourth in which the same markers were concatenated per specimen via PhyloSuite v1.2.2 [25]. Optimal nucleotide substitution models were identified with ModelFinder [26] under the Bayesian Information Criterion. Maximum-likelihood phylogenies were inferred in IQ-TREE v1.6.12 [27] and Bayesian topologies in MrBayes v3.2 [28]. Finally, FigTree v1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree>, accessed on 10 June 2024) was used to edit and visualize the ML and BI trees.

2.4.3. Statolith Shape Sampling and Statistical Analysis

To mitigate the potential impact of individual developmental variations on statolith morphology, a selection process was implemented, focusing solely on mature specimens (at Stages III, IV, and V) with body lengths falling within a specified, suitable range. These were then earmarked for subsequent morphological examination of their statoliths [29]. Prior research has highlighted the near-identical microstructural characteristics exhibited by the left and right statoliths. Consequently, for the purposes of photography and image analysis, one statolith was randomly chosen from each paired set [30]. The statoliths were arranged on a matte black substrate and imaged through a Nikon SMZ 18 stereomicroscope coupled to a digital camera. Leveraging the “shapeR” package [31] for the analysis of statolith images, the contours of sampled statoliths were extracted. Subsequently, shape data was visualized and generated, with the contours being converted into 45 normalized elliptical Fourier coefficients. This particular package facilitated the measurement of statolith length, width, and area, as well as the computation of Sw/SI (the ratio of statolith width to length) and the standard statolith area ($Ssa = 1000 \times (\text{statolith area}/DML^2)$). Species discriminant analysis was performed utilizing version 4.7 of the Random Forest (RF) package in R, with the out-of-bag (OOB) evaluation employed to gauge the accuracy of the random forest algorithm [32].

3. Results

3.1. Genetic Analysis

A total of 117 COI sequences, 110 16S sequences and 113 ODH partial sequences of *T. pacificus* were obtained (Table 2). Across all three genes, thymine consistently dominated the nucleotide profile, producing a pronounced AT skew ($A + T > G + C$). The ODH sequences exhibited the highest proportion of variable sites and the highest degree of variation (20.58%), whereas mitochondrial 16S was the most conserved (0.88%). The mitochondrial gene sequences showed low levels of variation compared to the nuclear gene.

Table 2. Molecular characterization based on mitochondrial and nuclear gene sequences.

Mt/Nu.	Gene	Length/bp	T/%	C/%	A/%	G/%	A+T/%	G+C/%	S	Proportion of Variable Sites/%
Mt	COI	439	43.20	20.86	21.49	15.36	63.79	36.22	15	3.36
Mt	16S	458	26.58	0.00	48.42	25.00	75.00	25.00	4	0.88
Nu	ODH	517	28.13	31.30	26.63	13.93	54.76	45.23	106	20.58

Across the two cohorts of *T. pacificus*, 18 COI haplotypes, 6 16S haplotypes and 21 ODH haplotypes were defined. Based on the ODH and COI genes, the haplotype diversity and nucleotide diversity of all individuals were generally high (ODH:0.6010 and 0.0053; COI:0.4560 and 0.0013), whereas the 16S sequences displayed low haplotype diversity and low nucleotide diversity. The analysis of the three genes basically revealed that the autumn cohort had higher nucleotide diversity, while the summer cohort had lower haplotype diversity (Table 3).

Table 3. The genetic diversity indices for different seasons of *Todarodes pacificus*.

Group	COI				16s				ODH			
	N	Hap	Hd	π	N	Hap	Hd	π	N	Hap	Hd	π
summer	59	9	0.4610	0.0014	55	3	0.230	0.0005	57	12	0.6020	0.0020
autumn	58	11	0.4740	0.0013	55	4	0.109	0.0002	56	13	0.6070	0.0059
Total	117	18	0.4650	0.0013	110	6	0.171	0.0004	113	21	0.6010	0.0053

The 18 haplotypes of COI sequences were 3 shared haplotypes and 15 unique haplotypes. These haplotypes formed a star-shaped network centered around Hap1, respectively, with Hap1, Hap5,

Hap8, and Hap10 being identified as shared haplotypes, with deletion variants also detected (Figure 2). The 16S gene exhibited the lowest haplotype diversity, with Hap1 and Hap2 being the most frequently shared haplotypes (Figure 3). For the ODH gene, there were five shared haplotypes, including Hap1, Hap2, Hap3, Hap4, with a higher distribution of haplotypes and the presence of deletion haplotypes (Figure 4). Compared to mitochondrial genes, nuclear genes demonstrated fewer mutational steps between the two lineages.

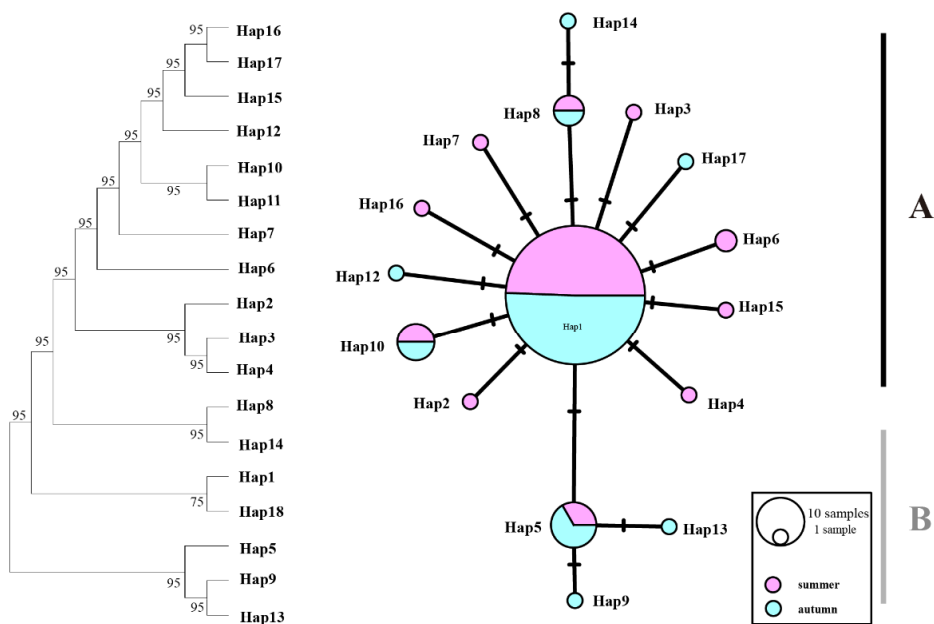


Figure 2. Maximum likelihood (ML) phylogenetic tree and haplotype network of *T. pacificus* based on COI haplotypes. Branch numbers are bootstraps (left) and posterior probability (right). Black dots represent hypothetical missing intermediates.

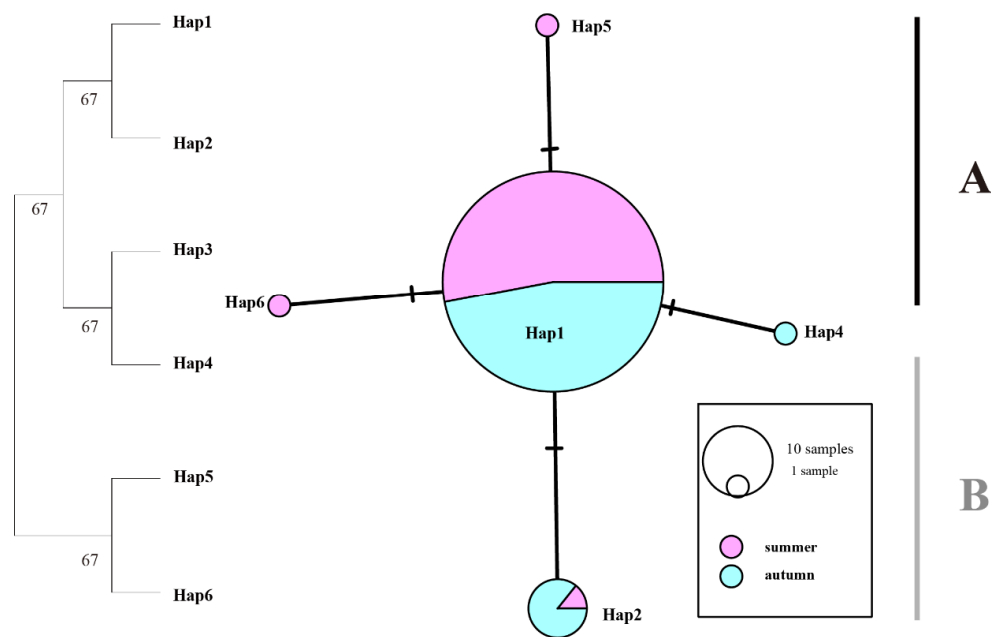


Figure 3. Maximum likelihood (ML) phylogenetic tree and haplotype network of *T. pacificus* based on 16S haplotypes. Branch numbers are bootstraps (left) and posterior probability (right). Black dots represent hypothetical missing intermediates.

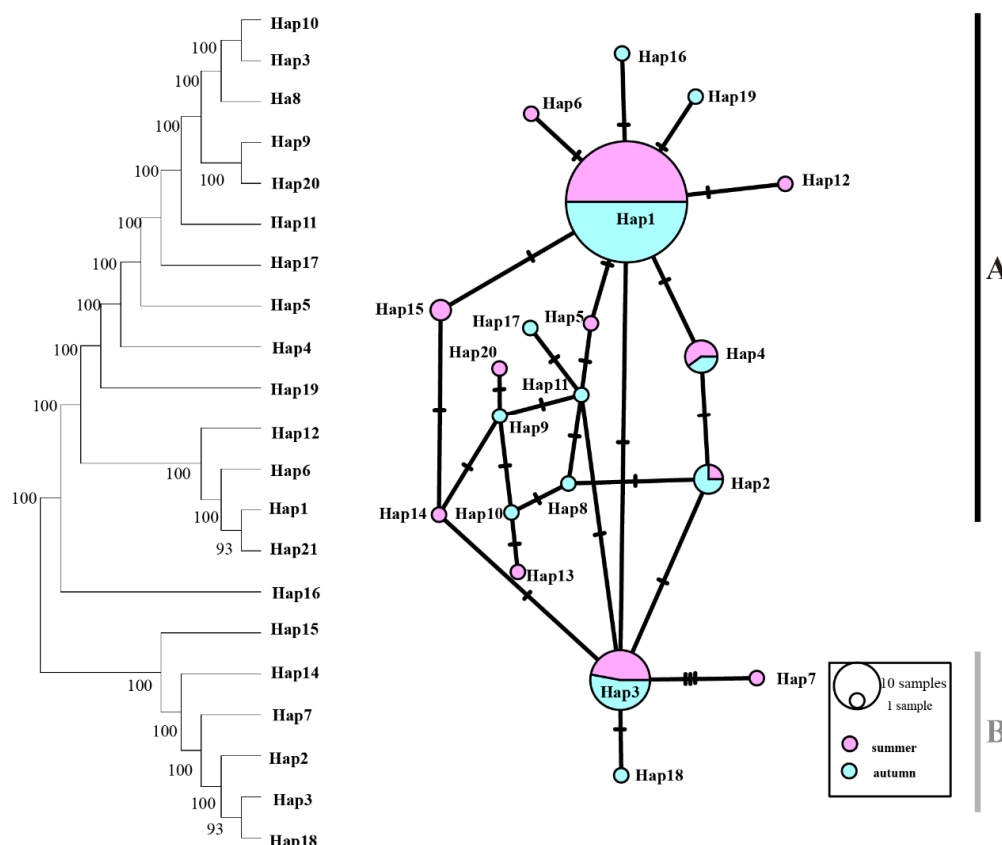


Figure 4. Maximum likelihood (ML) phylogenetic tree and haplotype network of *T. pacificus* based on ODH haplotypes. Branch numbers are bootstraps (left) and posterior probability (right). Black dots represent hypothetical missing intermediates.

Both the trees and haplotype networks of the three genes informed no distinct branches (Figures 2–4). The results of the combined phylogenetic analysis of multiple genes based on the ML and BI methods were in agreement with the single-gene results (Figure 5).

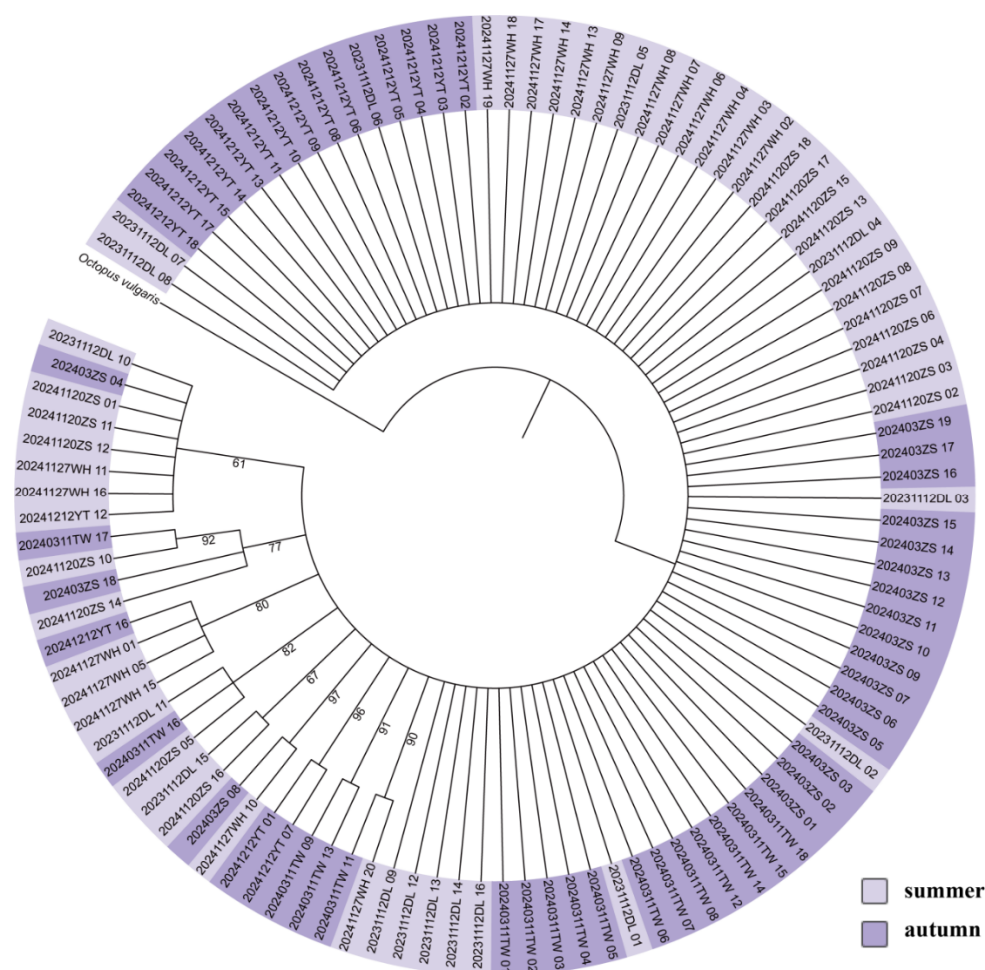


Figure 5. Phylogenetic trees constructed using the concatenation of mitochondrial and nuclear genes. The numbers in each node represent bootstraps of maximum likelihood (ML) and posterior probabilities of Bayesian inference (BI) analyses, respectively. Lavender represents the summer population and purple represents the autumn population.

AMOVA results derived from the three genes demonstrated consistent patterns, indicating a lack of genetic differentiation among *T. pacificus* populations ($p \geq 0.1$), and mitochondrial genes exhibited a higher proportion of variation compared to nuclear genes (Table 4). The Fst analyses of all the genes indicated that there is almost no differentiation between autumn and summer cohorts, and the mutation mainly exists within the population ($0 < F_{st} < 0.05$) (Table 5).

Table 4. AMOVA analysis of *T. pacificus* populations based on mitochondrial and nuclear gene sequences.

Gene	Source of Variation	df	Sum of squares	Variance Component	Percentage of Variation (%)	F Statistic
COI	Among populations	2	0.463	-0.00280 Va	-0.85	FST : -0.00853 (P-value = 0.83187+0.01114)
	Within populations	114	37.811	0.33167 Vb	100.85	
	Total	116	38.274	0.32887	100	
Cyt b	Among populations	2	0.203	-0.00066 Va	-0.54	FST : -0.00537 (P-value = 0.57478+0.01351)
	Within populations	114	37.811	0.33167 Vb	100.85	
	Total	116	38.274	0.32887	100	

16S	Within populations	107	13.251	0.12385 Vb	100.54	FST : 0.00148 (P-value = 0.37634+-0.01613)
	Total	109	13.455	0.12385 Vb	100	
ODH	Among populations	2	1.133	0.00080 Va	0.15	
	Within populations	111	59.815	0.53887 Vb	99.85	
	Total	113	60.947	0.53967	100	

Table 5. Fst between populations of *T. pacificus* based on COI, 16S, ODH genes for different seasons.

	Groups	autumn	summer
COI	autumn	0.00000	
	summer	0.00291	0.00000
16S	autumn	0.00000	
	summer	0.03191	0.00000
ODH	autumn	0.00000	
	summer	0.00075	0.00000

* Significant at *p* < 0.01.

3.2. Statolith Morphological Analysis

There are significant differences in the statolith morphology coefficients between summer and autumn cohorts (*p*<0.01). The major morphology difference appeared in posterior indentation (Figure 6). Random forest revealed a high discrimination success rate between cohorts (Table 6).

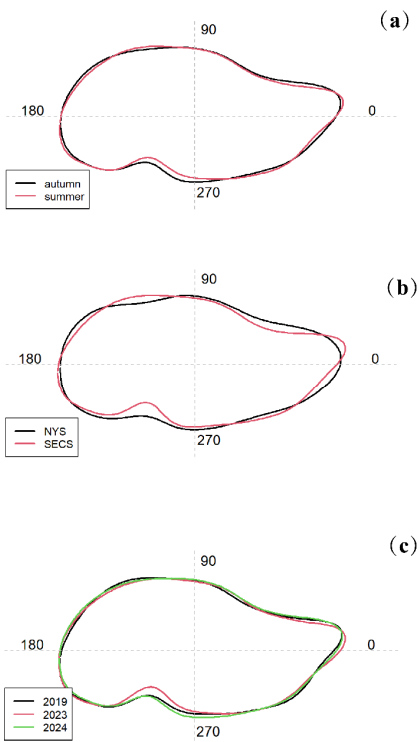


Figure 6. Reconstructed statolith outlines for different cohorts (a), different regions in autumn (b) and different years in summer (c).

Table 6. Out-of-Bag (OOB) estimation matrix of the random forest among the three seasons in BBG.

Season	autumn	summer	Classification Success Rate (%)
autumn	119	21	85.00
summer	10	182	94.79

Significant spatial difference in statolith morphology was only detected between NYS and SECS in autumn cohort ($p<0.01$). The statolith difference appeared in overall outlines. Additionally, the average statolith outlines revealed significant differences in the rostrum and posterior indentation regions among years (Figure 6). The statolith difference appeared in overall outlines. There are significant differences in the statolith morphology between cohorts ($p<0.01$) (Table 7).

Table 7. Results of Variance analysis on *T. pacificus* in different seasons based on the statolith shape coefficient.

Groups	<i>p</i>	<i>Sw/Sl</i>	<i>Ssa</i>
autumn		0.5710	1763.0060
summer		0.5483	1706.8217
summer VS autumn	1.351e-10 *	5.342e-06 *	0.09057

* Significant at $p < 0.01$.

4. Discussion

The study combines statolith with genetic analysis in *T. pacificus* and provides a comprehensive insight into the population structure in the China Seas. The result of statolith microstructure first reveals the cohort composition of *T. pacificus* commercially explored in China. The winter cohort, a major component, was not detected in the commercially collected specimen. The result is inconsistent with Rosa et al. [33], only under unfavourable spawning area distribution pattern, the winter cohort would be transported more successfully into the Yellow Sea and occupy certain proportion. In contrast, despite lacking specific proportion, summer cohort contributes a more important part in China compared to Korea and Japan [34]. Such condition should be emphasized in future management since summer cohort is a small-sized group that is more vulnerable to fishing pressure and local extinction [35].

The results indicated that *T. pacificus* has low haplotype and nucleotide diversity, with no significant genetic differentiation among different populations. This is similar to the conclusions drawn by Zhang et al. [7] in recent years. However, there’s a big difference with results of Kim et al. [36], who identified 49 haplotypes in all with high nucleotide diversity. This may be due to the severe decline in *T. pacificus* population caused by overfishing in recent years, which has reduced genetic diversity along with the population size [21]. In addition, this study identified only six haplotypes of the 16S gene in the Pacific umbrella squid. This is because the 16S gene in cephalopods, including the Pacific umbrella squid, may have maintained a high level of conservation over long-term evolution, resulting in fewer variable sites within the population and, consequently, lower haplotype diversity [37].

The present study reveals relatively low genetic diversity in *T. pacificus* across Chinese coastal waters, with no significant genetic differentiation detected among NECS, NYS, and SECS populations. This pattern may be closely tied to the species’ extensive migratory behavior and the geographical distribution of its spawning grounds [38]. Pacific flying squid undertake long-distance migrations, moving between spawning, feeding, and nursery grounds throughout their life cycle. These migrations facilitate substantial gene flow among spatially separated populations, thereby preventing genetic divergence and maintaining genetic homogeneity across a broad geographical range [39]. The spawning grounds of *T. pacificus* in the Northwest Pacific Ocean serve as critical hubs

for genetic exchange. The aggregation of individuals from various regions during the spawning season enhances the opportunity for genetic recombination and mixing. Subsequent larval dispersal by ocean currents further contributes to the widespread distribution of genetic material, reinforcing the genetic connectivity among different coastal populations. Similar patterns have been observed in other Ommastrephidae species. *Ommastrephes bartramii* demonstrated that despite potential geographical segregation of spawning areas, high levels of gene flow occur among different populations, maintaining genetic homogeneity across the species' range. In contrast, species with more limited dispersal capabilities or isolated spawning grounds tend to exhibit greater genetic differentiation among populations [40].

Compared to genetic markers, significant differentiation was detected in the statolith shape, highlighting the fact that ecologically separated groups can be identified in this *T. pacificus*. The statolith shape difference further emphasizes the ecological difference between summer and autumn cohorts. The high discrimination success rate between cohorts provides an effective tool for evaluating cohort composition and support effective management. Similar with fish otoliths, upon environment-induced overall outline change and genetically locally induced changes in prominent parts [41]. Since none significant genetic structure in *T. pacificus*, the observed statolith difference could be mainly resulted by environmental factors. Consistently, the major difference locates in posterior indentation, a prominent part. Because of strong swimming ability of *T. pacificus*, it is difficult to track its experienced environmental factor [9]. However, compared to significant seasonal difference, spatial difference was only observed between NYS and SECS of autumn cohort. Thus, the morphology difference could be mainly resulted by environmental conditions during early life stage [42]. In addition, the position of oceanic fronts played an important role in restricting paralarvae distribution along the inshore edge of the Tsushima Warm Current (TWC) [36]. Therefore, the inter annual distribution and difference result in the detected morphology difference among years.

5. Conclusions

The study combines statolith with genetic analysis in *T. pacificus* and provides a comprehensive insight into the population structure in the China Seas. The result of statolith microstructure first reveals the cohort composition of *T. pacificus* commercially explored in China. This study revealed there is almost no significant genetic differentiation among summer and autumn cohorts based on the mitochondrial genes (COI and 16S) and nuclear gene (ODH) sequences. The overall shapes of statoliths in summer and autumn cohorts are quite different, along with high classification success, could be used as a classification marker on the basis of no significant differences in genetics, providing an important reference basis for the subsequent classification and a theoretical basis for the rational development and utilization of fishery resources of *T. pacificus*.

Supplementary Materials: Table S1.: The primers of mitochondrial (mt) and nuclear (nu) genes. Figure. S1: Age of statolith diagram.

Author Contributions: X.Z., C.Z., Sonthaya Phuynoi,J.L. and X.L. conceptualized and designed the research framework. C.Z. was responsible for sample collection. X.L. conducted the experimental procedures and drafted the initial manuscript. X.Z.,J.L. and C.Z. provided critical revisions to the paper. All authors reviewed the final manuscript and approved its submission for publication.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Conflicts of Interest: The authors declare no conflicts of interest.

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