

Article

Population Structure of *Todarodes pacificus* (Cephalopoda: Ommastrephidae) in the Northwest Pacific: Insights from Integrated Genetic and Statolith Morphology Analyses

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Abstract

Accurate identification of population structure is crucial for the sustainable management of cephalopod fisheries. This study integrated genetic and morphological approaches to investigate the population structure of *Todarodes pacificus* in the northwest Pacific. Specimens were collected from the northern Yellow Sea, northern East China Sea, and southern East China Sea between 2019 and 2024. Based on two mitochondrial genes (*COI* and *16S rRNA*) and one nuclear gene (*ODH*), population genetic analyses revealed no significant genetic differentiation among geographical regions or between summer- and autumn-spawning cohorts, suggesting high gene flow and possible panmixia. In contrast, geometric morphometric analysis of statoliths revealed significant differentiation between spawning cohorts, particularly in the posterior indentation region. Random Forest classification achieved high discriminatory accuracy (85.0–94.8%), supporting the robustness of statolith shape as a phenotypic marker. Interannual variation in statolith morphology was also detected, potentially linked to climatic events. Although no winter-spawning cohort was identified in the commercial samples examined, the summer-spawning cohort appears to constitute a major component of the Western Pacific stock. These findings demonstrate that statolith morphology can serve as a reliable tool for cohort discrimination even in the absence of genetic differentiation, offering critical insights for the cohort-specific management and conservation of *T. pacificus* resources.

Keywords: *Todarodes pacificus*; Cephalopoda; population structure; cohort; statolith morphology



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1. Introduction

The global expansion of cephalopod fisheries in recent decades has positioned them as viable alternatives to depleted traditional finfish stocks [1]. Increased cephalopod landings have helped maintain the overall stability and yield of global marine fisheries. However, this has led to intensified fishing pressure, with global catches leveling off at 3.6 million tons in 2018 after peaking at 4.9 million tons in 2014 [2]. Identifying the population structure of these commercial species is therefore critical as the population serves as the fundamental unit for ecosystem assessment and fishery management [3]. Cephalopods exhibit high species diversity and generally adopt a “fast-turnover” life history strategy, characterized

by annual reproduction, rapid growth, and short life cycles [4,5]. This strategy renders them highly sensitive to environmental conditions, enabling swift responses to fluctuations in hydrology, temperature, and food resources across specific spatiotemporal scales. Moreover, their extensive geographic distribution, diverse reproductive strategies, and population replenishment dynamics, which are strongly influenced by environmental variability, often result in highly dynamic and complex population structures [6]. Effectively governing these populations thus necessitates integrating diverse stock identification methods, a challenge compounded by the vast oceanic migrations of nektonic squids across multiple ecosystems, which complicates the alignment of biological and operational management strategies.

Todarodes pacificus (Cephalopoda: Ommastrephidae) is distributed in the northwest Pacific, ranging from 20° N to 60° N [7]. It is one of the earliest developed and utilized species worldwide and largely exploited by Asia-Pacific countries including China, Japan, and Korea [8]. At present, research on *T. pacificus* mainly focuses on resource abundance and its assessment, basic biology, genetic diversity of populations, and the spatiotemporal distribution of spawning ground [9]. The species has three cohorts with different peak spawning seasons: summer, autumn and winter. The three cohorts have different distribution, migration routes, spawning ground, and resource dynamics [9]. Although studies have evaluated long-term abundance variability and its relationship with environmental changes to support improved fisheries management [10], complex migration patterns cause cohorts to intermix in certain fishing grounds. This necessitates accurate assessment of stock composition, highlighting the need for effective cohort discrimination methods. Furthermore, while *T. pacificus* remains relatively abundant in the East China Sea and the Yellow Sea, biological knowledge of the stocks in these areas remains limited. The East China Sea serves as a key spawning and overwintering ground [11], whereas the Yellow Sea provides an important feeding area [12]. Given that these regions represent a substantial portion of the overall population, there is an urgent need to clarify cohort composition to advance a comprehensive understanding of the *T. pacificus* population structure.

The challenges associated with identifying squid stocks have led to the development of a wider range of investigative tools [13]. A multidisciplinary approach that integrates several methods is particularly beneficial. Molecular markers are essential for assessing genetic diversity and phylogenetic relationships in marine organisms [14]. Currently, two common types of markers (mitochondrial DNA (mtDNA) and nuclear DNA (nuDNA)) play critical roles in revealing population genetic structure and dynamics [15]. While mtDNA markers are widely applied in cephalopod population genetics [16], nuclear genes offer distinct advantages in molecular phylogenetic studies of animals, chiefly manifested in their more uniform substitution rates and consistent patterns of nucleotide replacement across different sites [17]. In addition, statolith is also an effective tool for studying population structure due to its stable morphology and its recording of important biological and ecological information [18]. 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 behavior and demographic subdivision in oceanic squid [14].

Integrating molecular markers with statolith morphology provides a more robust approach for analyzing the life history traits, mechanisms underlying the spatial population structure, and patterns of genetic differentiation [19]. In this study, we adopted a strategy combining two mitochondrial genes (*cytochrome c oxidase I* (COI) and *16S ribosomal RNA* (16S rRNA)) with a nuclear gene, *octopine dehydrogenase* (ODH). As a biparentally inherited nuclear marker with a moderate evolutionary rate, ODH has become an important tool in cephalopod population genetics and phylogeography [20]. For example, Muhammad et al. used two nuclear genes (*rhodopsin* and ODH) to characterize the population structure of

Octopus minor in Chinese waters [20]. Similar integrated multi-marker approaches have been successfully applied in studies of various cephalopod species [21]. The above analysis is conducive to a better understanding of the migration patterns and gene flow of *T. pacificus*. Furthermore, by examining differences in statolith morphology between summer and autumn spawning cohorts, this study aims to establish more reliable classification criteria and enhance our knowledge of cephalopod resource dynamics in Chinese seas.

2. Materials and Methods

2.1. Population Sampling

Between 2019 and 2024, *T. pacificus* specimens were acquired across three sectors of the northwest Pacific: the northern Yellow Sea, the northern East China Sea, and the southern East China Sea (Table 1; Figure 1). Samples were collected by Chinese squid jigging vessels during production surveys, immediately placed on ice, and transferred to the laboratory.

Table 1. Sample data of *T. pacificus* from the western Pacific.

	Region	Number of Analyzed Individuals	Cohort Composition (Summer:Autumn)	Sampling Time	Mantle Length Range/mm
Statolith samples	NYS	48	48:0	November 2019	162~204
		26	26:0	November 2023	235~305
		68	68:0	November 2024	160~320
	NECS	51	0:51	December 2024	150~317
		24	0:24	March 2024	205~280
		49	49:0	November 2024	215~296
	SECS	60	0:60	March 2024	165~283
Genetic samples	NYS	20	20:0	November 2023	235~305
		20	20:0	November 2024	160~320
		20	0:22	December 2024	150~317
	NECS	20	0:20	March 2024	216~296
		20	20:0	November 2024	215~296
	SECS	20	0:20	March 2024	165~284

Mantle muscle specimens were preserved in ethanol (100%) and maintained at −30 °C for genetic analysis. Mantle length and body weight were measured to the nearest 1 mm and 0.1 g, respectively. Gonadal maturity phases were determined through visual examination [14]. Squid statoliths were extracted and then cleaned in an ultrasonic bath, after which samples were dried and placed into centrifuge tubes.

2.2. Statolith Sample Collection and Processing

2.2.1. Analysis of Statolith Shape Sampling

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 [22,23]. These were then earmarked for subsequent morphological examination of their statoliths [24]. Previous research has demonstrated a high degree of similarity in the microstructural morphology between left and right statoliths. Therefore, one statolith from each bilateral pair was chosen at random for subsequent imaging and analytical processing [25–27]. Analysis of statolith shape sampling detailed protocol is provided in Supplementary Material (Text S1).

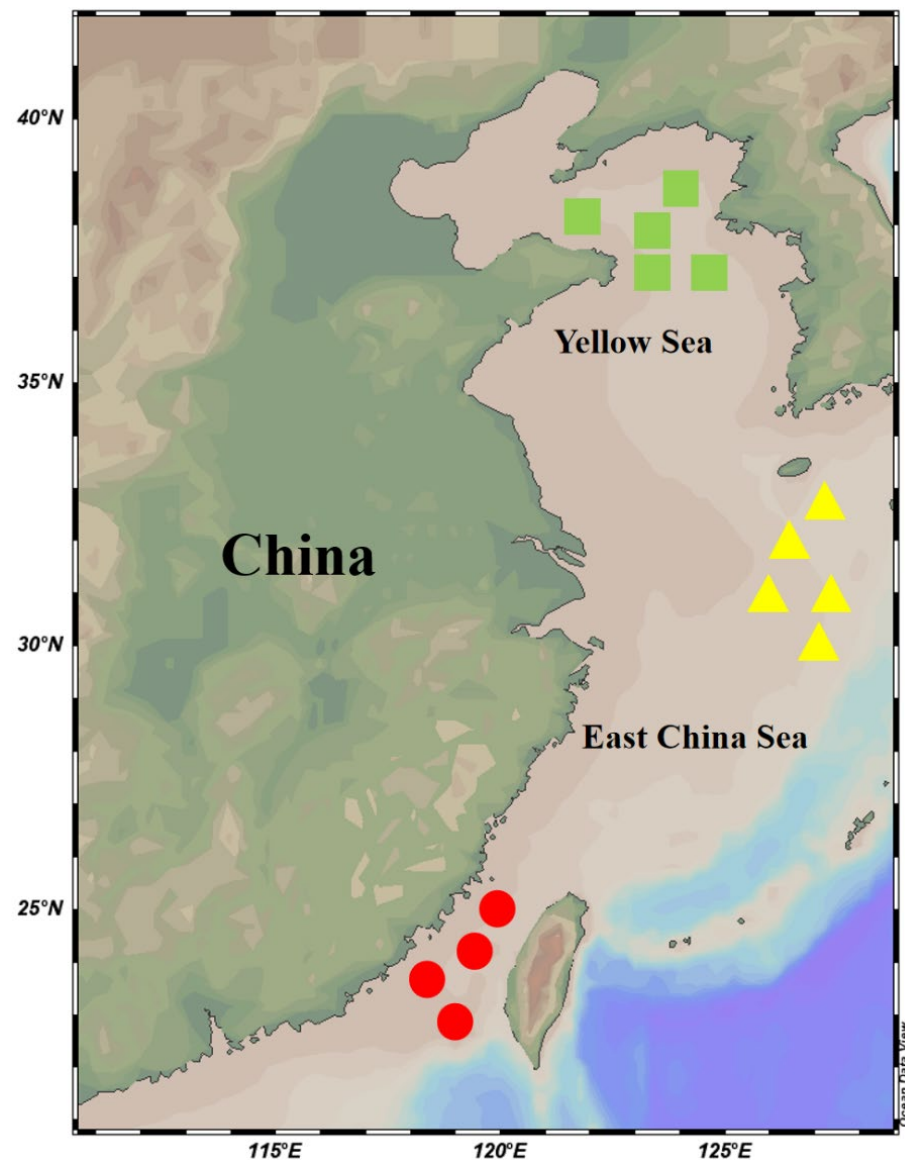


Figure 1. Locations of different sites of *T. pacificus*. The green squares represent the northern Yellow Sea, the yellow triangles represent the northern East China Sea, and the red circles represent the southern East China Sea.

2.2.2. Analysis of Statolith Age Identification

Statoliths were embedded in epoxy resin (Buehler, Epothin) on glass slides with their posterior side facing up. After the resin hardened, the posterior side was ground manually using progressively finer waterproof sandpaper (Buehler; 600 to 4000 grit) until the nucleus was fully exposed and then polished with 0.3 μm alumina powder [28]. For larger statoliths, the grinding direction was adjusted during the process to avoid over-grinding the dorsal dome. The polished statoliths were photographed at 200 $\times$ magnification using an Olympus BX53 microscope equipped with a DP74 camera (Olympus Corporation, Tokyo, Japan) (Figure A1). The age of each specimen was estimated by enumerating all dark rings. For individual statoliths, increment counts were performed from the hatch check to the dorsal dome margin, representing the total number of growth increments. Increments were counted three times by an experienced reader, with a one-month interval between counts. If the variation between counts was less than 10%, the mean of the three readings was used to estimate daily age. The hatch date for each individual was calculated by subtracting the estimated age from the capture date, after which specimens were assigned to a cohort.

2.3. Methods of Population Genetics

Detailed protocols of DNA extraction and PCR amplification are provided in Supplementary Material (Text S2). The primers used for amplification are listed in Appendix A (Table A1). Detailed protocols of population genetics and the phylogenetic relationship are provided in Supplementary Material (Texts S3 and S4).

3. Results

3.1. Genetic Analysis

Sequence data were successfully generated for *T. pacificus*, yielding 117 *COI*, 110 *16S rRNA*, and 113 partial *ODH* sequences, as summarized in 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 (20.58%), demonstrating the most substantial among all sequences analyzed, whereas mitochondrial *16S* was the most conserved (0.88%), reflecting the overall pattern of lower sequence variation in mitochondrial genes compared to the nuclear gene.

Table 2. Molecular analyses utilizing three gene sequences.

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

In total, 18, 6, and 21 distinct haplotypes were identified for the *COI*, *16S rRNA*, and *ODH* loci, respectively, across the two sampled cohorts of *T. pacificus*. Based on the *ODH* ($H_d = 0.6010 \pm 0.0025$, $\pi = 0.0053$ (95% CI: 0.0050–0.0055)) and *COI* genes ($H_d = 0.4560 \pm 0.0032$, $\pi = 0.0013$ (95% CI: 0.0011–0.0014)), haplotype diversity and nucleotide diversity across all individuals were generally high. In contrast, the *16S* sequences exhibited low haplotype diversity ($H_d = 0.1710 \pm 0.0024$) and low nucleotide diversity ($\pi = 0.0004$ (95% CI: 0.0002–0.0005)). Analysis across the three markers indicated that the autumn cohort generally had higher nucleotide diversity, while the summer cohort showed lower haplotype diversity (Table 3).

Table 3. Genetic diversity metrics across seasonal cohorts of *T. pacificus*.

Group	<i>COI</i>				<i>16S</i>				<i>ODH</i>			
	N	Hap	Hd	π	N	Hap	Hd	π	N	Hap	Hd	π
summer	59	9	0.4610	0.0014	55	3	0.2300	0.0005	57	12	0.6020	0.0020
autumn	58	11	0.4740	0.0013	55	4	0.1090	0.0002	56	13	0.6070	0.0059
total	117	18	0.4650	0.0013	110	6	0.1710	0.0004	113	21	0.6010	0.0053

Among the 18 *COI* haplotypes, three were shared and 15 were unique. These formed a star-shaped network centered on Hap1, with Hap1, Hap5, Hap8, and Hap10 identified as shared haplotypes; deletion variants were also observed (Figure 2). The *16S* gene displayed the lowest haplotype diversity (Figure 3). Analysis of the *ODH* gene revealed five shared haplotypes (Hap1, Hap2, Hap3, and Hap4), which exhibited a high-frequency distribution across populations (Figure 4). Both phylogenetic trees and haplotype networks of the three genes revealed no distinct branches, indicating an absence of clear genetic subdivision (Figures 2–4).

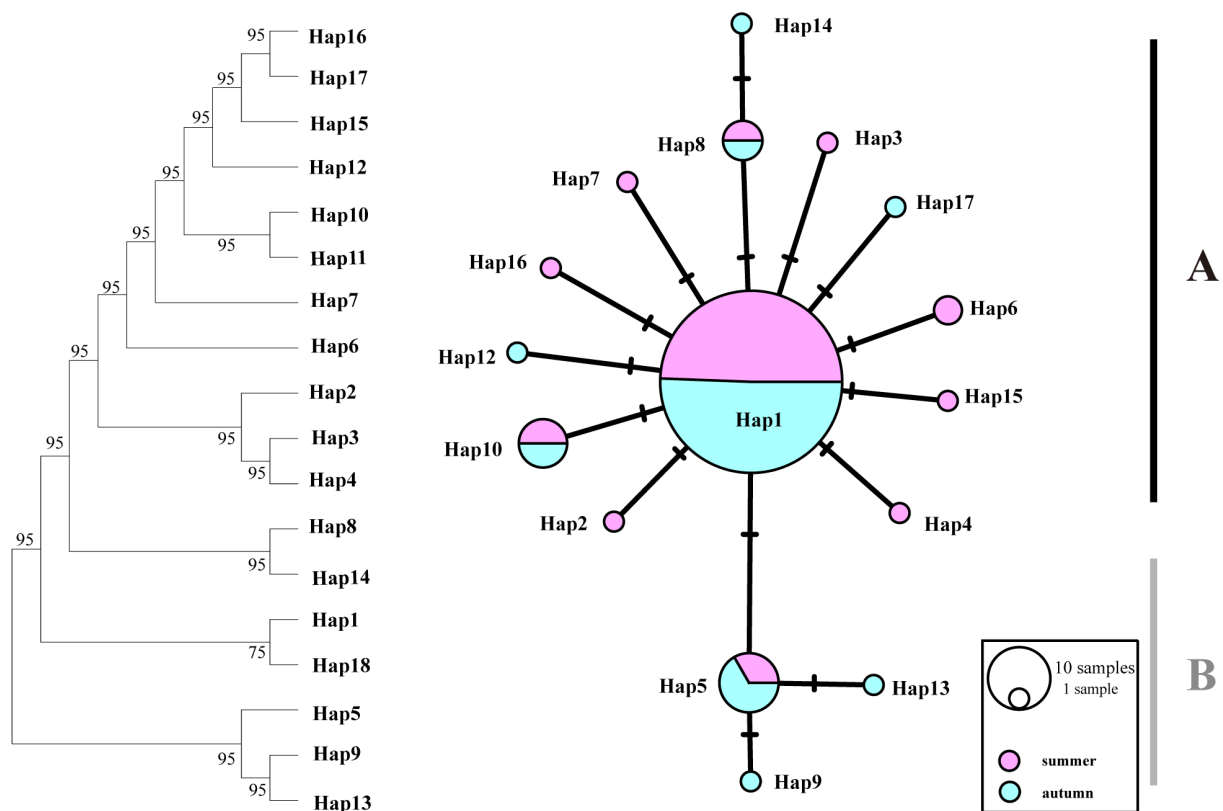


Figure 2. Maximum likelihood (ML) tree and haplotype network based on 18 COI haplotypes of *T. pacificus*. Branch numbers are bootstraps.

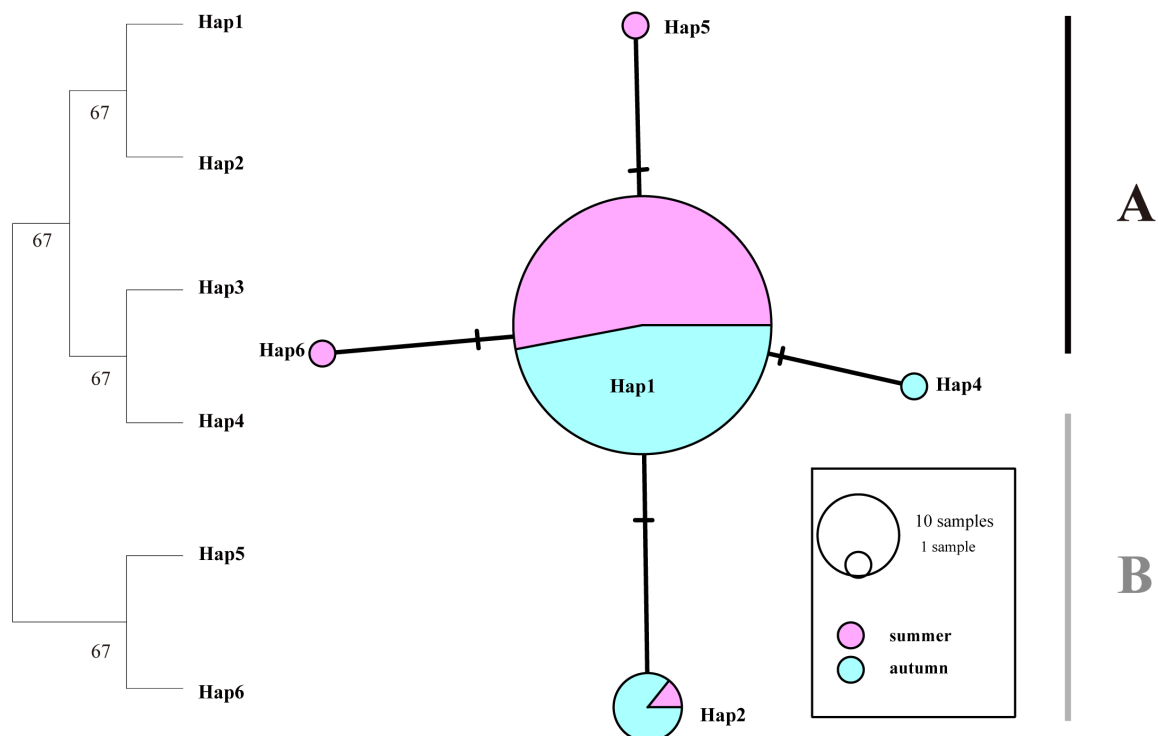


Figure 3. Maximum likelihood (ML) tree and haplotype network based on 6 16S haplotypes of *T. pacificus*. Branch numbers are bootstraps.

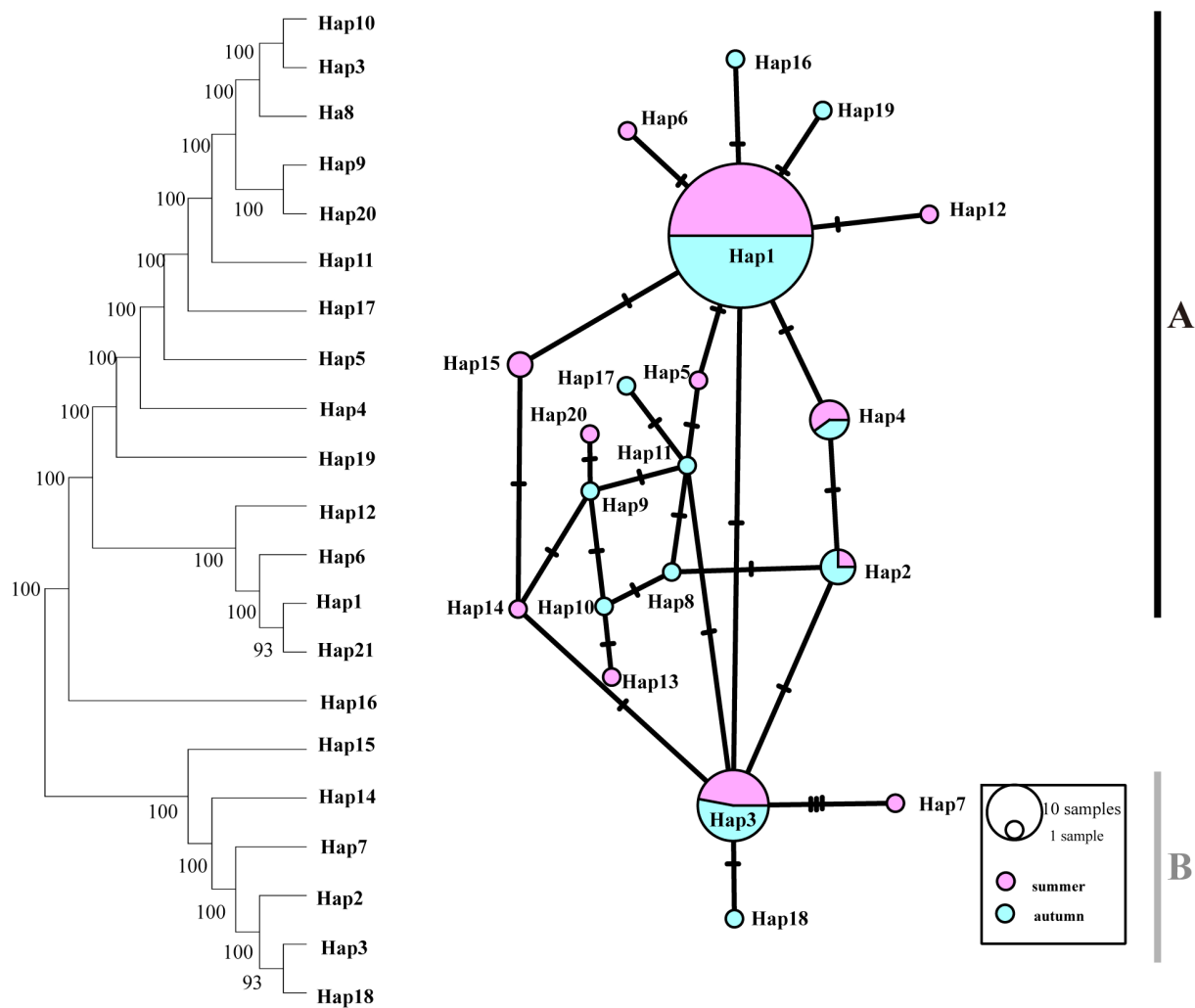


Figure 4. Maximum likelihood (ML) tree and haplotype network based on 21 *ODH* haplotypes of *T. pacificus*. Branch numbers are bootstraps.

The AMOVA results from all three genes were consistent, revealing no significant genetic differentiation among *T. pacificus* populations ($p \geq 0.1$). Mitochondrial genes accounted for a higher proportion of variation than the nuclear genes (Table 4). Pairwise F_{ST} analyses for all markers indicated minimal differentiation between the autumn and summer cohorts, with genetic variation existing almost entirely within populations ($0 < F_{ST} < 0.05$) (Table 5).

Table 4. AMOVA of mtDNA and nuDNA 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 (<i>p</i> -value = 0.83187 ± 0.01114)
	Within populations	114	37.811	0.33167 Vb	100.85	
	Total	116	38.274	0.32887	100.00	
16S	Among populations	2	0.203	−0.00066 Va	−0.54	FST: −0.00537 (<i>p</i> -value = 0.57478 ± 0.01351)
	Within populations	107	13.251	0.12385 Vb	100.54	
	Total	109	13.455	0.12385 Vb	100.00	

Table 4. Cont.

Gene	Source of Variation	df	Sum of Squares	Variance Component	Percentage of Variation (%)	F Statistic
ODH	Among populations	2	1.133	0.00080 Va	0.15	FST: 0.00148 (<i>p</i> -value = 0.37634 ± 0.01613)
	Within populations	111	59.815	0.53887 Vb	99.85	
	Total	113	60.947	0.53967	100.00	

Table 5. F_{ST} between populations of *T. pacificus* based on three 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

The statolith morphology coefficients differed significantly between summer and autumn cohorts ($p < 0.01$), with the greatest difference occurring in the posterior indentation (Figure 5). Random forest analysis revealed a high discrimination success rate between these cohorts (Table 6).

Table 6. Random forest out-of-bag (OOB) error estimation matrix for the two seasonal cohorts.

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

Within the autumn cohort, a significant spatial difference in statolith morphology was detected only between the northern Yellow Sea (NYS) and the southern East China Sea (SECS) ($p < 0.01$), primarily reflected in overall outline shape. Moreover, the average statolith outlines displayed significant annual variation in the rostrum and posterior indentation regions across years (Figure 5). Additionally, significant morphological differences in statolith morphology were found 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/SI</i>	<i>Ssa</i>
autumn		0.5710	1763.0060
summer		0.5483	1706.8217
summer vs. autumn	$1.351 \times 10^{-10} *$	$5.342 \times 10^{-6} *$	0.09057

* Significant at $p < 0.01$.

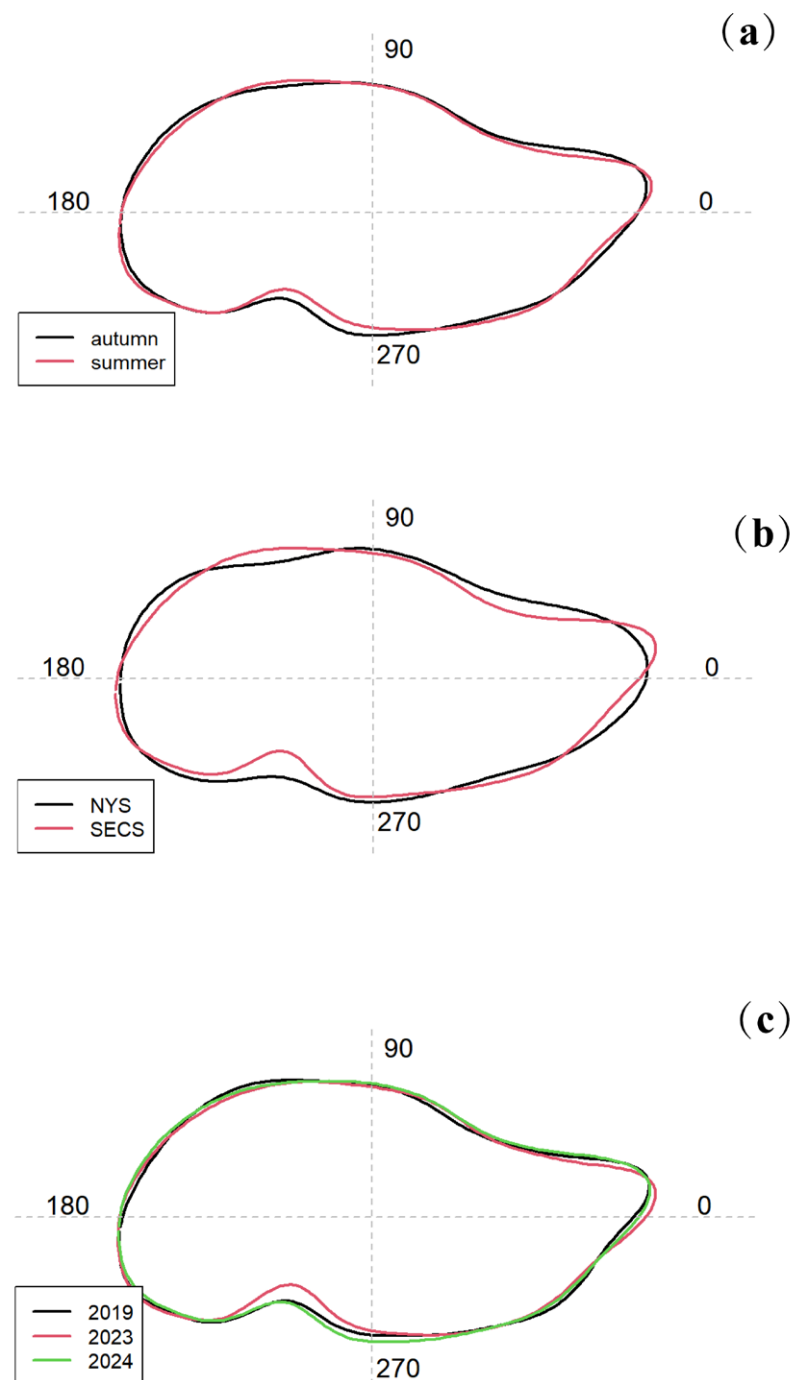


Figure 5. Reconstructed outlines of statoliths for different cohorts (a), different regions in autumn (b) and different years in summer (c).

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 results of statolith microstructure analysis initially indicate that the cohort composition of *T. pacificus* in China consists of two spawning cohorts—summer and autumn. The winter cohort, a major component, was not detected in the commercially collected specimen, which is consistent with Guo et al. [29]. This phenomenon may be attributed to environmental suitability, as the sampling period of this study coincided with El Niño events [30], suggesting that population fluctuations in the *T. pacificus* winter cohort are closely linked to the extent of winter spawning grounds. Cold climatic conditions tend to contract the winter spawning

areas in the East China Sea, subsequently reducing the adult population size [31]. Furthermore, studies by Rosa confirmed that the decline in fishery catches from Japan and South Korea also contributes to the spatial discontinuity of spawning grounds in the East China Sea during the winter spawning season, thereby affecting the overall scale of the winter cohort [32]. In contrast, despite lacking specific proportion, the summer cohort plays a more important role in China compared to Korea and Japan [33]. Such condition should be emphasized in future management processes since the summer cohort is a small-sized group that is more vulnerable to fishing pressure and local extinction [31].

The genetic analysis revealed low haplotype and nucleotide diversity in *T. pacificus* and an absence of significant population structure, consistent with the findings of Zhang et al. [9]. Furthermore, only six 16S haplotypes were identified in this species. This low haplotype number is likely because the 16S in cephalopods, including *T. pacificus*, is highly conserved with few variable sites, leading to reduced haplotype diversity [34].

The present study reveals relatively low genetic diversity in *T. pacificus* across Chinese coastal waters, with no significant genetic differentiation detected among the NECS, NYS, and SECS populations. This pattern may be closely linked to the species' extensive migratory behavior and the distribution of its spawning grounds [35]. Throughout its life cycle, *T. pacificus* undertakes long-distance migrations between spawning, feeding, and nursery areas. These movements facilitate substantial gene flow among geographically separated populations, counteracting genetic divergence and maintaining genetic homogeneity across a wide geographical range [36]. The spawning grounds of *T. pacificus* in the Northwest Pacific Ocean serve as key hubs for genetic exchange [37]. During the spawning season, individuals from different regions aggregate, enhancing opportunities for genetic recombination and mixing. Subsequent larval dispersal by ocean currents further promotes the broad distribution of genetic material, reinforcing connectivity among coastal populations. Similar patterns have been reported in other ommastrephid squids. For example, *Ommastrephes bartramii* exhibited high gene flow among populations despite potential geographical separation of spawning areas, resulting in maintained genetic homogeneity across its range. In contrast, species with more restricted dispersal ability or isolated spawning grounds tend to show stronger genetic differentiation among populations [7].

Compared to genetic markers, significant differentiation was detected in the statolith shape, highlighting the fact that ecologically separated groups can be identified in *T. pacificus*. The difference in statolith shape 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 supports effective management. Similar to fish otoliths, morphological variation in cephalopod statoliths is influenced by both environmental and genetic factors [38]. Since there is no significant genetic structure in *T. pacificus*, the observed statolith difference could mainly be a result of environmental factors. Consistently, major differences are observed in posterior indentation. Because of the strong swimming ability of *T. pacificus*, it is difficult to track its environmental factors in the long term [14]. However, only spatial differences were observed between NYS and SECS in the autumn cohort. Thus, the morphology differences could mainly be a result of environmental conditions during early life stages [39]. In addition, the interannual distribution in morphology showed differences among years. A study [29] has demonstrated that differences in marine environmental factors and climatic anomalies can influence the growth of the external morphology of cephalopod hard tissues. The years 2019 and 2023 were characterized by El Niño events, during which the sea surface temperature was relatively low. In contrast, 2024 was marked by a La Niña event, with higher sea surface temperatures observed in these regions. These variations may have contributed to the morphological differences observed in the statoliths of *T. pacificus*.

5. Conclusions

The study combines statolith analysis with genetic analysis in *T. pacificus* and provides comprehensive insights into the population structure in the China Seas. The statolith microstructure first reveals the cohort composition of *T. pacificus* that is commercially explored in China. Based on these findings, it is recommended to comprehensively investigate the proportion of the summer cohort, which may serve as a potential alternative to the winter cohort under extreme conditions. Currently, the winter cohort population of *T. pacificus* is highly sensitive to environmental fluctuations. It is essential to monitor the declining trend in biomass resulting from the combined effects of fishing pressure and environmental stressors to ensure the sustainable utilization of this resource. This study revealed that there is almost no significant genetic differentiation among summer and autumn cohorts based on the mtDNA (*COI* and *16S*) and nuDNA (*ODH*) sequences. The overall shapes of statoliths in summer and autumn cohorts exhibit significant differences, and their high classification success enables them to serve as reliable classification markers. Given the absence of significant genetic differentiation, these morphological markers provide an important reference for subsequent cohort classification and a theoretical foundation for the rational development and utilization of *T. pacificus* fishery resources.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d18020123/s1>, references [40–53] are cited in the Supplementary Materials Texts S1–S4.

Author Contributions: X.Z., C.Z., and X.L. conceptualized and designed the research framework. C.Z. was responsible for sample collection. X.L. and S.P. conducted the experimental procedures and drafted the initial manuscript. X.Z., C.Z. and J.L. made revisions to the paper. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All experiments were approved by the Institutional Animal Care and Use Committee of Ocean University of China.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available upon 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.

Appendix A

Table A1. The primers of mitochondrial (mt) and nuclear (nu) genes.

Gene	Mt/Nu	Primers	PCR Conifion	Reference
<i>COI</i>	Mt	<i>COI</i> -F: 5'-CCTATCTCGGCAGCACTA-3' <i>COI</i> -R: 5'-CCAATAAAATCGCTCTAATA-3'		[9]
<i>16S</i>	Mt	<i>16S</i> -F: 5'-GCCTCGCCTGTTTACCAAAAAC-3' <i>16S</i> -R: 5'-CGGTCTGAACTCAGATCACGT-3'	95 °C 5 min, (95° C 10 s, 55 °C 20 s, 72 °C 30 s) × 35, 72 °C 5 min	[54]
<i>ODH</i>	Nu	<i>ODH</i> -F: 5'-ATGAGGGTTACTTTAGAGCCA-3' <i>ODH</i> -R: 5'-GCAGGTCTTCATTGCCATAC-3'		[23]

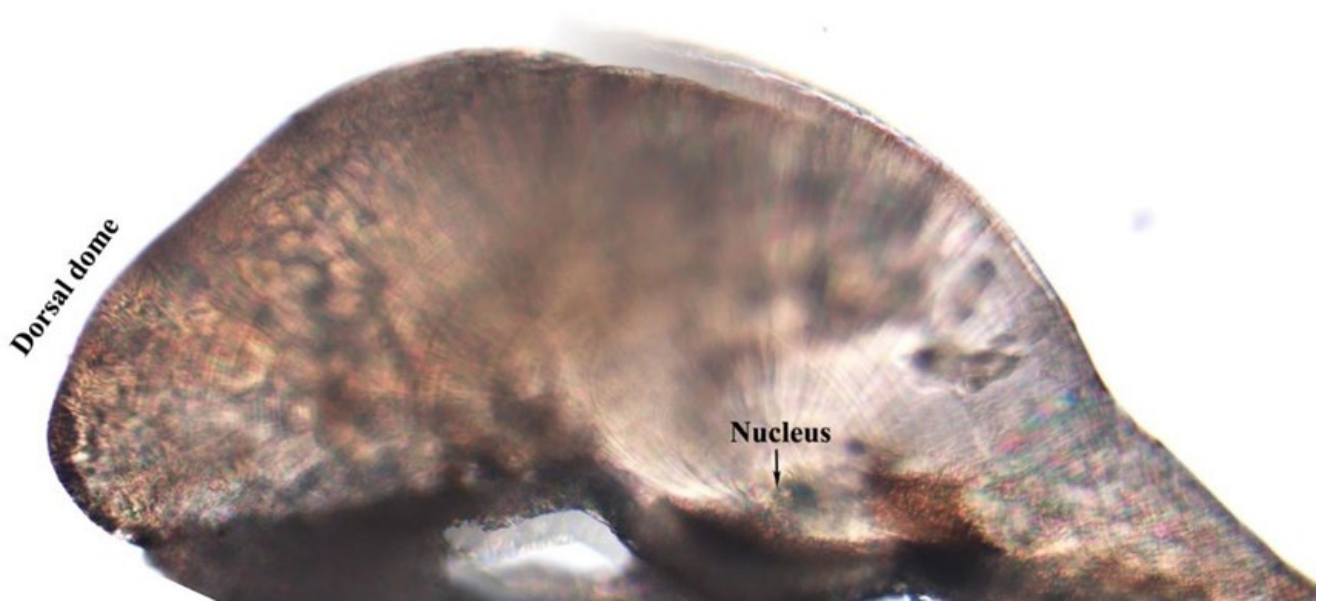


Figure A1. Age of statolith diagram.

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