

Phylogeography of sea pen *Cavernularia obesa* in the East China Sea: Historical expansion and Changjiang outflow barrier

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Abstract

Sea-level fluctuations in the marginal seas of the northwestern Pacific Ocean during the Pleistocene have profoundly influenced the genetic structure of marine organisms. Previous phylogeographic studies have mainly focused on fish and molluscs; other taxonomic groups have been poorly studied, which restricts a comprehensive understanding of the geogenetic patterns of marine species in this area. To precisely understand how environmental factors and historical events shape the population structure of the sea pen *Cavernularia obesa* in the East China Sea, we determined partial nucleotide sequences of the mitochondrial cytochrome oxidase subunit I (COI) gene in 71 individuals from five sites. Results of population Genetic and demographic analyses revealed a low genetic diversity within each population. Moreover, the historical population size change showed that the populations experienced bottlenecks during the Pleistocene. The sea level of the East China Sea decreased by >100 m when the glacial advanced during the Pleistocene, which caused the shrinking of habitats for marine species and eventually resulted in low genetic diversity within the populations in this area.

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¹ School of Marine Sciences, Ningbo University, Ningbo 315211, P. R. China² Key Laboratory of Marine Ecosystem Dynamics, Second Institute of Oceanography, Ministry of Natural Resources, Hangzhou 310012, P. R. China³ Observation and Research Station of Yangtze River Delta Marine Ecosystems, Ministry of Natural Resources, Zhoushan 316022, P. R. China* Correspondence: lichenghua@nbu.edu.cn**Abstract:** Sea-level fluctuations in the marginal seas of the northwestern Pacific Ocean during the Pleistocene have profoundly influenced the genetic structure of marine organisms. Previous phylogeographic studies have mainly focused on fish and molluscs; other taxonomic groups have been poorly studied, which restricts a comprehensive understanding of the geogenetic patterns of marine species in this area. To precisely understand how environmental factors and historical events shape the population structure of the sea pen *Cavernularia obesa* in the East China Sea, we determined partial nucleotide sequences of the mitochondrial cytochrome oxidase subunit I (COI) gene in 71 individuals from five sites. Results of population Genetic and demographic analyses revealed a low genetic diversity within each population. Moreover, the historical population size change showed that the populations experienced bottlenecks during the Pleistocene. The sea level of the East China Sea decreased by >100 m when the glacial advanced during the Pleistocene, which caused the shrinking of habitats for marine species and eventually resulted in low genetic diversity within the populations in this area.**Keywords:** phylogeography; marginal sea; *Cavernularia obesa*; sea level fluctuation;

Changjiang diluted water 1. Introduction Phylogeography is the study of the geographic distribution of genetic lineages within a species and provides a means for inferring historical and contemporary processes that influence population distribution and abundance. The phylogeographic distribution of marine species is driven by historical events and modern environmental factors (Dong et al., 2012; Lima-Junior et al., 2021). Among these factors, oscillations of climate and oceanography have caused drastic changes in subtropical and temperate coastal environments (Avice, 2000). The marginal seas of the northwestern Pacific Ocean experienced sea-level fluctuations during late Quaternary glacial cycles (Wang, 1999). When the glacier advanced during the Pleistocene, the East China Sea (ECS) declined by approximately 130–150 m than present (Waelbroeck et al., 2002). The ECS shelf was completely exposed as the coastline migrated approximately 1200 km seaward (Wang, 1999). Consequently, the ECS was reduced to an elongated enclosed sea (Okinawa Trough) with an area $<1/3$ of its present size (Ni et al., 2014). During interglacial periods, the ECS was inundated by rising sea levels. These repeated transgression–regression cycles, together with ocean currents and recent anthropogenic activities, have greatly impacted the phylogeographic patterns and population genetic differentiation in this area. Therefore, the ECS has been proposed as an ideal model for studying how sea level oscillations are caused by repeated glaciations, structured distribution ranges, spatial population genetic diversity, and marine phylogeography (Ni et al., 2014). The Changjiang River is the largest river in Asia and the third-largest river in the world. It enters the ECS with about 9×10^{11} cm³ of freshwater discharge annually. This huge freshwater flow, named the Changjiang diluted water (CDW), causes significant shifts in various ecological and environmental parameters in the ECS and acts as a barrier to the genetic connectivity of marine species. In other marine realms, some well-known biogeographic boundaries, such as the Bering Isthmus (York et al., 2008), Central American Isthmus (Coppard and Lessios, 2017), and Cape Hatteras (Mccartney et al., 2013), have proven to be intraspecific barriers restricting the gene flow of some wide-ranging taxa. Recently, several studies have tested the phylogeographic barrier effect of CDW in addition to biogeographic boundary [1,10–12]. However, the results of these studies are inconsistent, and controversy has arisen as different genetic patterns have emerged. Previous phylogeographic studies on the ECS have mainly focused on commercially exploited fish and molluscs [12,13]. Although the genetic structure of commercial species may be affected by invasion genomics (Jaspers et al., 2021; Rius and Turon, 2020), species from other diverse taxonomic groups are good candidates for such comparative studies (Ni et al., 2017). The sea pen *C. obesa* is a widely distributed colonial cnidarian found in the northeastern Pacific Ocean (López-González et al., 2000). Sea pens undergo a two-phase life cycle of planktonic larvae and sessile adult forms. Mature adults of *C. obesa* half-buried in the sediments of the seabed and barely move, and their dispersion mainly relies on planktonic larvae. The commercial value of *C. obesa* is low; therefore, the genetic structure of the ECS community was unaffected by commercial aquaculture activities. These characteristics make *C. obesa* an ideal model species for phylogeographic studies of the ECS. In this study, we collected 71 individuals of *C. obesa* from five sites in the ECS and used phylogenetic analyses based on mitochondrial cytochrome oxidase subunit I (COI) to analyze the range-wide phylogeographic structure and determine the potential drivers of spatial genetic variation in *C. obesa*.

2. Materials and Methods

2.1. Sample collection

A total of 71 individuals of *C. obesa* were collected from five sites using Agassiz trawl in the ECS from 2016 to 2019. The samples were frozen at -20 °C. As *C. obesa* is not an endangered or protected species, and collection was only carried out from public access areas, no specific permits were required to collect this species from these locations. The locations of the sampling sites are shown in Figure 1 and Table 1. **Figure 1.** Map of Northwest Pacific showing sampling sites and haplotypes of *C. obesa*; YSCC, Yellow Sea Coastal Current; CDW, Changjiang Diluted Water; and ECSCC, East China Sea Coastal Current **Table 1.** Sampling sites and diversity indices for the five populations of *C. obesa*

Sample site	Abbr.	Geographic coordinates	N	n	h	π
Nantong	NT	32°24'07"; 122deg53'28" E	6	3	0.600	0.0317
Shanghai	SH	30°58'02"; 122deg12'21" E	14	1	0.000	0.0000
Sanmen	SM	29°11'18"; 121deg34'24" E	19	4	0.298	0.1096
Yueqing	YQ	28°08'05"; 121deg05'30" E	17	2	0.118	0.1115
Nanji	NJ	27°33'35"; 120deg56'44" E	15	2	0.133	0.1266

¹ N , number of individuals analyzed; n , number of haplotypes; h , haplotype diversity; and π , nuclear diversity

2.2 DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing Total genomic DNA was isolated from frozen samples. The COI was amplified with the primers COI8068F (5'-CCATAACAGGACTAGCAGCATC-3') and COIOCTR (5'-ATCATAGCATAGACCATAACC-3') (McFadden et al., 2006). All the reactions were performed in 50 μ L volume total volume, with 1 μ L DNA extract, 5 μ L 10 \times buffer (2.5 mM Mg²⁺), 1 μ L Taq DNA polymerase (5 U/ μ L), 1 μ L dNTP (10mM), 1.5 μ L F-primer (10 μ M), 1.5 μ L R-primer (10uM), and 39 μ L ddH₂O. The amplification protocol for COI and ITS consisted of 5 min of initial denaturation at 95 °C followed by 35 cycles of 30 s at 95 °C, annealing at 58 °C for 30 s, extension at 72 °C for 90 s and a final extension at 72 °C for 7 min. The resulting PCR products were purified and sequenced using an ABI 3730XL Genetic Analyzer (Applied Biosystems) with the same forward and reverse PCR primers. The initial sequences were assembled using SeqMan and aligned and trimmed using BioEdit.

2.3 Phylogenetic analyses The number of haplotypes for each locus was determined using DnaSP software. The number of polymorphic sites, haplotype diversity, and nucleotide diversity were estimated using the Arlequin (Excoffier et al., 2005). The distribution of characteristic nucleotide mutations in each haplotype was calculated using MEGA 6.06 (Kumar et al., 2004). Bayesian inference (BI) and maximum likelihood (ML) trees were constructed using BEAST v.2.6.7 and MEGA 6.06, respectively.

2.4 Population analyses Analysis of molecular variance (AMOVA) was performed for the COI data using Arlequin with 1000 permutations to examine the amount of genetic diversity partitioned among populations (Excoffier et al., 1992). In addition, pairwise genetic differentiation (F_{ST}) among populations was calculated using the Arlequin software. Mantel's test with 10000 randomizations for isolation-by-distance (IBD) was performed between the linearized F_{ST} ($F_{ST}/(1-F_{ST})$) and geographical distances (Slatkin, 1995) using IBD v 1.52 (Bohonak, 2002) to determine whether this pattern meets the expectation of genetic similarity decaying with geographic distance (Novembre et al., 2008).

2.5 Historical demography analyses Extended Bayesian Skyline Plots (EBSPs) were used to examine population size changes during BEAST. Tajima's D (Tajima, 1989) and Fu's F_s (Fu, 1997) neutrality tests were performed using Arlequin, and a mismatch distribution was constructed for each geographic population to test the exponential population growth model.

3. Results

3.1. Sequence variation An alignment of the 892 bp COI gene fragment was analyzed for 71 individuals, with 494 variable sites yielding a total of six unique haplotypes named H1-H6 (Figure 1). The central haplotype, H1, was the most common, with 64 copies, and was dominant at all five sites. The genetic variation of COI mtDNA in the five populations of *C. obesa* from the ECS was low, with haplotype diversity ranging from 0.000 in SH to 0.600 in NT, and nucleotide diversity ranging from 0.0000 in SH to 0.1266 in NJ (Table 1).

3.2. Population structure The AMOVA results for COI showed that the differentiation among populations was not significant ($P > 0.05$) (Table 2), with F_{ST} values ranging -0.0774–0.1532. IBD analysis also revealed a non-significant ($P > 0.05$) correlation between genetic and geographic distances for all populations in the ECS. The ML tree showed that most of the *C. obesa* clustered into one clade (Figure 2), with the exception of a few individuals.

Table 2. AMOVA comparing genetic variation based on COI sequences of *C. obesa*

Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation indices
Among populations	4	30.831	-0.980	-4.83	-0.067
Within populations	66	1404.634	21.282	104.83	-0.032
Total	70	1435.465	20.302	\	\

Figure 2. ML phylogenetic tree for COI of 71 individuals of *C. obesa*.

3.3. Historical demography The mismatch distribution analyses display a skewed unimodal distribution (Figure 3), indicating that they experienced demographic expansion and a recent bottleneck. All populations, except SH, yielded significant negative values in Tajima's D test (Table 3), indicating that each population experienced demographic expansion under the neutral model.

Figure 3. Mismatch distribution of *C. obesa* based on COI

Table 3. Tajima's D and Fu's F_s , and corresponding P values for five populations of *C. obesa*

Population	Tajima's D	Tajima's D	Fu's F_S	Fu's F_S
	D	P	F_S	P
NT	-1.5289	0.0010	6.22507	0.9930
SH	\	\	\	\
SM	-2.6013	0.0000	28.6375	1.0000
YQ	-2.6020	0.0000	35.6381	1.0000
NJ	-2.51875	0.0000	34.2518	1.0000

Reconstruction of population size over time using the EBSP approach indicated that *C. obesa* in the ECS experienced significant population size changes. After a prolonged stable period, the ECS population decreased from approximately 20000 years ago and increased from approximately 2000 years ago (Figure 4). The subsequent goodness-of-fit tests also supported the null hypothesis of the sudden expansion model, with non-significant values for SSD. **Figure 4.** Historical population size change of *C. obesa* in the ECS based on EBSPs 4. Discussion In the present study, we used the mitochondrial sequence COI to examine the genetic diversity and phylogeographical structure of the sea pen *C. obesa* in the ECS. Genetic variation among the ECS populations was generally low, perhaps due to a combination of high contemporary gene flow and the recent common ancestry of haplotypes. Larvae of sea pens, called planulae, usually drift freely for approximately 1 week before settling in sediments (López-González et al., 2000). If appropriate sediments are not available, settlement can be delayed by up to 1 month. The long planktonic larval stage in *C. obesa* may facilitate gene flow by current-driven dispersal of pelagic larvae, and consequently decrease the genetic structure among distant populations in the ECS. Ocean currents play an important role in the genetic flow connections between populations (York et al., 2008). In summer, the ECSCC enters the ECS from the South China Sea and the YSCC from the Yellow Sea. These prevailing currents transport large numbers of planktonic larvae across the marginal seas of the northwest Pacific. The CDW has been reported to act as a physical barrier for some marine species. However, the influence of the CDW on population differentiation is taxonomically variable; some species show no genetic breaks between populations from each side of the CDW (Ni et al., 2017). Species with a long planktonic larval stage usually show substantial mitochondrial homogeneity across the CDW, whereas species with a sessile life history are more prone to biogeographic and historical barriers. The genetic structure of *C. obesa* in the ECS seems to be uninfluenced by the CDW and may benefit from the long planktonic larval stage. Mismatch distribution analyses indicated that the ECS population recently experienced a bottleneck, which may have resulted in low genetic diversity. Rare variants in small populations are predicted to be eliminated through genetic drift. Recent population isolation and fragmentation during the Pleistocene glacial age increased the role of genetic drift in COI variation in *C. obesa* (Luo et al., 2012). The mitochondrial gene evolution rate in sea pens is relatively slow (Williams, 2011), which causes them to exhibit lower genetic diversity than other species at the same time. We speculated that the mitochondrial genetic structure of *C. obesa* in the ECS may have retained the genetic features of the historical population during the Pleistocene. The Pleistocene glacial age, particularly the last glacial maximum (LGM), approximately 20,000 years ago, has had an important influence on the evolution and genetic structure of marine organisms. Some marine species appear to have undergone dramatic population expansion during the LGM when the sea level fluctuated. The population size of *C. obesa* decreased rapidly when sea level decreased and the ECS shrunk. When sea level rose, *C. obesa* experienced demographic expansion and reoccupied the seabeds reflooded by the ECS. In that case, the theoretical genetic homology of *C. obesa* populations in the ECS was high, which is consistent with our results. Most phylogeographic studies in the ECS have focused on economically important species such as fish and mollusks, and more comparative phylogeographic studies using additional species pairs are needed to explain the underlying mechanism of these discrepancies. Our results imply that sea pens such as *C. obesa* are good candidates for such comparative studies, as they are abundant in this region and suffer less from human-mediated activities than commercially exploited fish and mollusks. **Funding:** This research was funded by the National Key Research and Development Program of China (grant number 2021YFC3101702). **Data Availability Statement:** The data that support the findings of this study are available on request upon reasonable request. **Acknowledgments:** The authors thank

Wang Hangjun for collecting the samples. **Conflicts of Interest:** The authors declare no conflicts of interest.

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