Pakistan J. Zool., vol. 53(5), pp 1669-1676, 2021 DOI: https://dx.doi.org/10.17582/journal.pjz/20191015071041

Wild Resource Diversity of *Mytilus unguiculatus* Reduced by Aquaculture in the Southeast China Sea

Yahong Guo[1,3], Zeqin Fu[1,3], Jiantong Feng[1,3], Chengrui Yan[1,3], Yingying Ye[1,3]*, KaidaXu[2] and Baoying Guo[1,3]

[1]*National Engineering Research Center for Marine Aquaculture, Marine Science and Technology College, Zhejiang Ocean University, No.1 Haida South Road, Changzhi Island, Zhoushan, Zhejiang, 316022, PR. China*
[2]*Scientific Observing and Experimental Station of Fishery Resources for Key Fishing Grounds, MOA, Key Laboratory of Sustainable Utilization of Technology Research, Marine Fisheries Research Institute of Zhejiang, Zhoushan 316021, PR China*
[3]*Key Laboratory of Informatization of Habitat Monitoring and Fishery Resource Conservation Research in the East China Sea of Zhejiang Province, Zhejiang Ocean University, Zhoushan 316021, PR China*

Article Information
Received 15 October 2019
Revised 02 January 2020
Accepted 28 January 2020
Available online 16 June 2021

Authors' Contribution
YY conceived and designed the experiments. YG, ZF, JF and CY performed the experiments. YY and KX analyzed the data. BG provided analysis tools. YY wrote the paper. YG and JF collected field material and processed the samples.

Key words
***Mytilus unguiculatus*, Genetic variation, Aquaculture, Genetic protection unit, 16S rRNA**

ABSTRACT

The hard-shelled mussel, *Mytilus unguiculatus*, is one of main aquaculture bivalve species in Southeast China Sea, and excellent growth characteristics. For mussel breeding, farmers use wild individuals to multiply the cultured populations. However, blind selection of wild parents has inevitably resulted in inbreeding and decreased genetic variation. In this study, four wild specimens groups (ZSW, WZW, NNW, and FZW) and four cultured specimens groups (ZSC, WZC, NDC, and FZC) of *M. unguiculatus* were used to analyze their genetic diversity, population structure and understand the relationship between the wild and cultured specimens groups. The results showed that haplotype diversity (*h*) of the cultured specimens groups (from 0.621 to 0.797; average, 0.717) was higher than that of three wild specimens groups (ZSW, WZW, and NNW; from 0.428 to 0.668; average, 0.560). The pairwise F_{ST} values suggested that only the FZW sample showed significant divergence from the others. The wild and cultured ZS and WZ specimens groups were similar, and the wild and cultured ND specimens groups were crossed. Thus, we need to establish genetic protection units for *M. unguiculatus*, limit the selection of breeding parents, and maintain a high-quality germplasm bank for *M. unguiculatus*.

INTRODUCTION

The hard-shelled mussel, *Mytilus unguiculatus*, is one of the four main economic bivalve used in the aquaculture industry in China. It has a wide distribution in the coast of Japan, Korea, and China (Shen *et al*., 2009). In China, *M. unguiculatus* is distributed in the temperate coastal zone from the Bohai Sea to the East China Sea. It is one of the main aquaculture mollusk species in Zhejiang Province, Shandong Province, Jiangsu Province and Fujian Province (Chang *et al*., 2007). The dried visceral mass is used in traditional food therapy and recorded in Materia Medica. Although attempts have been made to improve its breeding, the natural resources of *M. unguiculatus* are decreasing because of commercial exploitation. In previous studies, *M. unguiculatus* was selected as an ecotoxicology model to monitor marine pollution in China (Wang *et al*., 2010; Xu *et al*., 2016; Yang *et al*., 2014). Yang *et al*. (2015) suggested that the wild resources are under the risk of depletion around Taishan Islands in Fujian Province. Yuan *et al*. (2017) assessed the resources of Yushan Island using *COI* and suggested that the divergence of samples from those in five other locations was low and only the Yushan sample showed differentiation. Because of limited information on the genetic resources of *M. unguiculatus*, it is difficult to set a genetic baseline for mussel aquaculture.

For genetic conservation, the adaptive potential of a population is correlated with the phenotypic variation of genes (Hoffmann and Sgrò, 2011). In natural populations, inbreeding and gene drift result in neutral genetic variation and reduce their viability (O'grady *et al*., 2006). For an effective population size, neutral genetic diversity could show the long-term potential of evolution and species adaptability (Kahilainen *et al*., 2014; Reed and Frankham,

* Corresponding author: yeyy@zjou.edu.cn
0030-9923/2021/0005-1669 $ 9.00/0

2001). Thus, estimation of the genetic variation between wild and cultured samples is useful for establishing a genetic protection unit for wild populations (Xiong *et al*., 2014). Protection of the genetic diversity of wild populations and separation of wild and farmed communities are necessary to maintain a good gene stock for the breeding industry. Vigorous development of mussel aquaculture had led to the invasion of the habitats of wild populations by the cultured stock, and the genetic diversity of the wild populations and potential for evolution and adaptability have decreased. A decrease in genetic variation would affect the quality of germplasm resources.

Molecular markers are widely used to identify species and measure interspecific and intraspecific genetic divergence. Because the oceans lack sufficient genetic barriers, it is easy for marine species to exchange genes material (Palumbi, 1994). The 16S rRNA gene is a commonly used molecular marker (Rochelle *et al*., 1992) that can provide sufficient evolutionary information to confirm the phylogenetic relationships of both close and distant species, and it has been used in many studies of aquatic organisms (An *et al*., 2005; Thanh *et al*., 2015; Pan *et al*., 2006).

In this study, the 16S rRNA gene was used to analyze wild and cultured specimens groups of *M. unguiculatus* along the southeast coastal areas of China and obtain basic evolutionary information on the phylogenetic relationships of both wild and cultured *M. unguiculatus* and accumulate raw genetic data for establishing a genetic baseline. We hope that this study would provide insight for establishing a genetic protection unit for *M. unguiculatus* and maintaining a high-quality genetic resource base for breeding and fisheries management.

MATERIALS AND METHODS

Specimen collection

A total of 157 wild and cultured specimens were collected from four locations: Zhoushan, Wenzhou, Ningde, and Fuzhou. The wild specimen groups were named ZSW, WZW, NNW, and FZW, and the cultured specimen groups, ZSC, WZC, NNC, and FZC. The sampling details are shown in Table I and Figure 1. The mussel individuals were applied morphological identification and the adductor muscle was dissected and stored in 100% alcohol until DNA extraction.

DNA extraction was performed using the improved salting-out method reported by Aljanabi *et al*. (1997). The DNA quality was tested using 2% agarose gel electrophoresis. The DNA concentration was evaluated with the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, MA, USA) at an absorption ratio of 260/280 nm. Then, the DNA was diluted to a concentration of 40–60 ng/µl and stored at -20°C.

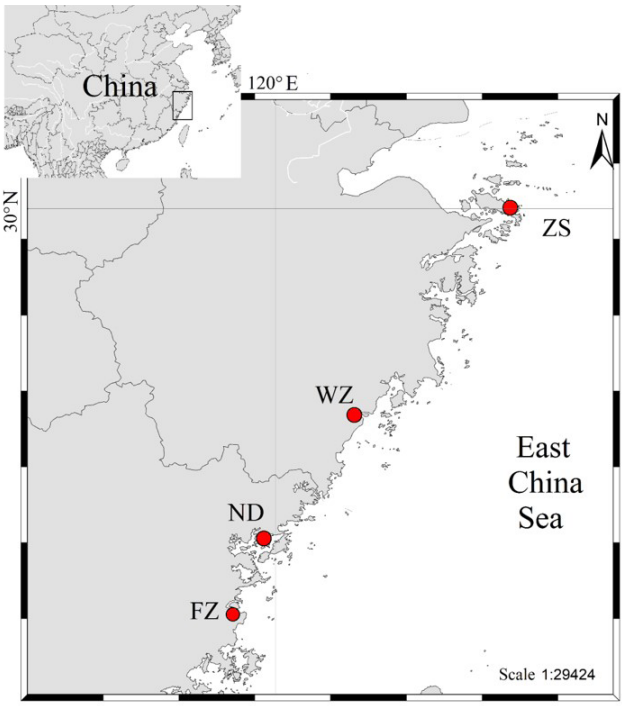

Fig. 1. The map shows the sampling locations in the East China Sea.

Design and sequencing of the 16S rRNA gene

The mitochondrial (mt)-DNA sequences of *M. unguiculatus* were used for amplifying fragments of the 16S rRNA gene by using the primers 16S3L, 5′-TGAGCGTGCTAAGGTAGC-3′, and 16S4H, 5′-AGCCAACATCG AGGTCGC-3′ (Lydeard *et al*., 1996). The PCR volume was 25 µl, containing 2.5 µl of 10× buffer, 2 µl of Mg^{2+} (20 mmol/l), 2 µl of dNTPs (2.5 mmol/l), 0.2 µl of *Taq* DNA polymerase (5 U/µl), 1 µl of the template DNA, 1 µl of each forward and reverse primer, and 16.3 µl of ddH_2O. The reactions were performed at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 52°C for 30 s, and 72°C for 1 min and 72°C for 10 min. The PCR products were obtained using 1.5% agarose gel electrophoresis. The sequencing was performed by Shanghai Invitrogen Biotechnology Co. Ltd., Shanghai, China.

Sequence analysis

The DNA fragment was assembled using ClustalX v2.1 (Larkin *et al*., 2007). For identifying the species and fragment positions, all fragments were compared using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The software MEGA v7.0 was used to align the fragments and perform

Table I. Sampling details for the wild and cultured specimens of *Mytilus unguiculatus*.

Samples	Sampling date	Latitude, longitude	N	n	np	h	π
ZSC	Oct. 2010	123°25′E 30°32′N	18	6	2	0.745±0.079	0.0042±0.0009
ZSW	Oct. 2010	123°25′E 30°32′N	20	5	3	0.668±0.097	0.0033±0.0007
WZC	Oct. 2010	120°25′E 27°33′N	18	3	0	0.621±0.067	0.0024±0.0004
WZW	Oct. 2010	120°25′E 27°33′N	26	3	1	0.428±0.095	0.0015±0.0004
NNC	July 2009	119°25′E 26°34′N	18	8	6	0.797±0.074	0.0114±0.0028
NNW	July 2009	119°25′E 26°35′N	20	7	4	0.584±0.127	0.0047±0.0000
FZC	Dec. 2010	119°52′E 26°03′N	17	6	3	0.706±0.106	0.0032±0.0007
FZW	Dec. 2010	119°52′E 26°03′N	20	14	11	0.916±0.055	0.0508±0.0100
Total			157	33		0.694±0.033	0.0116±0.0027

the model test (Kumar *et al*., 2016). DnaSP v6.0 was performed to group and define the haplotypes and estimate the following genetic parameters: *N*, number of individuals; *n*, number of haplotypes; *np*, number of private haplotypes; *h*, haplotype diversity; and π, nucleotide diversity (Rozas *et al*., 2017). All the fragments were analyzed using the software Arlequin v3.5 (Excoffier and Lischer, 2010; Excoffier *et al*., 1992), and all fragments (using the GTR model, +G = 0) were subjected to hierarchical analysis of molecular variance (AMOVA); the pairwise F_{ST} values were calculated with 10,000 permutations. The formula $F_{ST} = 1 / (4N_m + 1)$ was used to calculate the number of migrants (N_m). An unweighted pair group method with arithmetic mean (UPGMA) tree was constructed on the basis of the pairwise genetic distances by using MEGA v7.0 with 1,000 bootstrap values (Kumar *et al*., 2016). A heatmap of F_{ST} and N_m with the UPGMA tree was plotted using the R program. The plot for mismatch distribution was constructed using the software DnaSP v6.0 (Rozas *et al*., 2017). Network v5.1 was used to construct the haplotype network (Röhl and Mihn, 1997).

To evaluate the genetic divergence between each location, the fragment data were converted from FASTA to STR format. Using the package GenAlEx v6.5, principal coordinate analysis (PCoA) was performed on the basis of the genetic distance between populations (Peakall and Smouse, 2006). The software STRUCTURE v2.3.4 was used to cluster the samples (Evanno *et al*., 2005). Twenty replications were performed using the admixture model, correlated allele frequencies and the prior population information with a burn-in period of 100,000 followed by 1,000,000 iterations and *K* = 1-8. The most suitable *K* value was determined using the statistical ΔK with STRUCTURE HARVESTER v0.6.92 (Earl *et al*., 2011). The software CLUMMP v1.1.2 (Jakobsson *et al*., 2007) was used to summarize parameters across 20 runs, and the website "POPHELPER (www.pophelper.com)" was used to acquire the line chart for each *K* value and the population structure plot for the best ΔK.

RESULTS

Genetic diversity

A 303-bp fragment of the 16S rRNA gene was analyzed and 33 haplotypes (Genbank under accession numbers: MG888480-MG888512) were obtained from 157 specimens groups of *M. unguiculatus*. The sampling size and genetic parameters of each specimen are listed in Table I.

The number of haplotypes (*n*) was between 3 (WZC) and 14 (FZW). The number of private haplotypes was between 0 (WZC) and 11 (FZW). Hap3 was the high-frequency haplotype and observed in all specimens groups, accounting for 50.32% (79/157) of the total haplotypes identified (Fig. 2). Hap2 and hap3 were detected in most specimens groups, accounting for 73.25% (115/157) of the total haplotypes. The haplotype (*h*) and nucleotide (π) diversity were 0.428 ± 0.095 (WZW) to 0.916 ± 0.055 (FZW) and 0.0015 ± 0.0004 (WZW) to 0.0508 ± 0.0100 (FZW), respectively.

Genetic structure

The pairwise F_{ST} values ranged from -0.009 to 0.216, and N_m ranged from 1.22 to 384.87 (Fig. 3). AMOVA (Table II) showed that 13.94% of the genetic variation was observed among populations, and the genetic difference within populations explained 86.06% of the total variation. For the fixation index, F_{ST} value was 0.139 (Table II). According to Nei's standard genetic distance, the eight populations were gathered into two clusters (Fig. 4). The *K* values were estimated and ΔK =2 (Fig. 4). In the UPGMA tree of the haplotypes (Fig. 2), FZW had the highest number of private haplotypes. The plot of the haplotype network (Fig. 5) showed that these haplotypes were gathered into

one cluster. PCoA (Fig. 6) indicated FZW had diverged from the others. The estimation of genetic structure also showed that FZW had diverged from the other samples. Except for FZW, the seven specimens groups showed non-significant divergence.

Table II. AMOVA for the eight location specimens of *Mytilus unguiculatus*.

Source of variation	d.f.	Sum of squares	Variance components	% of variation
Among populations	7	44.82	0.25	13.94%
Within populations	149	228.69	1.53	86.06%
Total	156	273.51	1.78	
Fixation Index			F_{ST}	0.139**

** $P < 0.01$

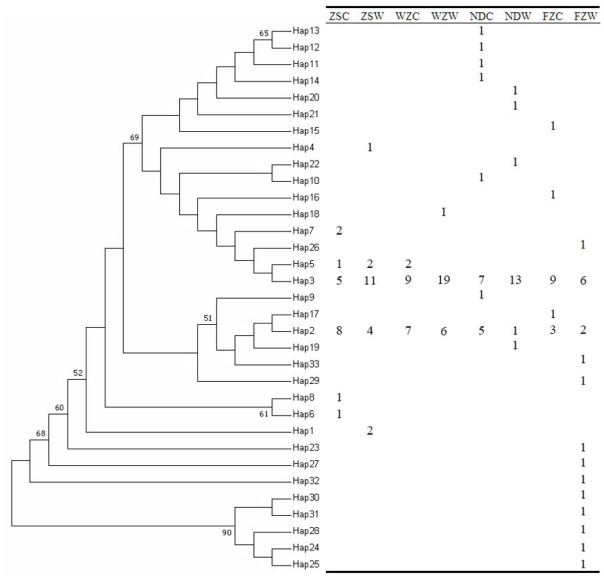

Fig. 2. UPGMA tree of 33 haplotypes of the 16S rRNA gene and the number of haplotypes of the eight specimens groups.

DISCUSSION

Genetic diversity between specimens groups

The haplotype diversity of the cultured specimens (from 0.621 to 0.797; average, 0.717) was higher than that of three wild specimens groups (ZSW, WZW, and NNW; from 0.428 to 0.668; average, 0.560). Expect for FZW, the wild specimens showed non-significant genetic diversity. Both haplotype and nucleotide diversities were low. The mtDNA diversity was significantly reduced in the cultured specimens groups; this has been observed in other fish and mussel aquaculture species (Shu *et al.*, 2008; Lundrigan *et al.*, 2005). With human intervention, the cultured specimens groups expanded from a small number to several successful breeders, which is analogous to a previous occurrence of a bottleneck in a population of *M. unguiculatus* (Allendorf, 1986). Furthermore, to reduce the production cost, most mollusk farmers collect the natural spat from wild populations of *M. unguiculatus*. This may be why the haplotype number of the cultured specimens groups was less than that of the wild specimen groups.

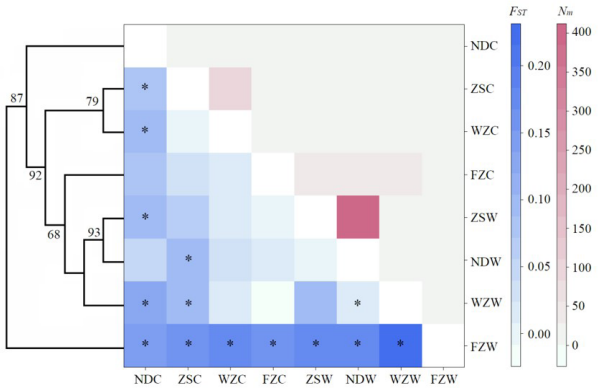

Fig. 3. Heatmap of FST and Nm with the UPGMA tree for the eight specimens groups. The pairwise F_{ST} values ranged from -0.009 to 0.216, and N_m ranged from 1.22 to 384.87.

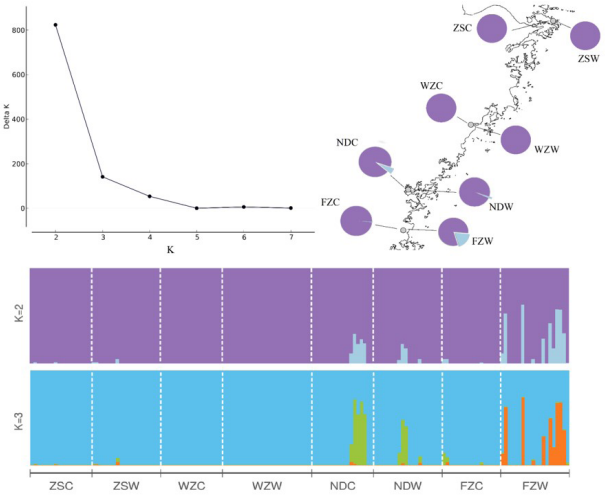

Fig. 4. STRUCTURE analysis for the 16S rRNA of *Mytilus unguiculatus*.

Genetic structure among the specimens groups

The AMOVA showed that F_{ST} was significant and most of the total variations were explained by the genetic differences within populations (Table II). The pairwise F_{ST}

values of FZW were significantly divergent from the others. FZW was divided from the others in the UPGMA tree (Fig. 3). This result was also observed in the haplotype network (Fig. 5), in which most haplotypes of FZW were assembled. The PCoA indicated that only FZW was different from the others. With frequent gene flow and development of the wild populations, the genetic divergence between wild and cultured specimens groups would recede. Several studies on wild and cultured populations have reported similar results (Chen *et al*., 2016; Jiang *et al*., 2007; Xiao *et al*., 2017). However, FZW was significantly different from the other specimens groups, possibly because of geographic factors. For the aquaculture of *M. unguiculatus*, Zhejiang Province in China is the main production area for mussel juveniles. The spawning season of *M. unguiculatus* is between November and January; at this stage, the planktonic larva is not affected by the Yangtze River plume. Thus, the larvae could spread widely without any barriers, which is consistent with the results obtained by Shen *et al*. (2009).

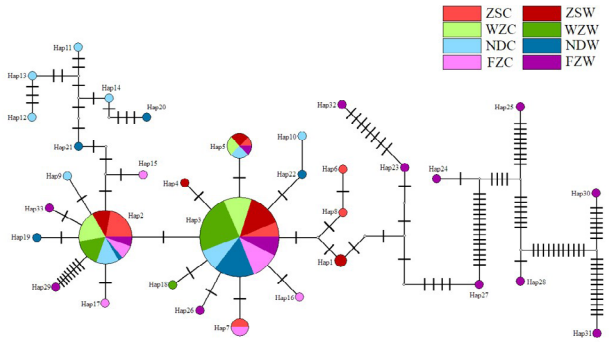

Fig. 5. Haplotype network for the eight specimens of *Mytilus unguiculatus*. The plot of the haplotype network showed that these haplotypes were gathered into one cluster.

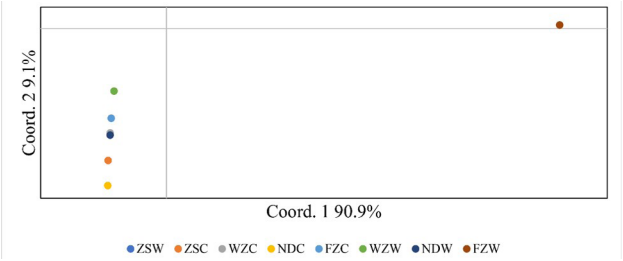

Fig. 6. Principal coordinate analysis of population differentiation on the basis of the 16S rRNA.

Management implications

Among the cultured specimens groups, high haplotype diversity and low nucleotide diversity indicated that the parents of the cultured specimens groups would be from a single population of *M. unguiculatus*, and, then, the sample size expanded rapidly. The haplotype number of the cultured specimens groups was less than that of the wild specimens (Fig. 3). Therefore, the cultured specimens groups possibly developed from the natural spat of a single or several wild populations. These results were confirmed using the genetic structure analysis. The gene flow between the cultured and wild specimens groups was low, and most of the pairwise F_{ST} values were non-significant.

In sessile marine species, a long pelagic larval duration increases the dispersal of the larvae and gene flow between populations over large spatial scales. Usually, a shortened larval duration is linked to frequent gene exchange (Cowen *et al*., 2009). Remarkably, the ZS and WZ specimens groups were not divergent; this suggested that the wild and cultured specimens groups were crossed. The mussel breeding was evolving without consideration of fisher farmers, causing the confusedly mussel seeding cultivation. The gene exchange in wild populations is obviously affected by human activities. Inbreeding reduces the genetic variation of wild populations and causes a decline in the quality of seeding. Because of traditional farming methods (using a rope or deadman anchor to fix the mussels), the cultured specimens groups travel off the farm and interbreed with wild specimens groups. Artificial movement of animals between sites results in a limited genetic structure (Pérez-Portela *et al*., 2013). In the absence of distinct genetic protection units, the wild and cultured populations would become more similar, which is why the germplasm resources of *M. unguiculatus* deteriorated in Zhejiang. NDC showed significant differentiation from the other cultured specimens groups, possibly because NDC interbred with the wild specimens groups. This is a sign that the barriers between wild and cultured populations are broken; under the risk of depletion, the wild population in ND may have been assimilated by the cultured mussels.

On the basis of some insights for the maintenance of genetic stock, on-shore farming would be a better substitution model to reduce the number of cultured larvae that sneak into the natural environment because the larvae would be caught by the water filtration system (Appleford *et al*., 2012). On-shore farming would also be able to easily control the production chain and enhance mussel quality. The genetic information obtained in this study forms the basis for further studies on *M. unguiculatus* as a seeding resource in aquaculture. Our findings could provide genetic insights for selecting the first generation. Farming practice should reduce the effect of natural habitat of wild populations to insure the wild populations that could keep the primary evolution as a cultured stock with rich genetic variability. As a preventive measure, the farming area should be limited, and genetic diversity levels of the

cultured and wild populations should be monitored (De Girolamo *et al*., 2017). Appropriate population management can help to prevent inbreeding and maintain heterozygosity levels and breeding that promote genetic diversity and reduce inbreeding (Barroso *et al*., 2005). Focus on the genetic protection units is also necessary to ensure the wild populations are not affected by human activities and the cultured organisms are isolated on the basis of international regulations (Diversity, 2010).

CONCLUSION

In this study, high diversity in cultured specimens groups was estimated than wild groups in hard-shelled mussel, *M. unguiculatus*. It was suggested to establish genetic protection units for *M. unguiculatus*.

ACKNOWLEDGEMENTS

This work was financially supported by the Fundamental Research Funds for Zhejiang Provincial Universities and Research Institutes (Grant No.: 2019J00021), the Natural Science Foundation of Zhejiang Province (Grant No.: LQ18D060004), the Zhoushan technology office program (Grant No.: 2019F12004), theSpecial Fund for the Key Research and Development Project of Zhejiang Province (Grant No.: 2019C02056) and the Open Foundation from Marine Sciences in the First-Class Subjects of Zhejiang Province.

Statement of conflict of interest

The authors have declared no conflict of interest.

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