International Journal of Biology; Vol. 6, No. 4; 2014
ISSN 1916-9671 E-ISSN 1916-968X
Published by Canadian Center of Science and Education

Comparison of the Internal Transcribed Spacer (ITS) Regions Between Hybrid and Their Parents in Scallop

Biao Wu[1], Aiguo Yang[1], Jiakun Yan[1], Wandong Xu[3], Tao Yu[2], Jiteng Tian[1], Zhihong Liu[1], Liqing Zhou[1] & Xiujun Sun[1]

[1] Key Laboratory of Sustainable Development of Marine Fisheries, Ministry of Agriculture, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, PR China

[2] Changdao Enhancement and Experiment Station, Chinese Academy of Fishery Science, Changdao 265800, PR China

[3] Kenli Prefecture Ocean and Fisheries Bureau, Dongying 257500, PR China

Correspondence: Aiguo Yang, Key Laboratory of Sustainable Development of Marine Fisheries, Ministry of Agriculture, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, PR China. E-mail: yangag@ysfri.ac.cn

Received: July 28, 2014 Accepted: August 15, 2014 Online Published: August 17, 2014

doi:10.5539/ijb.v6n4p103 URL: http://dx.doi.org/10.5539/ijb.v6n4p103

Abstract

The remarkable heterosis of the heterozygous F_1 derived from Zhikong scallop *Chlamys farreri* (♀) and Japanese scallop *Patinopecten yessoensis* (♂) has been proved during cultured process, and some genetic characteristics analysis have also been conducted using some molecular markers ISSR, SSR, MSAP, and so on. However, no study about the comparison between hybrid offspring and parents based on the nuclear ribosomal DNA internal transcribed spacer (ITS) was explored. In this study, the ITS-1 and ITS-2 of the *C. farreri*, *P. yessoensis* and their hybrid were amplified by PCR using specific primers and characteristics of those ITS regions, genetic regularity were analyzed. The results suggested that ITS-2 (334 bp- 352 bp) were a little longer than ITS-1 (241 bp- 256 bp) in length while the average GC content were lower than AT in most individuals, and they all had high genetic variation. Also, the gene flow between the hybrid and female parents were higher than that of hybrid and male parents. Our data implied a commonality that the ITS regions of offspring shared more similarity with that of female parents. This data in the present investigation will help for further studies in heterosis mechanism and utilization of hybrid scallop in China.

Keywords: *Chlamys farreri*, *Patinopecten yessoensis*, hybrid, internal transcribed spacer (ITS)

1. Introduction

Scallop is enjoyed as a good taste food source by humans in China, and four species are widely cultured. The Zhikong scallop, *Chlamys farreri,* a native species in China*,* is one of the most economically scallops, whose production had almost reached approximately 80% of the total scallop production before introducing the Bay Scallop (*Argopecten irradians*) and Japanese scallop (*Patinopecten yessoensis*) from overseas. It has a wide distribution along the coasts of North China, Korea, Japan, and Eastern Russia (Zhang et al., 2011). However, in recent years, because of high temperature in summer, deterioration of water quality, and low quality seeds, the mass mortality of *C. farreri* have frequently broken out in summer season in the main cultured areas, and the mortality reached even 80% in some areas (Guo et al., 1999; Zhang & Yang, 1999), which had seriously affected the development of its industry (Xiao et al., 2005). It is imperative for us to actively breed new varieties with high adversity resistance and fast-growing through using traditional and new breeding methods. Hybridization is a very effective means of breeding for improving the germplasm resources. The Japanese scallop *P. yessoensis*, a cold-tolerant species inhabiting coastal waters of the northern islands of Japan, the northern part of the Korean Peninsula, and Russian Primorye, was introduced to China about two decades ago from Japan (Li et al., 2007). The intercross of *C. farreri* (♀) and *P. yessoensis* (♂) has been proved to be a good way to improve the scallop quality, comparing to *C. farreri*, the hybrid F_1 had similar appearance traits, higher survival rate of 95% and growth rate was improved by 23%, while there was large scale death of *C. farreri* in high water temperature season, which revealed that the heterosis of the heterozygous F_1 was very apparent (Yang et al., 2004).

Molecular technique supplies a good tool for effective investigation on genetic analysis. Many researches showed that the molecular basis of heterosis may be attributed to the increased gene expression level in the hybrid or to the altered regulation of gene expression in the hybrid either at the global level or specific classes of genes (Leonardi et al., 1991; Romagnoli et al., 1990; Tsaftaris, 2006). As to the hybridization of *C. farreri* and *P. yessoensis*, some analyses were conducted based on some molecular technologies like random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), sequence-related amplified polymorphism (SRAP), and methylation-sensitive amplification polymorphism (MSAP) in our previous study and all the results showed that the hybrids have rich genetic diversity level (Cheng et al., 2009; He et al., 2007; Yang et al., 2009; Yu et al., 2010). However, only limited information is available regarding ribosomal DNA in hybridization study.

The internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA) is non-coding DNA sequence. It has a high degree of mutation, made it become one of the most extensively sequenced molecular markers (Won & Renner, 2005). This region is part of the rDNA cistron, which consists of 18S, ITS-1, 5.8S, ITS-2 and 28S, and is present in several hundred copies in most eukaryotes (Aktas et al., 2007; Cheng et al., 2006; Fernández-Tajes et al., 2010). To date, the ITS sequences have been successfully employed to distinguish related species and infer phylogenetic relationships from populations to families and even higher taxonomic levels. Genetic approaches based on DNA sequencing are widely used nowadays, but the level of genetic variation between the hybrid scallop offspring and their parents based on ITS sequence rDNA remains few. Thus, it is valuable to study this region for better understanding on the genetic variation of the hybrid offspring and their parents.

The aim of this study was to amplify and sequence the ITS-1 and ITS-2 regions of *C. farreri, P. yessoensis* and their hybrid to provide basic characteristics data of these sequences, then to assess the genetic variation among the populations. These results are expected to provide molecular biological basis for the protection and utilization of scallop resources in China.

2. Materials and Methods

2.1 Sample and DNA Extraction

Healthy *P. yessoensis* with the averaging shell length of 100 mm from Changdao Bay (Shandong Province), and *C. farreri* with 65 mm from Qingdao Bay (Shandong Province), were collected as the parent scallop, respectively. The parent scallop were cultured under the hatchery-reared condition until gonads mature by increasing the water temperature and then hybridization between male *P. yessoensis* and female *C. farreri* was implemented randomly by artificial breeding in Changdao Enhancement and Experiment Station, Chinese Academy of Fishery Science. The offspring were cultured to adult in Qingdao Bay. The adductor muscle of parents and hybrid offspring were collected from the fresh bodies and stored at -80 °C for genomic DNA extraction. The DNA extract process was performed as described previously by Aljanabi (Aljanabi & Martinez, 1997) with slight modification. Approximate 100 mg of adductor muscle was digested for 4 h at 56 °C in the lysis buffer (10 mM Tris-Cl, 10 mM EDTA, 0.5 % SDS, 2 % proteinase K). The extracted DNA quantity, purity and integrity were tested by spectrophotometry (A_{260}/A_{280}) and 1.5% agarose gel electrophoresis, and the DNA was stored in the DEPC-treated water at -80 °C for use.

2.2 PCR Amplification and Sequencing

The PCRs were carried out to amplify the complete ITS regions of *P. yessoensis, C. farreri* and their offspring. Primers, ITS-1-F and ITS-1-R for amplifying ITS-1 region, ITS-2-F and ITS-2-R for ITS-2 region, were synthesized by BGI and used for PCR amplify as described by Hedgecock (Hedgecock et al., 1999) and. PCRs were performed in 30 μl volume reaction mixture composed of 100 ng genomic DNA, 3.0 μl 10 × buffer, 1.6 μl Mg^{2+} (20 mM), 2.0 μl dNTP (10 mM each), 2 μl each primer (10 μM), 1 U DNA polymerase (TianGen). PCR amplifications were carried out in a Master cycler (Eppendorf), using the reaction condition settings as follows: initial denaturation at 94 °C for 5 min, followed by 38 cycles of denaturing for 40 s at 94 °C, annealing for 40 s at 52 °C for ITS-1 and 56 °C for ITS-2, extension for 45 s at 72 °C, and a final extension for 10 min at 72 °C. The target amplified fragments were selected by 1.5 % agarose gel electrophoresis and cloned into pMD18-T vector (TaKaRa) according to the instruction and then transformed into competent cells *E. coli*. The positive recombinants were detected by PCR and selected for sequencing (Sangon Biotech). The primers used in this study were listed in Table 1.

Table 1. Primers used in this study

Primer name	Sequence (5'-3')
ITS-1-F	GGTTTCTGTAGGTCAACCTGC
ITS-1-R	CTGCGTTCTTCATCGACCC
ITS-2-F	GGGTCGATGAAGAACGCAG
ITS-2-R	GCTCTTCCCGCTTCACTCG

2.3 Data Analysis

Sequences obtained were edited using EditSeq model of DNAStar version 7.1 and revised manually according to the results of Base calling and NCBI blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence length and nucleotide composition were computed by MEGA 5.01 (Tamura et al., 2011). Number of Haplotypes, Haplotype (gene) diversity (*HD*), Nucleotide diversity (*PI*), and genetic divergence (F_{st}) were calculated by DNASP V5 (Librado & Rozas, 2009). Gene flow (N_m) was calculated by formula, $N_m = (1- F_{st})/2F_{st}$ (Xu et al., 2011).

Table 2. Summary of length variation (bp) and GC content (%) of the nuclear ribosomal ITS

Parameter	ITS-1						ITS-2					
	T	C	A	G	Length	G+C	T	C	A	G	Length	G+C
C1	20.7	25.9	34.3	19.1	251	45.02	23.1	24.9	30.2	21.9	334	46.7
C2	22.9	29.2	30.0	17.8	253	47.04	24.0	24.9	29.3	21.9	334	46.7
C3	22.1	30.4	28.9	18.6	253	49.01	24.0	24.9	29.3	21.9	334	46.7
C4	24.4	25.2	31.5	18.9	254	44.09	23.4	24.9	30.2	21.6	338	46.4
C5	20.9	25.7	34.0	19.4	253	45.06	21.6	26.6	32.2	19.5	338	46.2
C6	25.8	23.8	31.0	19.4	252	43.25	23.4	24.9	30.2	21.6	338	46.4
C7	20.2	32.5	30.6	16.7	252	49.21	23.4	24.9	30.2	21.6	338	46.4
C8	19.7	33.1	28.0	19.3	254	52.36	24.0	26.3	29.3	20.4	338	46.7
C10	19.4	28.5	34.8	17.4	253	45.85	24.9	25.1	28.7	21.3	334	46.4
C11	22.0	31.5	31.1	15.4	254	46.85	23.4	24.9	30.2	21.6	338	46.4
C12	22.9	28.1	32.0	17.0	253	45.06	24.0	24.9	29.3	21.9	334	46.7
F1	21.0	25.8	33.7	19.4	252	45.24	23.1	25.1	30.2	21.6	338	46.7
F2	21.0	25.8	33.7	19.4	252	45.24	23.4	24.9	30.5	21.3	338	46.2
F3	17.9	31.3	31.3	19.4	252	50.79	23.4	24.9	30.2	21.6	338	46.4
F4	22.7	25.4	34.4	17.6	256	42.97	24.3	25.4	30.2	20.1	334	45.5
F5	20.9	26.0	34.3	18.9	254	44.88	23.1	25.1	30.2	21.6	338	46.7
F6	20.9	27.6	33.1	18.5	254	46.06	23.1	25.1	30.2	21.6	338	46.7
F7	20.9	26.0	33.9	19.3	254	45.28	23.1	25.1	30.2	21.6	338	46.7
F8	22.5	27.3	32.4	17.8	253	45.06	22.8	26.0	31.1	20.1	338	46.2
F10	26.5	24.9	29.6	19.0	253	43.87	23.4	24.9	30.2	21.6	338	46.4
F12	20.9	28.3	31.5	19.3	254	47.64	22.8	26.0	30.5	20.7	338	46.7
P1	23.7	27.8	30.3	18.3	241	46.06	22.6	24.6	30.2	22.6	341	47.2
P2	23.8	25.0	33.2	18.0	244	43.03	22.9	24.9	30.4	21.7	345	46.7
P3	25.3	24.5	32.2	18.0	245	42.45	22.5	25.1	31.1	21.4	351	46.4
P4	24.8	29.8	28.9	16.5	242	46.28	21.4	25.7	33.5	19.4	346	45.1
P5	24.8	29.8	28.9	16.5	242	46.28	22.6	24.6	29.6	23.2	341	47.8
P6	22.7	26.4	33.9	16.9	242	43.39	23.8	26.1	30.5	19.6	341	45.7
P7	23.0	24.7	35.0	17.3	243	41.98	22.9	24.6	30.2	22.3	341	46.9
P8	21.4	28.4	33.3	16.9	243	45.27	23.2	25.8	30.5	20.5	341	46.3
P9	22.2	26.7	32.1	18.9	243	45.68	24.2	26.2	28.9	20.7	343	46.9
P10	28.1	26.4	28.5	16.9	242	43.39	22.7	25.0	31.3	21.0	352	46.0
P11	25.0	24.2	32.4	18.4	244	42.62	23.4	24.3	31.4	20.9	350	45.1
P12	23.0	26.3	33.7	16.9	243	43.20	***	***	***	***	***	***

Note: C, *C. Farreri*; F, hybrids; P, *P. yessoensis.*

3. Results

3.1 Length and GC Content

The ITS regions containing partial sequences of 18S, 5.8S and 28S ribosomal RNA genes were obtained by PCR in at least 10 individuals from each of the three populations. The compete sequence length and GC content of ITS-1 and ITS-2 regions after deleted the 18s, 5.8s and 28s regions were shown in Table 2. It revealed that the length of ITS of the offspring were more similar to their female parents *C. farreri* in both ITS-1 (ranged from 251 bp to 254 bp) and ITS-2 (ranged from 334 bp to 338 bp), while their male parents *P. yessoensis* were shorter in ITS-1 (ranged from 241 bp to 245 bp) and longer in ITS-2 (ranged from 341 bp to 352 bp). Average GC content in ITS-1 of *C. farreri* (43.25 % - 52.36 %) and offspring (42.97 % - 50.79 %) were a little higher than that in *P. yessoensis* (41.98 % - 46.28 %), in comparison, the average GC contents in ITS-2 were nearly equal among the three populations (45.1 % - 47.2 %).

3.2 Gene Polymorphism Analyses

Genetic variation parameters based on the data of ITS-1 and ITS-2 from two parental populations and their offspring were shown as Table 3. As the haplotype number, it displayed that 11/11 ITS-1 from *C. farreri*, 9/10 from hybrids, and 11/12 from *P. yessoensis* were haplotype, compared to that of ITS-2 were 5/11, 6/10, 11/12, which showed that it had higher haplotype's proportion in ITS-1 than that in ITS-2 of all the three populations. The haplotype diversity of ITS-1 from hybrids was lower than that from their parent, however, the nucleotide diversity of ITS-1 from hybrids was the highest among three populations. While in ITS-2, haplotype diversity of hybrids was higher than *C. farreri* but lower than *P. yessoensis*, and nucleotide diversity of hybrids was the lowest.

Table 3. Number of Haplotypes, Haplotype (gene) diversity (*HD*), Nucleotide diversity (*PI*)

Parameter		Sample size	Number of Haplotypes	Haplotype diversity(*HD*)	Nucleotide diversity(*PI*)
C	ITS-1	11	11	1.00	0.39208
	ITS-2	11	5	0.8182	0.41796
F	ITS-1	10	9	0.9449	0.41217
	ITS-2	10	6	0.8444	0.30373
P	ITS-1	12	11	0.9848	0.36904
	ITS-2	11	11	1.00	0.57240

Note: C, *C. Farreri;* F, hybrids; P, *P. yessoensis.*

As shown in Table 4, the gene flow was found to be varied significantly. The gene flow of ITS-2 between *hybrid offspring* and *P. yessoensis* was the minimum (0.2906), and the maximum appeared in the ITS-1 between the hybrid and *C. farreri.* The gene flow between offspring and their female parent *C. farreri*, 7.5645 of ITS-1 and 1.9452 of ITS-2, were higher than other interspecies. Besides, compared to ITS-1, the gene flow of ITS-2 were lower than ITS-1 between offspring and their female and male parents. The genetic divergence of ITS-1 and ITS-2 among different populations had at least significance difference except that of ITS-1 between hybrids and female parents, what's more, two of them reached extreme significant difference ($P < 0.01$). Comparatively speaking, the genetic difference between the hybrid and *C. farreri* were lower than other interspecies in both ITS-1 and ITS-2.

Table 4. Gene flow and genetic divergence

Parameter	C		F		P	
	ITS-1	ITS-2	ITS-1	ITS-2	ITS-1	ITS-2
C	——	——	7.5645	1.9452	0.6543	0.3394
F	0.062	0.044	——	——	0.6430	0.2906
P	$0.43314^{※}$	$0.5105^{※※}$	$0.43743^{※}$	$0.632^{※※}$	——	——

Note: C, *C. farreri*; F, hybrids; P, *P. yessoensis*. Figures below diagonal represent genetic divergence (F_{st}) corresponding significance test of divergence between populations ($P < 0.05$ means statistical significance; $P < 0.01$ means extreme significance, while figures above diagonal represent gene flow (N_m) between populations.

4. Discussion

In recent years, there has been an increased interest in understanding ITS regions research (Chen et al., 2010; Freire et al., 2011; Shafiei et al., 2013; Sudheer Pamidimarri et al., 2009; Sum et al., 2014). Comparing to the encoding regions, the ITS rDNA sequences belong to the non-coding regions, and they own some special property such as relatively higher variability, more rich divergence information, easily amplification, thus they are commonly applied in many fields and have been proven to be a powerful and useful genetic maker for genetic breeding and infer evolutionary relationships. Many studies were conducted in those fields. For example, the ITS-1 regions of wild giant clam population were amplified, and showed high polymorphism with 29% variation arising from base substitutions (Sudheer Pamidimarri et al., 2009; Yu et al., 2000); the ITS-2 regions of orient clam *Meretrix meretrix* Linnaeus were used for phylogenetic study among clam populations with different stripe color, which ITS were proved to be a good tool for genetic analysis (Li et al., 2006). These reported studies indicated that most previous research focused on variability and phylogenetic and taxonomic relationships, so far, knowledge about the relationships characterization between hybrids and parents based on ITS is not well known.

In the present study, the two internal spacers, ITS-1 and ITS-2 of the scallop hybrid and their parents were obtained successfully. The results showed that the original sequences included some partial sequence of conserved 18S, 5.8S and 28S rRNA genes, indicating the validity of ITS region were obtained. According to the reports about ITS sequences, the length of ITS sequences and GC content were variable in different species. The amplified ITS-2 fragments were about 500 bp in Pearl Oyster, spanning the partial sequences of 5.8S and 28S rRNA genes, whereas rhodnius presented a less variable ITS-1 with around 300 bp, and the species of the Triatoma and Panstrongylus genera presented an amplified ITS-1 fragment between 600 and 1000 bp (He et al., 2005; Tartarotti and Ceron, 2005). Both ITS-1 and ITS-2 of the three populations in our study had normal length of approximately 300 bp, and GC contents were a little lower than AT contents. What's interested was that the ITS length size and GC contents of the hybrid offspring was found to have more similarity with their female parents than male parents. What's more, the same results were also found in the following gene polymorphism analyses in this study. Especially, the gene flow and genetic divergence all obviously showed the higher identical between the hybrid offspring and their female parents in both ITS-1 and ITS-2 regions. In the previous study, we found the hybrid F_1 were similar to the female parents in the genetic analyses via the molecular markers RAPD, SSR, ISSR and SRAP (Cheng et al., 2009; He et al., 2007; Yang et al., 2008; Yang et al., 2009), although the F_1 genome came from male and female parents, which might be the reason for that the hybrid F_1 had similar appearance and higher resistance traits with mothers. The results in this study are considered to be very parallel to those results we previously obtained using other molecular markers. This report about the ITS genetic regularity referring to the *C. farreri, P. yessoensis* and their hybrid offspring enhances our knowledge regarding to heterosis in scallop.

Acknowledgements

This work is supported by the grants from Independent Innovation Funds of Shandong Province (2013CXC80202) and Special Scientific Research Funds for Central Non-profit Institutes, Yellow Sea Fisheries Research Institutes (20603022013012).

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