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Parent-offspring relationship recognition based on SSR and mtDNA confirmed resource supplement effect of *Fenneropenaeus chinensis* release

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Abstract

The resource of *Fenneropenaeus chinensis* has declined sharply due to excessive fishing intensity, ecological changes and diseases. In order to supplement the fishing yield and restore resources of *F. chinensis*, the relevant authorities have carried out the activities of stock enhancement and releasing. It can increase biomass and recover resources. However, compared with increasing biomass, there were still few reports on its effect on the recovery of resources. Resource recovery is a process related to whether the released individuals can form a reproductive population. Up to now, there has been a lack of evidence whether the released *F. chinensis* can complete the entire life history, and form reproduction population. In this study, gravid female shrimp after spawning migration were captured from coastal waters of Haiyang, Qingdao, and Yellow Sea. After identifying parentage relationships using simple sequence repeat (SSR) and mtDNA haplotype, it was finally confirmed that there were eight released individuals in the recapture samples. It was confirmed for the first time that at least part of the released *F. chinensis* can complete overwintering and reproductive migration, and maintain the migration habits as their wild counterparts. Therefore, we infered that the released shrimp can reproduce under natural conditions, these *F. chinensis* can form reproductive populations theoretically if without human intervention. These results indicated that enhancenment and release activities have a positive effect on resource recovery.

Key words: *Fenneropenaeus chinensis*, release, simple sequence repeat (SSR), mtDNA, resource supplement

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1 Introduction

Chinese shrimp (*Fenneropenaeus chinensis*) is a large annual lukewarm-water economic shrimp with wintering and spawning migration. This species is widely distributed in the Bohai Sea, Yellow Sea and western coasts of Korea Peninsula (Deng et al., 1990). It is an important fishing object and mariculture species along the coast of northern China (Deng et al., 1990). Since 1980s, the resource of *F. chinensis* has declined sharply due to excessive fishing intensity, ecological changes and diseases (Shi and Deng et al., 2000; Wang et al., 2006). In order to supplement the fishing yield and restore resources of *F. chinensis*, the relevant authorities have carried out the activities of stock enhancement and releasing (Liu et al., 2022). At present, more than 90% part of *F. chinensis* landing in autumn fishing season was hatchery released, which has been confirmed in many studies (Wang et al., 2006; Li et al., 2019). These results confirmed that the stock enhancement and release of *F. chinensis* played a significant role in the supplement of biomass (Wang et al., 2006, 2020; Li et al., 2019), but there were still few reports on its effect on the recovery of resources. This was because the effect on biomass is only to increase the yield of *F. chinensis*, while resource recovery is a process related to whether the release individual can form a reproductive population (Wang et al., 2020). Increasing biomass and restoring resources were different levels in the mechanism of population size change (Tang, 2019). Up to now, there are a lot of research about accurate assessment of recapture rate, ecological habits, migration distribution, migration route, growth characteristics and genetic diversity of *F. chinensis* after release (Shi and Deng et al., 2000; Wang et al., 2006; Li et al., 2017; Song et al., 2020; Yang et al., 2020; Liu et al., 2022). However, there has been a lack of evidence whether *F. chinensis* can complete the entire life history after release, and form reproduction shrimp population.

Under natural conditions, the parent prawns of *F. chinensis* mate in late October. Then, in April of the following year, gravid female shrimp were captured during transported to hatcheries along the Bohai Sea and the Yellow Sea. The migration route and distribution of *F. chinensis* under natural conditions are shown in Fig.1. In consideration of gravid female shrimp should die immediately after spawning, and it is difficult to collect samples for parental tracing of wild offspring. Therefore, in the present study, gravid female shrimp completed spawning migration were cap-

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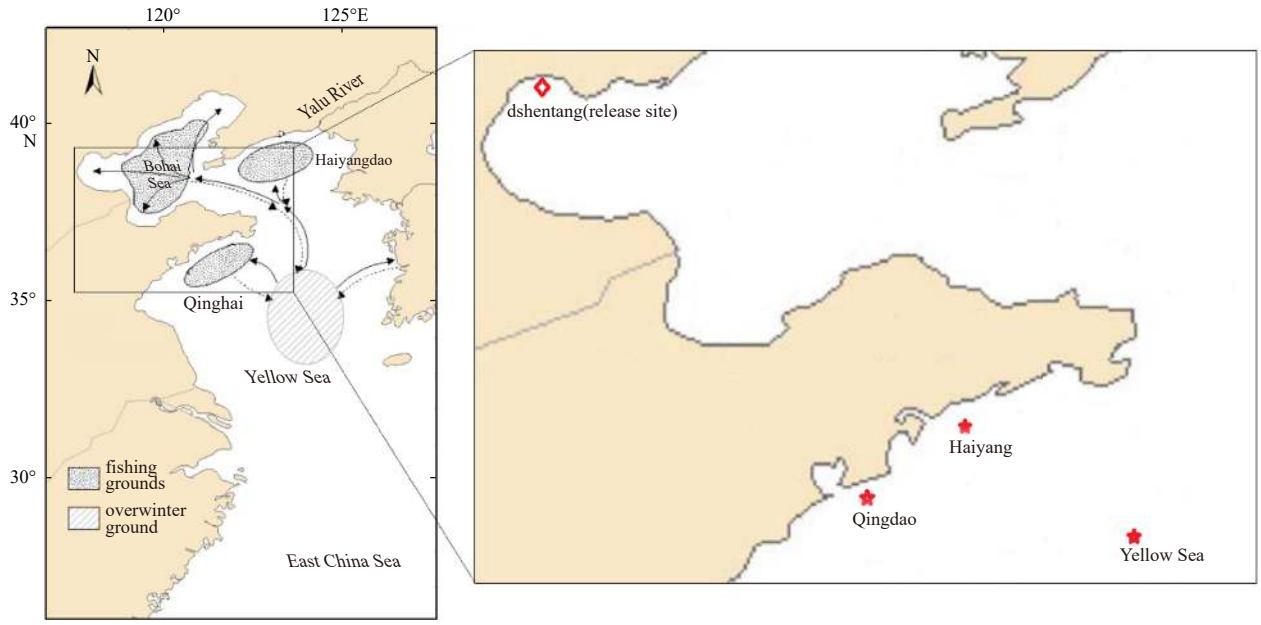

Fig. 1. Natural distribution and migration routes of *F. chinensis* in the Yellow Sea and Bohai Sea. Stars indicate locations of investigation, diamond indicate locations of hatchery releases modified from (Wang et al., 2006).

tured from coastal waters of Haiyang (HY), Qingdao (QD), and Yellow Sea (YS). Eleven simple sequence repeat (SSR) markers were used to recognize parent-offspring relationship. Then the haplotype identification obtained by mtDNA region sequencing (COI and 16S) were used to verify parentage assignment results based on SSR. This study will provide data support for whether the release population of *F. chinensis* can maintain the characteristics of natural migration, or form a supplementary effect on the reproductive population.

2 Materials and methods

2.1 *Experimental materials*

The egg-holding individuals were recaptured in April, 2022 from three sites: (1) 75 in Haiyang, (2) 160 in Qingdao, and (3) 80 in Yellow Sea (Fig.1), and the total was 315. Their candidate parents were 277 female parents, provided by Tianjin Dashentang Aquaculture Co., Ltd. The offspring of these parents were released in June 2021from Dashentang(Fig. 1). All samples were transferred with carbon dioxide ice to laboratory in the Yellow Sea Fisheries Research Institute, Qingdao, China and maintained in a –80℃ super-cold refrigerator for the following DNA isolation.

2.2 *Methods*

2.2.1 *SSR genotyping*

Genomic DNA was extracted from the swimming legs using standard phenol-chloroform procedures (Sambrook et al., 1989). Eleven SSR markers were used to parent-offspring relationship recognition of *F. chinensis*. These SSR were selected from loci previously developed in this laboratory and had high levels of polymorphisms and few genotyping errors (Zhang et al., 2015; Wang et al., 2016, 2020). The forward primer for each primer pair was labeled with one of four fluorescent dyes: 6-FAM, TAMRA, ROX, and HEX (Sangon Biotech, Shanghai). The specific primer information is shown in Table 1. PCR amplification was carried out according to Wang et al. (2016). The PCR products were separated using an ABI 3 730 automatic genetic analyzer (Applied Biosystems, USA). SSR alleles were sized with a GeneScanTM-500 LIZ size standard (Applied Biosystems, USA) and scored using GeneMapper™ V4.1 (Applied Biosystems, USA).

Cervus3.0 (Kalinowski et al., 2007) was used to obtain the allele frequency, observed heterozygosity (*Ho*), expected heterozygosity (*He*), polymorphic information content (*PIC*) and exclusion probability for each locus. According to the allele situation of each locus, the simulation analysis was carried out by Cervus3.0 to estimate the ability of genetic identification of each point. The Cervus3.0 was used to identify the parent-offspring relationship of parent shrimp and recapture shrimp based SSR genotyping date.

2.2.2 *mtDNA (COI and 16S) sequencing*

COI and 16S sequences were obtained from National Center for Biotechnology Information (NCBI) database, and primers were designed for PCR amplification by selecting highly variable regions within the sequences. The specific primer information is shown in Table 2. The products after PCR were detected by agarose gel electrophoresis and the unidirectional sequencing were performed by Sangon Biotech (Shanghai).

The results of mtDNA sequencing were compared by DNAman software to correct and remove the redundant sequences at both ends. COI and 16S sequence extraction and splicing are completed by DNAstar and R (Clewley, 1995; R Core Team, 2013). The software DnaSP6 is used to define haplotypes and calculate haplotype diversity, nucleotide diversity (*Pi*) and nucleic acid variation sites (Rozas et al., 2017). The haplotypes of mtDNA (COI and 16S) were used to further verify parent-offspring relationship based on SSR.

3 Results

3.1 *SSR genotyping results*

All parents and offspring were successfully genotyped. The genetic diversity parameter information of these loci between offspring group and female parent group was shown in Table 3. The

Table 1. Characteristics of eleven microsatellite loci

Locus	GenBank accession No.	Primer sequences (5'-3')	Annealingte mperature/℃	Labeled fluorescent dye
EN0033	AY132813	F: CCTTGACACGGCATTGATTGG R: TACGTTGTGCAAACGCCAAGC	64	6-FAM
RS0622	AY132778	F: CAGTCCGTAGTTCATACTTGG R: ACATGCCTTTGTGTGAAAACG	60	HEX
RS1101	AY132811	F: CGAGTGGCAGCGAGTCCT R: TATTCCCACGCTCTTGTC	52	ROX
RS0683	AY132823	F: CACTCACTTATGTCACACTGC R: ACACACCAACACTCAATCTCC	64	TAMRA
EN0113	AY132816	F: TGTCAAGAGAGCGAGAGGGAGG R: TGTCAAGAGAGCGAGAGGGAGG	65	6-FAM
BM29561	–	F: AACAGACCACATACGGGAC R: TTTTCGGAAGTAACATCACA	58	HEX
RS0916	AY132796	F: GGCTAATGATAATAATGCTG R: CGTTGTTGTTGCTGTTG	56	ROX
RS0779	AY132790	F: ATGACACTCAAATCAAAG R: CAGAATAACATCATTACTAC	50	TAMRA
FCKR009	JQ650352	F: GCACGAAAACACATTAGTAGGA R: ATATCTGGAATGGCAAAGAGTC	53	6-FAM
FCKR002	JQ650349	F: CTCAACCCTCACCTCAGGAACA R: AATTGTGGAGGCGACTAAGTTC	56	ROX
FC027	–	F: GCGTGTAATGCTTGCTGT R: TTTAGGACCTGCGGAGAA	53	TAMRA

Note: "–" represents no data.

Table 2. Characteristics of COI and 16s

	Primer sequences (5'-3')	Annealing temperature/℃
COI	F: TTTTTGACCCTGCAGGAGGT R: CCGTGGAGGGTTCCTATTCA	55
16S	F: GTAGCATAATCATTAGTCT R: GGATACCTTAATTCAACA	43

analysis results from Cervus 3.0 showed that the 11 markers shared 325 alleles, with an average allele number of 29.5 and a maximum of 75. The *Ho* ranged from 0.484 to 0.927 with average 0.760, and the *He* ranged from 0.590 to 0.968 with average 0.880. The average *PIC* was 0.860. The cumulative exclusion probability was 0.999 when one parent sex was known. All the loci used in current study showed high polymorphism.

3.2 *COI and 16S sequencing results*

The total size of the spliced sequence of COI and 16S was 501 bp. The analysis results from DnaSP showed that COI and 16S shared 15 variable (polymorphic) sites in all individuals, with the singleton variable sites of 5 and parsimony informative sites of 10. Haplotype (gene) diversity was 0.265 and variance of haplotype diversity was 0.000 57 ± 0.024 00. Nucleotide diversity was 0.000 58 and theta (per site) was 0.004 02(Fig.2). A total of 15 haplotypes were defined by DnaSP and detailed information was shown in Table 4.

3.3 *Contribution of breeding population resources*

Among the 315 recaptured individuals, nine individuals were assigned to a parent using SSR. Then one individual was excluded with no mtDNA haplotype shared, it was finally confirmed that there were eight released individuals in the recapture samples. Among them, 4 individuals were in QD; 3 in HY and 1 in YS. The details were listed in Table 5.

4 Discussions

4.1 *Genetic diversity analysis based on two molecular markers*

In this study, high *Ho*, *He* and *PIC* were obtained using SSR,

Table 3. Genetic diversity information of 11 SSR loci between offspring group and female parent group.

Locus	*K*		*Ho*		*He*		*PIC*		*AE-2P*		*HW*		F(Null)	
	Female parent	Offspring	Female parent	Offspring	Female parent	Offspring	Female parent	Offspring	Female parent	Offspring	Female parent	Offspring	Female parent	Offspring
EN0033	52	60	0.768	0.718	0.954	0.971	0.950	0.968	0.905	0.938	ND	ND	0.107	0.149
RS0622	35	37	0.931	0.924	0.946	0.953	0.941	0.949	0.887	0.902	ND	ND	0.006	0.014
RS1101	12	17	0.767	0.755	0.803	0.834	0.774	0.813	0.612	0.674	NS	NS	0.021	0.046
RS0683	35	42	0.695	0.760	0.890	0.935	0.878	0.929	0.781	0.867	***	***	0.123	0.103
EN0113	12	12	0.758	0.796	0.852	0.860	0.835	0.844	0.708	0.720	**	NS	0.057	0.036
BM29561	27	31	0.900	0.898	0.909	0.899	0.900	0.890	0.814	0.797	NS	NS	0.004	0.003
RS0916	3	4	0.516	0.514	0.587	0.588	0.504	0.498	0.299	0.294	NS	NS	0.063	0.067
RS0779	11	11	0.732	0.664	0.854	0.868	0.835	0.853	0.705	0.732	**	***	0.075	0.132
FCKR009	26	28	0.617	0.372	0.918	0.924	0.910	0.918	0.832	0.845	***	***	0.198	0.430
FCKR002	21	23	1.000	0.822	0.916	0.919	0.908	0.911	0.826	0.832	NS	ND	–0.045	0.054
FC027	25	27	0.945	0.841	0.932	0.943	0.926	0.939	0.859	0.882	NS	ND	–0.008	0.056
Mean	23.5	26.5	0.784	0.733	0.869	0.881	0.851	0.865	0.748	0.771	-	-	0.055	0.099

Note: *K*, number of alleles at the locus; *Ho*, observed heterozygosity; *He*, expected heterozygosity; *PIC*, polymorphic information content; *AE-2P*, average exclusion probability for one candidate parent given the genotype of a known parent of the opposite sex; *HW*, significance of deviation from Hardy-Weinberg equilibrium; NS. no significant difference ($p>0.05$); ND. not done; *, significant difference ($p<0.05$); **, extremely significant difference ($p<0.01$); F (Null), estimated null allele frequency. The significance level includes a Bonferroni correction if the Bonferroni correction option was selected.

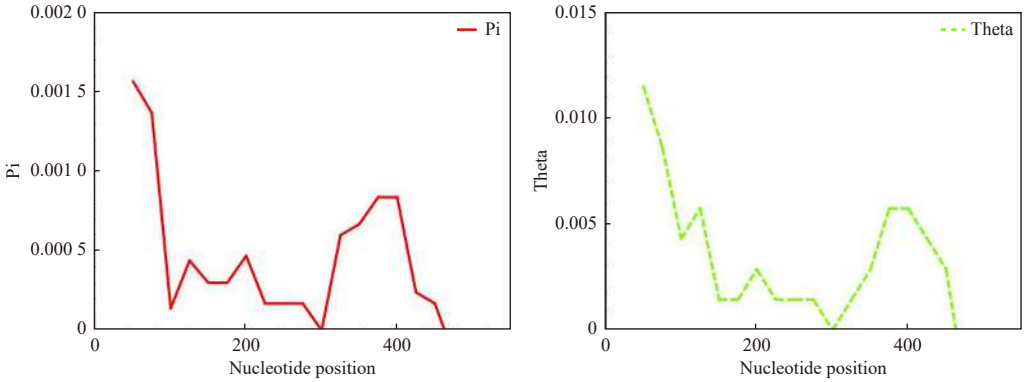

Fig. 2. Nucleotide diversity (Pi) and theta of DNA variation region.

Table 4. Number of haplotypes in different populations

Haplotype	Parents	QD	YS	HY
Hap01	25	2	5	3
Hap02	226	139	66	64
Hap03	10	2	2	4
Hap04	1	2	2	0
Hap05	8	1	0	0
Hap06	1	1	1	1
Hap07	1	0	0	0
Hap08	1	0	0	0
Hap09	1	0	1	0
Hap10	2	2	0	1
Hap11	0	2	0	0
Hap12	0	1	0	0
Hap13	0	1	0	0
Hap14	0	0	0	1
Hap15	0	0	1	0

Table 5. The released individuals in the recapture sample

Recapture individual ID	Mother ID	Shared haplotype	Lod score
HY-43	M181	Hap02	6.78
HY-71	M161	Hap02	6.19
HY-26	M221	Hap02	4.98
QD-152	M136	Hap02	7.66
QD-47	M123	Hap02	6.41
QD-14	M16	Hap02	5.39
QD-15	M59	Hap02	4.16
YS-53	M206	Hap09	6.18

which suggested the genetic diversity of *F. chinensis* natural population was at a high level. This result was consistent with those of previous studies (Sun et al., 2008; Zhang et al., 2015). The *He* was higher than the *Ho* at all 11 loci, indicating that there was a certain loss of heterozygosity in *F. chinensis* population. Similar results have been found in previous research (Zhang et al., 2015; Wang et al., 2016). The genetic diversity of the female parents and offspring was compared, and no significant difference was found, indicating that the enhancement and release had no significant effect on the genetic diversity of *F. chinensis* population. It is speculated that the main reason for this phenomenon is that the population of *F. chinensis* in China is subject to human intervention, the phenomenon of inbreeding, the homogenization of alleles and the loss of rare alleles in the process of gene recombination.

In this study, haplotype diversity and nucleotide diversity were 0.265 and 0.000 58 respectively using mtDNA, which showed a low level of genetic diversity. According to the study of Hedgecock et al. (1982), the low genetic variability of crustacean is a basic feature of its phylogeny, and many research on *F. chinensis* supported this conclusion (Yang et al., 2020; Liu et al., 2022). According to the standard proposed by Grant and Bowen (1998), the results in current study showed low diversity of mtDNA.

There may be some differences in the results of genetic analysis obtained by different molecular marker methods, which is mainly determined by the genetic characteristics and sensitivity of molecular markers. In this study, the genetic diversity calculated using SSR was high and the result obtained using mtDNA was low. This result may be because the mtDNA marker is relatively conserved in *F. chinensis* population.

4.2 *Effectiveness of SSR and mtDNA on parentage assignment*

Because of the advantages of SSR, our laboratory carried out the evaluation of *F. chinensis* release effect based on SSR for a decade (Zhang et al., 2015; Wang et al., 2016, 2020). The mtDNA is an effective tool for tracing matrilineal pedigree because that it follows the characteristics of matrilineal inheritance (Avise et al., 1987). The mtDNA has been applied in stock enhancement and release effect analysis of *F. chinensis* (Yang et al., 2020). However, there are few reports simultaneously using mtDNA and SSR in the evaluation on enhancement releasing of *F. chinensis*.

The results using 11 SSR showed that among the 315 recaptured individuals, 9 individuals were assigned to a parent, accounting for 2.86% of the total. Further, mtDNA (COI-16S) of 277 parents and 315 recapture individuals were analyzed, in which 10 haplotypes were found in parent shrimp and 13 haplotypes in recapture individuals, with the sequence exclusion rate of 2.54%. The results showed that the efficiency of excluding release individuals by mtDNA region sequence was low, which was consistent with the results of Yang et al. (2020) and Liu et al. (2022). In this study, COI-16S sequencing and SSR were used to evaluate the effect of proliferation and release of *F. chinensis*. Nine parent-offspring relationships were found by SSR, and one of them was excluded based on the results of COI-16S sequencing. This result indicated although the results of SSR were accurate, there is still a certain false positive result (Karaket and Poompuang, 2012), and the mtDNA (COI-16S) was also important in parentage identify.

4.3 *Assessment of release effect of F. chinensis*

Combined with the SSR and mtDNA, the final results showed that among the 315 recaptured individuals, 8 pairs of parent-offspring relationships were recognized, with a proportion of 2.54%. It was worth noting that considering that only 277 female parents

were sampled, and the number of female parents of the released individuals in the whole Bohai Sea were about 6 000 to 10 000 (Lyu et al., 2023), it is possible that there were still unidentified released individuals among the 315 recaptured individuals.

In the previous enhancement and releasing studies, all the shrimp were studied during the autumn. In this study, the samples were taken in April, when all the shrimp were egg-holding and about to release their eggs. Due to that female *F. chinensis* would die after spawning, it was hard to sample to verify whether the released *F. chinensis* produced offspring. Therefore, gravid female shrimp those have completed their winter migration and are about to spawn were sampled as substitutions to study resource supplement effect of stock enhancement and release.

In this study, it is confirmed for the first time that at least part of the released *F. chinensis* can complete overwintering and reproductive migration, and maintain the migration habits as their wild counterparts. Therefore, we infer that the released shrimp can form reproductive populations theoretically if without human intervention, and can reproduce themselves under natural conditions. These results indicate that multiplication and release activities have a positive effect on resource recovery.

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