Profile and development of microsatellite primers for *Acanthogobius ommaturus* based on high-throughput sequencing technology*

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Abstract   *Acanthogobius ommaturus*, a fish species of the Family Gobiidae, is a marine commercial fish perched on the bottom of seawater. In this study, Illumina high-throughput sequencing technology was applied to obtain the candidate microsatellite markers of *A. ommaturus*. A total of 4 746 microsatellite-rich fragments were found, of which 4 542 microsatellites are with primer fragments, containing 971 dinucleotide sequences, 2 643 trinucleotide sequences, 569 tetranucleotide sequences, 406 pentanucleotide sequences, and 212 hexanucleotide sequences. Based on the results of high-throughput sequencing, a total of 141 pairs of the microsatellite primers were designed and screened. And then 24 polymorphic primers were finally obtained by polyacrylamide gel electrophoresis. In total, 271 alleles were detected in the 24 pairs of primers. The number of alleles for different primers ranged from 5 to 19. The average number of effective alleles (N_a) was 11.292; the average observed heterozygosity (H_o) of the 24 pairs of primers was 0.665, the average expected heterozygosity (H_e) was 0.880, and the average polymorphic information content was 0.846. All sites were highly polymorphic (PIC>0.50).

Keyword: microsatellite; *Acanthogobius ommaturus*; high-throughput sequencing; polymorphic sites

1 INTRODUCTION

Microsatellite or simple sequence repeats (SSR) is a simple tandem repeat consisting of a few nucleotides (generally 1–6 repeat units) (Zane et al., 2002). The length polymorphism of these sequences is due to different repetition times and inconsistent repeat units. Microsatellites are widely distributed in the genomes of eukaryotes and prokaryotes. Microsatellite molecular marker technology is characterized by high abundance and reproducibility, codominant markers, and neutral selection (Zane et al., 2002; Zhang and Hewitt, 2003; Li, 2004). As a co-dominant molecular marker, microsatellite marker has the advantages of rich polymorphism, wide distribution range, high stability and easy detection. It has been widely used in various fields of conservation genetics, including population genetic diversity assessment, population

genetic structure analysis, paternity testing etc. (Arens et al., 2006; Uller et al., 2006; Barbará et al., 2007; Castoe et al., 2010).

Acanthogobius ommaturus, belonging to Osteichthyes, Perciformes, Gobioidei, Gobiidae, is a demersal fish that is widely distributed in coastal areas and estuaries of China, North Korea, Japan, and Indonesia. There are several formerly used names: *Synechogobius hasta*, *Acanthogobius hasta* and *Synechogobius ommaturus* (Zhao and Wu, 2008). Although microsatellite marker has been widely used in the studies on population genetics of marine
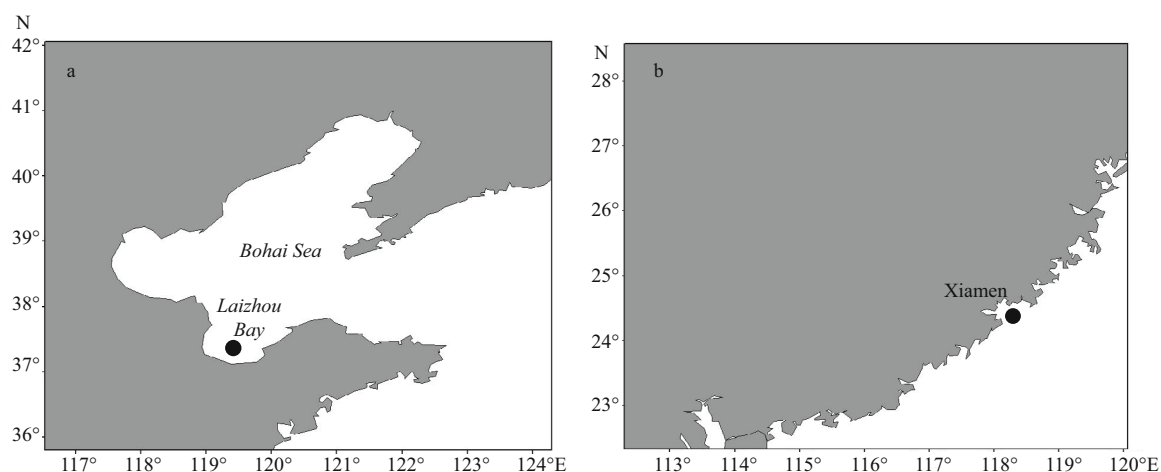
Fig.1 Map showing sampling sites of *A. ommaturus*
a. Laizhou Bay; b. Xiamen.

organisms (Winkelmann et al., 2013), no population genetic research has been conducted on the *A. ommaturus* until now. Studies on *A. ommaturus* mainly focused on the comparative analysis of individual fecundity and morphology (Feng et al., 2004), fishery biology (Fan et al., 2005), name of species (Song et al., 2010), DNA barcode (Gu et al., 2013), and genetic diversity and structure by other molecular markers (Song et al., 2010a, b, 2011). Bai et al. (2012) used magnetic bead enrichment method to develop 17 pairs of microsatellite primers. The number of microsatellite markers for *A. ommaturus* is far from enough for a more comprehensive population genetic analysis. High-throughput sequencing technology allows large-scale, accurate, inexpensive, and rapid sequencing of species (Holt and Jones, 2008). By analyzing the characteristics of the sequencing results in detail, we can provide a stronger basis for the following development of *A. ommaturus* primers to analyze the characteristics of microsatellite markers, screen more microsatellite primers, and lay foundation for further studies on the genetics and conservation of this species.

2 MATERIAL AND METHOD

2.1 Sampling and DNA extraction

One *A. ommaturus* sample was collected from Laizhou Bay (Fig.1a), China, and sent for high-throughput sequencing. Twenty-four individuals of *A. ommaturus* were collected from the southern coastal waters near Xiamen (Fig.1b), China, and were used for polymorphism detection and genetic diversity analysis. These samples were preserved in 95%

alcohol at the -20°C for DNA extraction. Genomic DNA was extracted from muscle tissue with the standard phenol-chloroform method described by Sambrook and Russell (2001).

2.2 High-throughput sequencing

DNA samples were detected by agarose gel electrophoresis, and the purity and integrity of DNA were analyzed. DNA samples with clear bands and no tail conform to the requirements of database construction. A microsatellite-enriched genomic library was constructed following the enrichment protocols of Ma and Chen (2009). Qualified DNA samples were digested and randomly interrupted. The preparation of the whole library needs terminal repair, A tail addition, sequencing connector addition (P5: 5′-AGATCGGAAGAGCGTCGTGTAGGGAAAGAG-TGTAGATCTCGGTGGTCGCCGTATCATT-3′; P7: 5′-TCGGAAGAGCACACGTCTGAACTCCAGTC-ACGCAGGAATCTCGTATGCCGTCTTCTGCTTG-3′), purification, PCR amplification, recovery of 300–700 bp sequence and other steps to complete. In the end, the constructed genomic library were sequenced by Illumina HiSeq™2000 (Illumina, USA) in the biological company (Personal Biotechnology Co., Ltd., Shanghai).

2.3 Acquisition of SSR sequence

The original sequences were clustered and assembled, and then the microsatellite sequences were detected by SSR search software. The standard of isolating SSR are as follows. The minimum length of repeating motif is 2 bp and the maximum length is 6 bp; the minimum length of the SSR sequence is

12 bp; The SSR upstream and downstream sequence length is 100 bp; the minimum distance between two microsatellite DNAs is 12 bp.

2.4 The detection of primer polymorphism

One hundred and forty-one PCR primer pairs were preferred to be designed using the PRIMER PREMIER 5 software (Premier Biosoft International, USA). The conditions are: repetition times of SSR units are more than 6; length of SSR units is 3–10 bp; expected length of PCR products is 130–300 bp; primers that repeat the same four bases continuously are excluded.

The polymorphism of these primer pairs were tested using 24 *A. ommaturus* samples. The PCR were conducted in volumes of 25 μL reaction mixtures containing 17.25-μL ultrapure water, 2.5-μL 10× PCR buffer, 2-μL dNTPs, 1-μL each primer (5 μmol/L), 0.25-μL Taq polymerase, and 1-μL DNA template. The PCR program was as follows: 5 min for denaturation at 95°C, followed by 35 cycles of 45 s at 94°C, 1 min at the annealing temperature (Table 1), 45 s at 72°C, and a final step for 10 min at 72°C. Then the PCR products were held at 4°C for insulation. After the amplification, the PCR products were electrophoresed on 8% non-denaturing polyacrylamide gel at 14 W for 3–4 h and visualized by silver staining (Lin et al., 2011). Allele size was identified according to the 20-bp DNA ladder (TaKaRa, China).

2.5 Data analysis

After statistical analysis of electrophoresis results, the results were input into the population genetic analysis software Genepop 4.0 (Rousset, 2008). The related parameters of SSR primers were calculated, including the average number of effective alleles (N_a), polymorphic information content (PIC), apparent heterozygosity (H_o) and expected heterozygosity (H_e), and Hardy-Weinberg equilibrium test and linkage disequilibrium test were carried out.

3 RESULT

3.1 Raw data quality

Raw reads 6.606 G was born and clean reads 6.511 G was filtered. The raw reads of each sequence were 6 606.008 M, with high quality (Q20≥95.6%, Q30≥93.92%). The GC content was 39.39%, and the RAD-Tag capture rate was 97.33%. Sequences were then spliced and assembled. The total contig base was
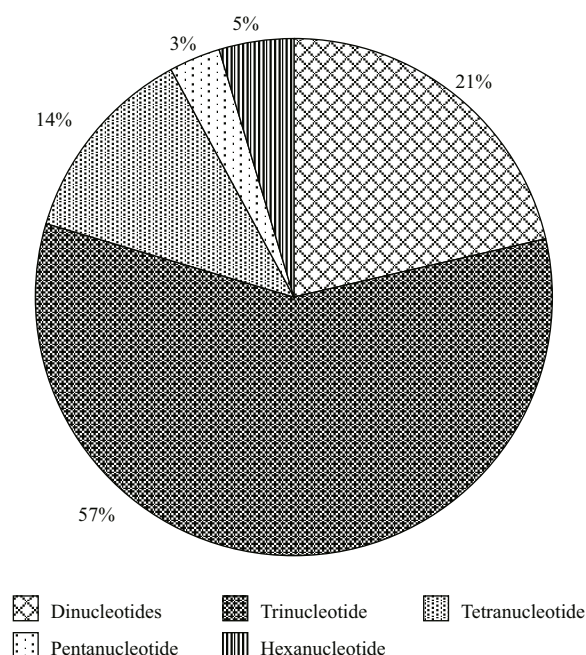

Fig.2 The proportion of different kinds repeat motif

96 594 144 bp, the total contig number of the sequences was 320 503, the average contig length of the assembly sequence was 301 bp, and the GC content of assembly result was 39.01%. Reads after de-duplication are compared to the assembly results. Variation detection showed that most of the heterozygous SNPs (Het rate (%): 83.73) and a few homozygous SNPs appeared in the test results, indicating that the assembly results were reliable.

### 3.2 Characterization of microsatellite markers

There are 4 756 microsatellite-rich fragments were examined to reduce redundancy and the number of microsatellites with primer fragments is 4 542. A total of 971 reads contained dinucleotide repeats, 2 643 contained trinucleotide repeats, 569 contained tetranucleotide repeats, 146 contained pentanucleotide repeats and 212 contained hexanucleotide repeats (Fig.2). The number of trinucleotide repeats was the highest. The number of eleven nucleotides was the lowest. Ttrinucleotide repeats had the greatest percentage of SSR mutation type distribution (Fig.2).

The AT repeat motifs was the most frequent among all ten types of dinucleotide repeats, whereas CT was the least frequent (Fig.3a). The ATT repeat motifs were the most frequent among all fifty-six types of trinucleotide repeats (Fig.3b). The CATG, AAAT, AATG repeat motifs were the three most frequent tetranucleotide motifs (Fig.3c), and 96% of all tetranucleotide motifs were less than 30 repeats. The

Table 1 Characteristics of microsatellite loci in *A. ommaturus*

Locus	Primer sequence (5′→3′)	Repeat motif	T_a (°C)	Allele size range (bp)	N_a	H_o	H_e	PIC	GenBank accession No.
BW4	F: ACGACTACACTGGGTCAGACTGT R: CTTACTGGTCTGAGTGGGAATTG	(CA)14	60.2	140–180	13	0.958	0.895	0.864	MK650129
BW11	F: ACACTCTGCATTTCCTCATGAAT R: CACATCATCTGTAGTTTGGCTCA	(CT)13	49.6	120–160	13	0.417	0.911	0.883	MK650128
BW12	F: ATTTCAAGAGGGATTGTTGGTTT R: CATTAATCCCAAAGCTAACCTCA	(TG)13	60.2	100–160	12	0.958	0.908	0.879	MK650127
BW15	F: AATGTTCAGGACAAACATGGAAT R: GAGAGGGACTGGACAATGTAGTG	(AG)12	58.3	120–180	10	0.708	0.892	0.860	MK650126
BW20	F: GTTGGAGATGTCAAAGACTCCG R: TGAGTCCATGAGTTTAACCGTCT	(GA)11	61.8	100–180	6	0.125	0.786	0.734	MK650125
BW28	F: GACACTGTGTGACACTCTTCTTTGT R: TTGAGAACACAGCCTTTGAATTT	(ATT)10	61.8	140–200	13	0.417	0.915	0.887	MK650124
BW34	F: GATGCAGTTATGGAGAGGTTACG R: CATTAAACCGAGGGGAATAAATC	(CGT)10	48.0	120–160	12	0.375	0.912	0.884	MK650123
BW44	F: CATCAACACAGAGTCAGTGAAGG R: TTTAACATCGCGTTATCGGTATT	(TAC)10	56.4	60–140	14	0.750	0.931	0.905	MK650122
BW48	F: CTGAAGAACGCTCACATTTCTCT R: GTAAGCCCTGCTCAGGTTACTG	(AC)10	56.4	120–140	12	0.917	0.904	0.874	MK650121
BW51	F: GTAATCCATTTGCCTCACTTCAT R: CCTCGTTCTGTGAGTCAATACAC	(CA)9	48.0	140–180	10	0.458	0.873	0.839	MK650120
BW74	F: GTCTCGCATTAAAGTCAGTGGTT R: CTTTATTTAAATGAGCAAGGGCA	(TG)8	56.4	100–200	14	0.292	0.918	0.891	MK650119
BW76	F: GCCGCACAGAAGAAATATGTAAC R: CAAAGCAGAGCTAACCGTATCTT	(TA)8	49.6	100–160	9	0.917	0.880	0.846	MK650118
BW82	F: CTTGTTTGTCTGAAATGTTGTGC R: TACCTTGAATGGCTCACATTCTT	(TAT)8	58.3	130–160	5	0.375	0.754	0.696	MK650117
BW91	F: AGAGAAGTCCAGAGTGTGAATGG R: CCAGTTTGTGTAGCCTCCTGA	(TGC)8	58.3	120–160	8	0.833	0.831	0.789	MK650116
BW115	F: GTCACTCTCCATTGTCCTGAATC R: GCATAGAGAAGTGTGCATGTGTG	(AC)7	61.8	100–160	10	1.000	0.841	0.804	MK650115
BW124	F: CATTGTTCACAGGAGTCACACTG R: GCGAAGCCATATTAGTTCACAAC	(GT)7	55.0	100–160	19	0.958	0.943	0.919	MK650114
BW126	F: GCACTCTTAGATACTTCATCAAGCC R: CGGCTCATATTTGAACAATGACT	(TAT)7	51.2	120–180	8	0.667	0.860	0.823	MK650113
BW127	F: AGGCTGTTATTTCCTGTAGACCG R: CCAGTTTGACAGCACATTTATCA	(CA)7	48.0	120–180	11	0.792	0.850	0.815	MK650112
BW129	F: CAGGTATGGCCACGAATTACTAC R: GACTCTGACTCCAGAGATGATCC	(CAA)7	64.0	100–160	19	0.792	0.923	0.896	MK650111
BW134	F: AAGAATGATGCAATGGGTTTATG R: TCAGAAATAAGTCACTGGAGGTTG	(TAT)7	53.1	120–180	11	0.708	0.895	0.863	MK650110
BW136	F: CCAGCAACTGAGTGAAGAACTTT R: CACTTCAGTTGAGAGTTCGGTTT	(AAC)7	58.3	120–160	12	0.792	0.910	0.881	MK650109
BW137	F: TGACAGAAGCTGCTAAGACTGTG R: TAGGTTTAGGCCTACCCAAATTC	(GA)7	55.0	120–140	8	0.500	0.769	0.729	MK650108
BW139	F: GAGGCAGATGTAGAGATGGTTTG R: GTTTGACACTGTCCACCATTAGG	(AG)7	51.2	120–180	12	0.583	0.924	0.897	MK650107
BW141	F: AAGAATAATGCAATGTGAAGCGT R: CAATTTGTTGACTGGAACACAGA	(TAT)7	53.1	120–160	10	0.667	0.887	0.854	MK650106

T_a: optimized annealing temperature; N_a: number of alleles; H_o: observed heterozygosity; H_e: expected heterozygosity; PIC: polymorphism information content.
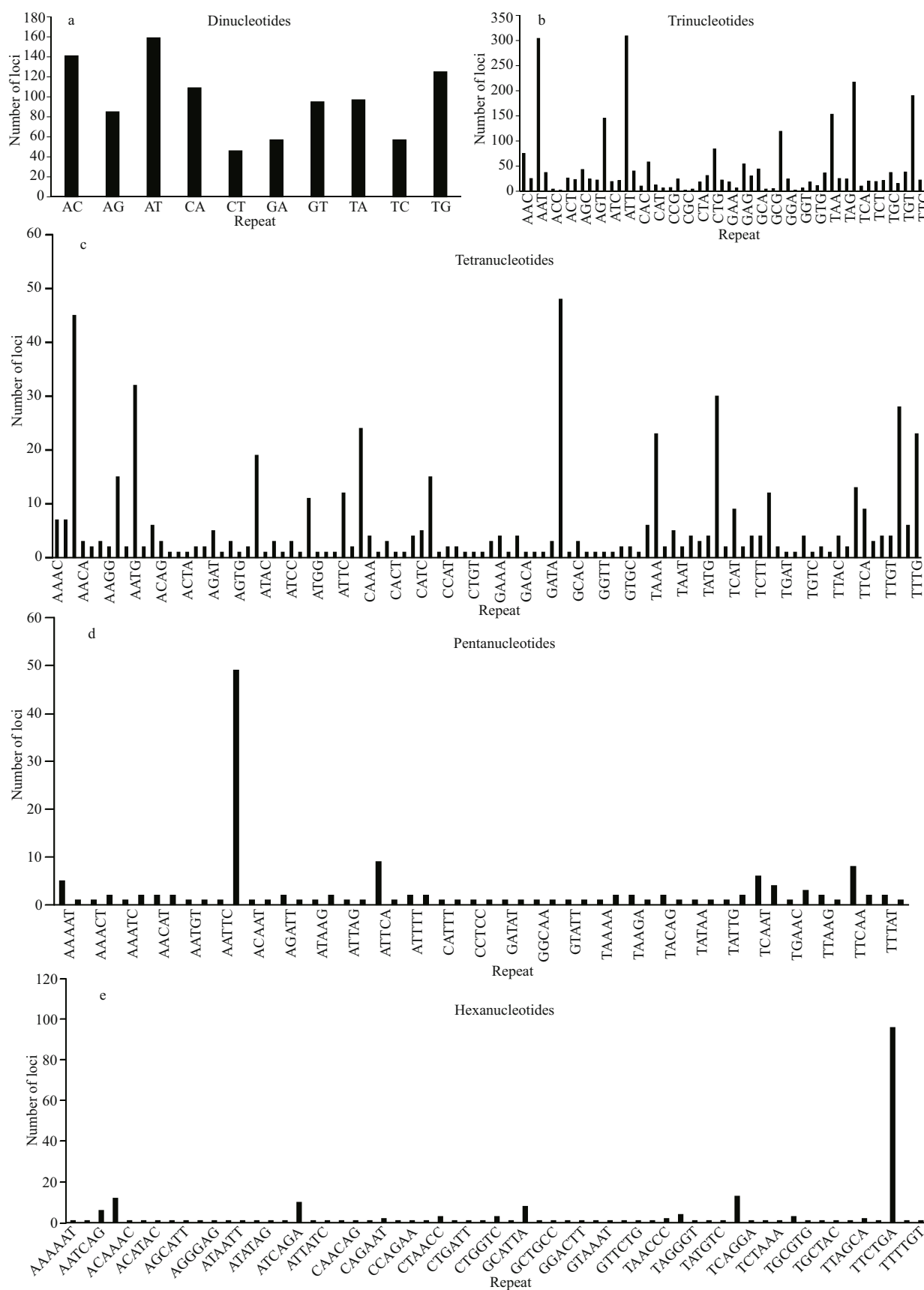
Fig.3 Type and number of repeat motif for dinucleotide (a), trinucleotide (b), tetranucleotide (c), pentanucleotide (d), and hexanucleotide (e)
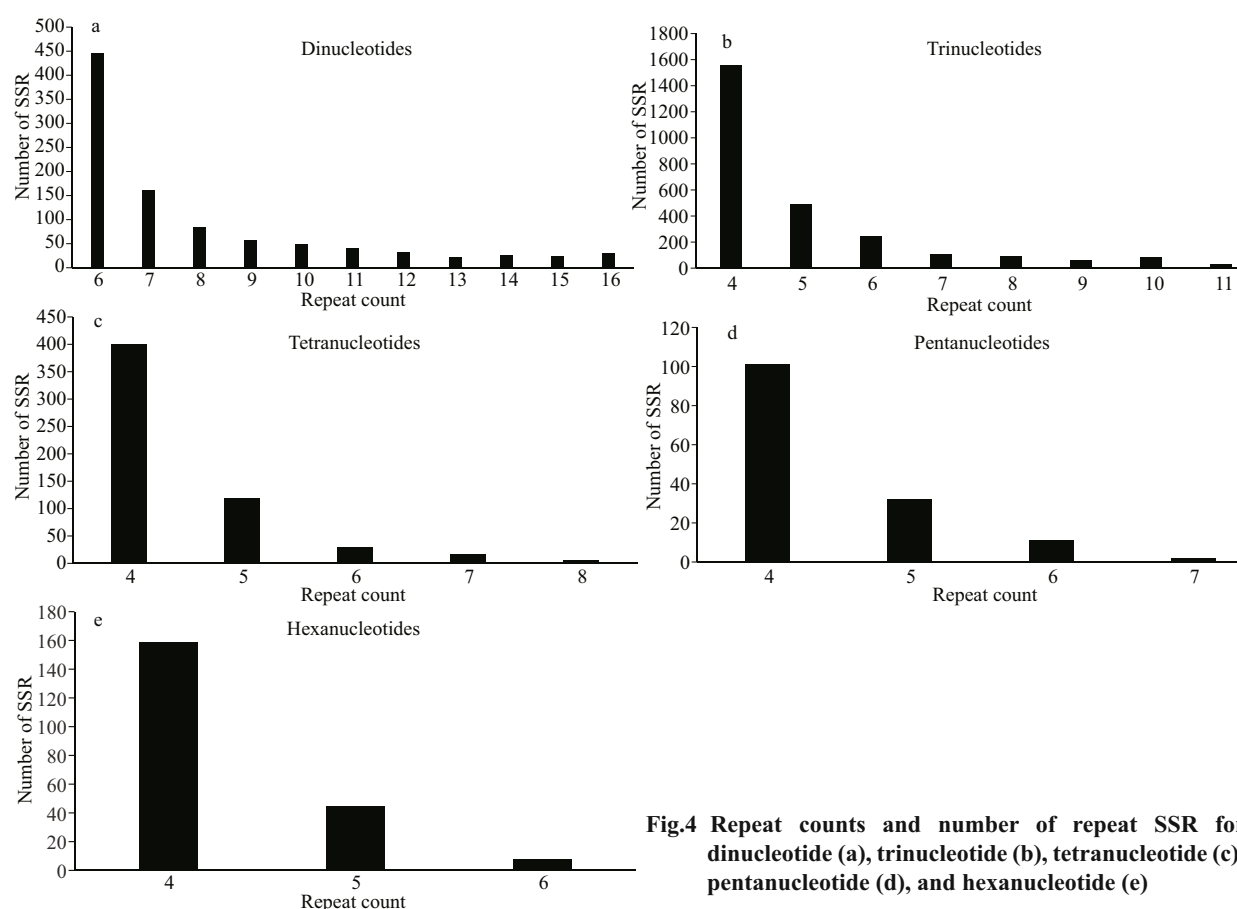
Fig.4 Repeat counts and number of repeat SSR for dinucleotide (a), trinucleotide (b), tetranucleotide (c), pentanucleotide (d), and hexanucleotide (e)

AATTG repeat motifswere the most frequent pentanucleotide (Fig.3d) and TTCTGA was the most frequent hexanucleotide (Fig.3e).

The number of SSRs in the dinucleotide type with 6 repeat units corresponds to an absolute dominant position, which is 45.83% of the total SSR (Fig.4a). Trinucleotide-type SSRs have the highest number of repeats 4 with repeat units, accounting for 58.76% (Fig.4b). Tetranucleotide-type SSRs have the highest number of 4repeats units, accounting for 70.30% (Fig.4c). The number of SSRs with 4 pentanucleotide type repeats was the highest, accounting for 69.18% (Fig.4d). The number of SSRs with the hexanucleotide type repeating sequence repetition number 4 is the highest, accounting for 75.00% (Fig.4e).

The number of dinucleotide repeats is 6–16, the repeat number of trinucleotides is 4–11, the repeat number of tetranucleotides is 4–8, the repeat number of pentanucleotides is 5–7, and the number of hexanucleotide repeats is 4–8, with a significant downward trend. As the number of perfect tandem repeat increased, the number of loci decreased (Fig.5). The perfect trinucleotide tandem repeat had the greatest numbers of loci (Fig.5b).

3.3 Detection of primer polymorphism

One hundred and forty-one primer pairs were tested by agarose gel electrophoresis and 24 primers showed polymorphic. The number of alleles per locus ranged from 5 to 19, with an effective average of 11.292. The observed (H_o) and expected (H_e) heterozygosities varied from 0.125 to 1.000 and from 0.754 to 0.943, respectively (Table 1). Eleven out of 24 polymorphic loci were significantly deviated from Hardy-Weinberg equilibrium ($P<0.05$). The polymorphism information content ranged from 0.696 to 0.919, all loci were showed highly polymorphic. Five loci (BW20, BW34, BW44, BW74, and BW129) were shown evidence of null alleles (null alleles frequency>5%). No genetic disequilibrium was detected in each locus.

4 DISCUSSION

4.1 Raw data quality

According to the sequencing results, the data quantity is good, the quality of sequencing is qualified, the GC distribution is normal, and the database is
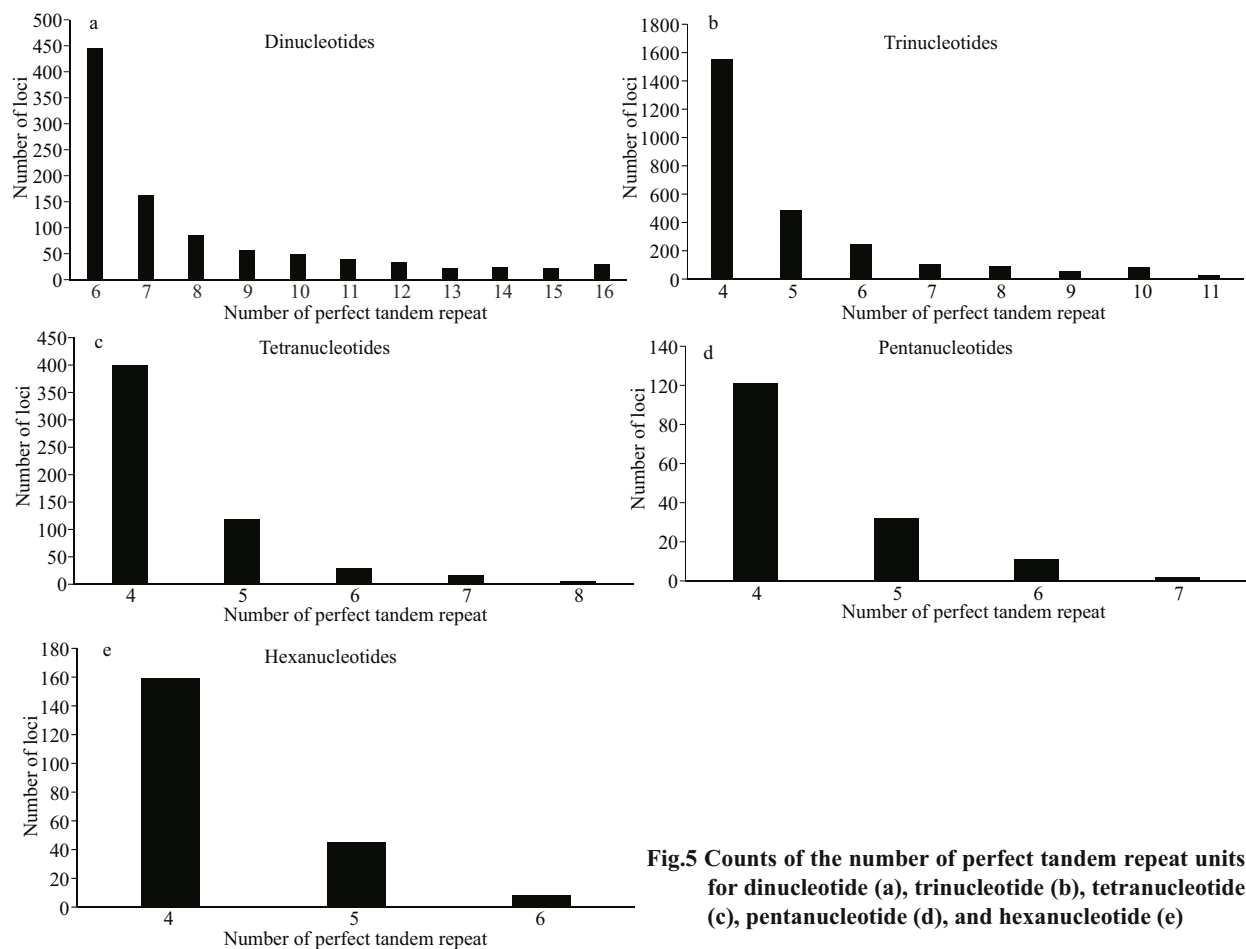
Fig.5 Counts of the number of perfect tandem repeat units for dinucleotide (a), trinucleotide (b), tetranucleotide (c), pentanucleotide (d), and hexanucleotide (e)

successfully constructed and sequenced (Zerbino and Birney, 2008). Yang et al. (2015) ever sequenced the genome of *Parargyropsedita* by random high-throughput sequencing, and the results show that the original sequence data were 2.80 Gbp. The difference of results may be caused by the different parameters of search software and software settings or the different genome size. The accuracy of assembly results can be reflected by the consistency between the GC content of assembly results and that of reads. The GC content of the assembly results is consistent with that of the sequencing data, which can reflect that the assembly results can represent part of the genome (Wang et al., 2017; Gao et al., 2018). The rate of designing fragment primer is 95.5%. To sum up, compared with traditional methods, high throughput sequencing technology can capture more microsatellite fragments more accurately in a shorter time.

4.2 Characterization of microsatellite markers

The number of repeated units tandemly in microsatellites is different from other species, which may be the result of natural selection and gene mutation (Katti et al., 2001). The results of the highest number of trinucleotide repeat units is consistent with other previous studies, such as in *Crassostreagigas* (Zhang et al., 2012), corals (Wang et al., 2009). The number of repeats decreases exponentially with repeat unit length in most eukaryotes because of higher gene mutation in longer repeats (Katti et al., 2001). It is reported that the number of repeats is negatively correlated with repeat length (Chen et al., 2010). The results of this study follow thispattern.

Some studies believe that the number of dinucleotide repeat unit of vertebrates is the most abundant (Wang et al., 2012). Therefore, this type of microsatellite loci has been widely used in molecular ecology, and AC repeat units are more commonly found (Tóth et al., 2000; Chistiakov et al., 2006). For example, the largest number of AC repeat units was found in the genome of *Patinopecten yessoensis* (Guo, 2014). The AG is the most common dinucleotide repeat unit in the *Exopalaemon carinicauda* microsatellite core sequence (Duan et al., 2016). The largest number of repeat units was AT in this study, which was different from common distributions.

Furthermore, the ATG (Guo, 2014) and the AAT (Duan et al., 2016) is the most common trinucleotide repeat unit in the genome of *Exopalaemon carinicauda*, while the types of trinucleotide repeats are different in the present study. Most of this result depends on the differences in species genomes, but it may also be related to the construction of cloned libraries and the length of sequencing fragments (Guo, 2014).

We found that the number of SSRs with a core sequence repeat number of four accounts for an absolute dominant position, followed by 6. However, the number of these two SSRs types is quite different. The number of SSR decreased with the increase of repetition times. The number of copies of the repeat unit and the number of microsatellite sequences are negatively correlated. The concentrated region of SSRs in genome may be the region where organisms respond rapidly under different environmental pressures (Li et al., 2004; Kashi and King, 2006). The organism can adapt to the variation of environmental factors by adjusting the repetition times of SSR units (Liu et al., 2006; Li et al., 2013).

4.3 Detection of primer polymorphism

With the reduction of sequencing cost and the expansion of sequencing platform throughput, large quantities of microsatellite sequences can be obtained immediately and effectively by random sequencing of whole genome using second generation sequencing technology (Kong et al., 2014; Lin et al., 2015; Gao et al., 2017). For example, Gao et al. (2017) successfully screened 60 pairs of microsatellite primers for *Epinephelus awoara* based on Illumina genome-wide random sequencing technology from 2.86 G of raw data. Yang et al. (2015) developed 15 polymorphic microsatellite loci in *Parargyrops edita* based on Illumina sequencing technology from 2.80 G of raw data. In this study, we obtained 24 polymorphic microsatellite primers for *A. ommaturus* based on Illumina genome-wide random sequencing technology from 6.60 G of raw data.

In this study, 11 polymorphic loci were found to deviate significantly from Hardy-Weinberg equilibrium ($P<0.05$). Deviation from Hardy-Weinberg equilibrium is a common phenomenon in fish populations, which may be caused by inbreeding, non-random sampling, subgroup structure, genetic drift, overfishing, the Wahlund effect, and invalid alleles (Bergh and Getz, 1989; Castric et al., 2002; Lu et al., 2017; Song et al., 2018). The main reason for

the null allele of microsatellite is that the mutation of the flanking sequence of microsatellite results in the abnormal amplification of the gene (Primmer et al., 1995). The generation of null alleles has a great influence on the parameters related to population genetic diversity, resulting in "pseudohomozygote" that causes excessive homozygotes and significantly reduces observed heterozygosity and expected heterozygosity (Paetkau et al., 1995). Five loci (BW20, BW34, BW44, BW74, and BW129) showed evidence of null alleles (null alleles frequency>5%) in this study which confirms the above hypothesis. As a dominant species, *A. ommaturus* may suffer from increasing fishing pressure (Gu et al., 2013; Han et al., 2013). Therefore, null alleles and overfishing may cause most sites to deviate from Hardy-Weinberg equilibrium.

Biodiversity-rich organisms can better adapt to various environments, and the analysis of genetic diversity is the basis for the conservation of germplasm resources (Lu et al., 2017). The parameters of genetic diversity obtained in this study showed higher genetic diversity as a whole. The polymorphic information content is an important indicator for evaluating the degree of genetic variation of the population. When PIC>0.5, the locus showed a high degree of polymorphism (Botstein et al., 1980). The polymorphism information content ranged from 0.696 to 0.919, all loci were showed highly polymorphic. Allele heterozygosity within population can reflect the genetic variation level of population (Leberg, 2002). The high proportion of heterozygotes in the population reflects a stable genetic structure. Generally speaking, expected heterozygosity is more accurate than observed heterozygosity in measuring population genetic diversity (Nei, 1978). When the value of heterozygosity is between 0.5 and 0.8, it can be considered that the population has high diversity (Takezaki and Nei, 1996).The average expected heterozygosity (H_e) was 0.880, which showed higher genetic diversity. The results show that the markers selected in this study had abundant polymorphism and could be used as effective genetic markers for the analysis of genetic diversity and phylogenetic relationships among populations of *A. ommaturus*.

Song et al. (2010a, b) carried out some researches on genetic diversity level and population genetic structure of *A. ommaturus* in Northwest Pacific Ocean by using mitochondrial DNA and AFLP. Mitochondrial DNA markers detected obvious geographic distance isolation patterns, while AFLP markers only detected

weak genetic differentiation among populations. There is currently no report on population genetics of *A. ommaturus* based on the description of microsatellite markers, and then the new developed 24 polymorphic primer pairs in the present study could be used to analyze the population genetic structure and evaluate the future genetic variation of *A. ommaturus*.

5 CONCLUSION

This is the first attempt to analyze the characteristics of *A. ommaturus* microsatellites using high-throughput sequencing, and the results provided an effective basis for our subsequent development of microsatellites primer pairs. The polymorphism primers obtained in this study will provide effectively basis for the comparison and analysis of the genetic structure and genetic characteristics of *A. ommaturus* in the future.

6 DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

7 ACKNOWLEDGMENT

8 AUTHOR DECLARATION

The authors declare that they have no conflict of interest.

The procedures implemented in this study follow all applicable international, national, and institutional guidelines on the research involved in human participants and/or animals.

Ethical approval was not required for this study because no endangered animal was involved. All handling of *A. ommaturus* specimens was conducted in strict accordance with Animal Care Quality Assurance in China.

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