Development and characterization of the microsatellite markers for the Neptune whelk, *Neptunea cumingii*

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Short Report

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Abstract

Background

The Neptune whelk, *Neptunea cumingii*, is an economic species endemic to the East Asia Warm Temperate Biotic Subregion. The development of microsatellite markers will be beneficial to the assessment of genetic diversity and conservation of resources on this decreasing gastropod.

Methods and Results

The microsatellite markers were developed and characterized through Illumina high-throughput sequencing and capillary electrophoresis techniques. 11 polymorphic microsatellite loci were screened and validated. The observed heterozygosity and expected heterozygosity of each locus ranged from 0.0600 to 0.6508 and from 0.7380 to 0.9375, respectively. The average Shannon's information index, polymorphism information content, F-Statistics and gene flow were 2.0828, 0.8325, 0.0519 and 4.5660, respectively. The genetic diversity analysis indicated a closer genetic distance between populations of Dalian and Lvshun.

Conclusions

The distribution pattern of polymorphic microsatellite loci reveals that the Yellow Sea Warm Current generates the high level of genetic connectivity in *N. cumingii*. The markers developed in this study are expected as informative for further analysis of genetic diversity in *N. cumingii*.

Introduction

The Neptune whelk, *Neptunea cumingii*, is a large gastropod distributed regionally within the areas of the Yellow Sea warm current and the Tsushima warm current [1, 2, 3]. As an economically important species, the muscle of *N. cumingii* is rich in essential amino acids and unsaturated fatty acids and valued by consumers [4], despite food poisoning case of tetramine has been reported [2]. With adult whelk mainly in gravel and sand bottom, the bottom trawlers have been widely used to catch *N. cumingii*, resulting in a sharp decrease and threatened status of wild resources [3]. Consequently, to relieve stress of overfishing and protect the wild resources, research on *N. cumingii* has been carried out, mainly focused on the feeding habits [5], the reproductive biology [1, 6, 7] and artificial breeding [8].

Genetic diversity is the scientific basis for germplasm resource protection [9]. Sui (2008) selected simple sequence repeats from close-relative gastropods and verified them in *N. cumingii* [10]. Later, short gene fragments, *COX1* and *CYTB*, were used to probe into genetic diversity of six geographic groups of *N. cumingii* in China, with no significant differences among populations detected [11]. Besides, polymorphic microsatellite loci were isolated from a congeneric whelk, *N. arthritica* [12]. Here in this study, we developed and characterized the microsatellite markers for *N. cumingii* using high-throughput sequencing and capillary electrophoresis techniques, aiming to assist in population genetic studies on this increasingly scarce gastropod.

Materials And Methods

Development of microsatellite markers

To construct the genomic library, nine individuals of *N. cumingii* from seas of Dalian (DL), Lvshun (LS) and Weihai (WH) were selected, with three individuals from each sampling site (Fig. 1) [13]. Genomic DNA was extracted from the cryopreserved foot muscles using the Tiangen DNA kit (DP324, Tiangen Biotech (Beijing) Co., Ltd., Beijing) following the manufacturer's instructions. The genomic DNA was then fragmented into ~ 400 bp to develop the genomic library. Eight probes, including p(AG)₁₀, p(AC)₁₀, p(AAC)₈, p(AGG)₈, p(ACG)₈, p(AAG)₈, p(ATCT)₆, as well as p(ACAT)₆, were adopted in capturing microsatellites from the library by the magnetic beads enriched method. The enriched genomic library was sequenced on the Illumina NovaSeq platform (Illumina, San Diego, CA, USA) at Shanghai Personalbio Biotechnology Co., Ltd using 2×150 bp paired-end sequencing mode. Raw reads were filtered by removing reads of which the length are ≤ 50 bp, or of low quality, or with 3' adaptors using AdapterRemoval 2.1.7 [14], and combined by FLASH 1.2.11 [15]. Microsatellites were calculated by motif types. The loci were then searched from all combined sequences by an online Microsatellite identification tool at <http://pgrc.ipk-gatersleben.de/misa/>. After deleting the microsatellites with flanking sequences < 20 bp, CDHIT software was used to cluster the remaining sequences. The SSR length polymorphism (SSLP) of each cluster was calculated by Perl program. Microsatellite markers for *N. cumingii* were developed from clusters with SSLP ≥ 2 and designed using Primer3 v2.3.6 [16]. The primer pairs were then screened by the following criterions: a) no motif types were the mononucleotide repeat motifs or compound repeat motifs, b) only one SSR in each read, c) the primer pairs should be within one cluster, d) each designed primer had to be supported by two primers in the clusters, and e) exact same primers were removed.

Screening And Characterization Of Microsatellite Markers

To check the amplified fragment length and specific amplification, 50 primer pairs were selected randomly. The PCR mixture contained 0.5 µL each primer, 1.0 µL template DNA, 10 µL 2 × Taq PCR MasterMix (PC1120, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), and 8 µL H₂O in a 20µL reaction system. Fragments were amplified using the following protocol: initial denature at 95°C for 5 min, 35 cycles of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 7 min. The agarose gel electrophoresis was used here to detect the PCR products. Primer pairs with single, bright bands agreeing with expected fragment sizes were selected to be further tested.

Genetic Diversity Analysis Of Microsatellite Markers

11 pairs of primers with high quality were selected (Table 1) and applied in three populations from Dalian (40 individuals), Lvshun (20 individuals) and Weihai (27 individuals). Genomic DNA was extracted as describe above and amplified with fluorescent labeled polymorphic microsatellite markers. The PCR mixture contained 1.0 µL each primer, 1.0 µL template DNA, 2.0 µL 10×buffer, 0.5 µL dNTP, 0.5 µL Taq DNA

Polymerase and 14 μL H_2O in a 20 μL reaction system. Fragments were amplified using the protocol described above with corresponding annealing temperature. The products were purified and capillary electrophoresis [17] was conducted on ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Table 1
Characteristics of eleven microsatellite loci from *N. cumingii* developed in this study

Locus	Primer sequence (5'-3')	Repeat motif	Size range (bp)	Ta (°C)	Fluorescence labeling
NC01	F: AAAACCCTTTGATGTTTCCAG R: TGCTGTCCAGCTTGGTTATG	(AAG) _n	141–162	59	HEX
NC02	F: CCTCGACCCAGATGCTATTG R: TTTTTCGGAAGAGTAGGCCA	(TCT) _n	269–299	60	FAM
NC03	F: TGGAGGAACGGGTGTCTAAT R: TTTACATCCCTCACCCGCTA	(TGTA) _n	202–234	60	FAM
NC04	F: CCCAATTTTCTTTCCCTTTCTT R: GCCTTTTTTCGGAATAAACCA	(AG) _n	163–189	59	HEX
NC05	F: TGGCAACTTGGATCAGTTCTC R: CACTTGATTGAGTCCTCGCA	(AG) _n	200–224	60	FAM
NC06	F: TCCACTGGCCCTGTATCTCT R: CATCCATCTGTCTATCTCTATCCA	(AGAT) _n	260–300	60	HEX
NC07	F: GTAGCTGTTGACCCAAACCC R: GAATGGACCAAAGCCAAAGA	(AAG) _n	272–302	60	TAMRA
NC08	F: CCTTCTTCGTCTTGTTCTTGTTT R: TTCATTTTGTTTCGTCAACCA	(TTC) _n	356–385	59	FAM
NC09	F: ATCCGCTCCCAAAGTTAGGT R: ACTGTTTCCCCAGCATGAGT	(AAG) _n	192 – 143	59	HEX
NC10	F: CCCTTGTTCTTCACCAAAAATC R: TGGTGCATAATGTGCTTTCTC	(AG) _n	218–230	60	TAMRA
NC11	F: TGAAGATATCTCCACACTTGG R: CTTGTTCCGCTTCCACTGTT	(AAG) _n	246–261	60	HEX

Results were analyzed by GeneMarker V2.2.0 to read the size and signal value of the fragments. The observed number of alleles (N_a), effective number of alleles (N_e), observed heterozygosity (H_o), expected heterozygosity (H_e), Shannon's information index (I) and F-Statistics (F_{st}) were evaluated by PopGene software version 1.31, as well as Nei's Unbiased measures of genetic identity and genetic distance among three populations [18]. The gene flow (N_m) was estimated following $N_m = 0.25(1 - F_{st}) / F_{st}$. And polymorphism information content (PIC) was calculated using the formula proposed by Bostein et al [19].

Results And Discussion

By Illumina high-throughput sequencing of nine individuals of *N. cumingii*, 8,776,062 raw reads were obtained with 1,316 million bp total bases. The average GC content was 41.86%. Totally, 7,339,720 high-quality reads were obtained, accounting for 83.63% raw reads. 2,497,115 combined pairs were assembled, from which a total of 30,31,455 microsatellites were identified. 851,479 combined pairs had more than one microsatellite. Most microsatellites were mononucleotide repeat motifs, accounting for 94.55% all microsatellites. 7875 microsatellites (4.50%) were dinucleotide repeat motifs, and 1135 (0.65%) and 347 (0.20%) were trinucleotide and tetranucleotide repeat motifs, respectively. The cluster analysis revealed 242,751 clusters in total, of which 51,467 clusters (21.20%) showed length polymorphism (≥ 2) across 9 individuals. Thereinto, 27,626 clusters (11.38%) had two lengths. After the primer pairs were filtered, 1297 primer pairs with SSLP from 2 to 4 were obtained.

A total of 11 primer pairs were validated in 87 individuals from three populations (Table 2). The N_a and N_e ranged from 6 to 13 and from 3.7478 to 14.4273, respectively. Both H_o and H_e were high, with means of 0.4355 and 0.8447 for each locus, respectively. The Shannon's information index values varied from 1.5955 to 2.4490. The PIC values of each locus varied from 0.7296 to 0.9301, with all greater than 0.5, suggesting that all the eleven microsatellite loci were highly polymorphic and valuable for genetic diversity studies [19]. The average F_{st} and N_m values were 0.0519 and 4.5660, indicating little genetic differentiation and abundant gene flow [20]. In addition, the maximum value (0.8332) and minimum value (0.6096) of Nei's genetic identity occurred between populations of Lvshun and Dalian and between Lvshun and Weihai, respectively, corresponding to the minimum and maximum genetic distance values of 0.1825 and 0.4949. The distribution pattern reveals that the Yellow Sea Warm Current is the drive that leads to the high level of genetic connectivity in *N. cumingii*. In conclusion, the 11 microsatellite markers developed and characterized in this study provide high level of genetics, and these markers will be useful for further analysis of genetic diversity in *N. cumingii*.

Table 2
Genetic diversity based on 11 microsatellite loci of *N. cumingii*

Locus	Sample size	<i>Na</i>	<i>Ne</i>	<i>Ho</i>	<i>He</i>	<i>I</i>	<i>PIC</i>	<i>Fst</i>	<i>Nm</i>
NC01	152	8	5.4516	0.5263	0.8220	1.8977	0.8113	0.0575	4.0993
NC02	154	9	3.7478	0.4545	0.7380	1.6868	0.7311	0.0522	4.5383
NC03	148	9	3.7909	0.3378	0.7412	1.7077	0.7296	0.0648	3.6100
NC04	126	13	10.2558	0.6508	0.9097	2.4490	0.9002	0.0449	5.3179
NC05	164	12	9.8089	0.5854	0.9036	2.3744	0.8968	0.0296	8.2066
NC06	114	11	7.3757	0.5088	0.8721	2.1666	0.8602	0.0302	8.0269
NC07	136	11	8.1337	0.3971	0.8836	2.2082	0.8733	0.0337	7.1696
NC08	132	11	9.2190	0.6364	0.8983	2.3018	0.8902	0.0404	5.9400
NC09	138	17	14.4273	0.3333	0.9375	2.7434	0.9301	0.0256	9.5205
NC10	100	7	5.2083	0.3000	0.8162	1.7794	0.7945	0.0657	3.5546
NC11	100	6	4.2017	0.0600	0.7697	1.5955	0.7403	0.1445	1.4805
Mean	133	10.3636	7.4201	0.4355	0.8447	2.0828	0.8325	0.0519	4.5660
<i>Na</i> : observed number of alleles, <i>Ne</i> : effective number of alleles, <i>Ho</i> : observed heterozygosity, <i>He</i> : expected heterozygosity, <i>I</i> : Shannon's information index,									
<i>PIC</i> : polymorphism information content, <i>Fst</i> : F-Statistics, <i>Nm</i> : gene flow.									

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

HW and TZ designed the experiment. Material preparation was performed by HL. Data collection and analysis were performed and the first draft of the manuscript was written by PM. All authors commented on previous versions of the manuscript and approved the final manuscript.

Ethics approval

This study did not require ethics approval.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent to publish

The authors consent to publish all the data in this study.

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Figures

Figure 1

A map of sampling sites of the Neptune whelk, *N. cumingii*, in this study. DL: Dalian, LS: Lvshun, WH: Weihai, YSWC: Yellow Sea Warm Current, BCC: Bohai Coastal Current, YSCC: Yellow Sea Coastal Current.