Pakistan J. Zool., vol. 51(2), pp 1-4, 2019. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2019.51.2.SC2

Short Communication

Utilizing Next-Generation Sequencing to Develop and Characterize Microsatellite Loci in Cuttlefish (*Sepia pharaonis*) and Cross-Amplification in Other Sepiidaes

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Article Information
Received 28 April 2017
Revised 27 July 2017
Accepted 22 December 2017
Available online 05 February 2019

Authors' Contributions
LL and MW designed the study and wrote this article. ZL and LG analyzed the sequence reads from S.pharaonis. CW, BG and LJ helped in sampling S.pharaonis.

Key words
***Sepia pharaonic*, Transcriptome sequencing, Microsatellites, Transferability.**

ABSTRACT

The cuttlefish *Sepia pharaonis*, known for its economic value, is distributed in the tropical coastal waters of the Indo-pacific region. In this study, we developed twenty-one microsatellite loci for *S. pharaonis* through next-generation sequencing technology. A total of 100 alleles were detected, and the number of alleles per loci ranged from 2 to 9. The observed and expected heterozygosities per loci ranged from 0.000 to 0.531 and from 0.031 to 0.751, respectively. Polymorphism information content (PIC) showed that six loci were highly informative (PIC > 0.5). Five loci (CL1142, CL1770, CL2683, CL3494, CL 3674) significantly deviated from the Hardy-Weinberg equilibrium after a Bonferroni correction ($P < 0.05$), and none of the loci showed linkage disequilibrium. In addition, these loci were cross-amplified in three closelyrelated species. Nineteen, fifteen, and thirteen loci were amplified in *Sepia lycidas*, *Sepia esculenta* and *Sepiella japonica*, respectively.

The cuttlefish *Sepia pharaonis*, known for its economic value, is distributed in the tropical coastal waters of the Indo-pacific region (Nabhitabhata and Nilaphat, 1999). Its characteristics include a large body type, fast growth rate and adaptability for high-density cultivation (Gabr *et al*., 1998). Over-exploitation and habitat degradation have led to a strong decline of its wild stocks since the 1980s. In recent years, stock enhancement programs have been initiated in China's coastal waters to address issues of wild population decline of this species (Domingues *et al*., 2001; Minton *et al*., 2001). To conserve and sustainably exploit this species, population genetic research is necessary. Microsatellite markers are widely used for a variety of applications in conservation and population genetics in many species because of their advantages, such as high intraspecific polymorphism, high reproducibility and co-dominant inheritance (Zhou *et al*., 2015; Brian *et al*., 2015).

Microsatellites, also called simple sequence repeats (SSRs), consist of short repeated DNA sequences of 1-6bp nucleotides and area abundant and randomly interspersed in eukaryotic genomes (Reid *et al*., 2007). The number of repeat units varied highly between individual caused the variability of the length of microsatellites (Weber and May, 1989). Microsatellites have proved to be useful markers in several genetic areas, including population genetics, evolution (Ren *et al*., 2015), paternity testing (Navarro *et al*., 2008), and genetic mapping (Ruan *et al*., 2010). However, the lack of available primers impeded the use of microsatellites for studying populations of endangered or non-model species (Yu *et al*., 2011). Screening primers in the past were time-consuming and costly (Wang *et al*., 2012). In recent years, these disadvantages have been overcome by the introduction of library enrichment and the emergence of next-generation sequencing technologies (Sahua *et al*., 2014; Du *et al*., 2017). The lack of sufficient microsatellite loci has limited studies on population genetic diversity, population structure and marker-assisted stock management. In this study, we developed twenty-one microsatellite loci in *S. pharaonis* using next-generation sequencing and investigated cross-amplification in closely related species, including *Sepia lycidas*, *Sepia esculenta* and *Sepiella japonica*.

Materials and methods

A total of 32 specimens of *S. pharaonis* were collected from Cangnan sea area (Fujian Province, China). Muscle tissues of *S. pharaonis* were obtained from each individual, preserved in 95% ethanol and stored at -20°C before DNA extraction. Total DNA was extracted from muscles using standard phenol–chloroform procedures (Sambrook *et al*., 1989).

An illumina-based RNA-Seq approach was used to characterize the novel microsatellite loci for *S. pharaonis*

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0030-9923/2019/0002-0001 $ 9.00/0

collected from the Cangnan Sea area in the The Beijing Genomics Institute (BGI, Shenzhen, China). Transcriptome contigs were obtained and screened for microsatellites using MISA (http://pgrc.ipk-gatersleben.de/misa/misa.html). Primers were designed for microsatellite loci using the program Primer3.0 (http://www.onlinedown.net/soft/51549.htm).

For SSR marker validation and population genetic analysis sixty primer pairs were arbitrarily chosen, synthesized and used to test for polymorphisms in 10 individuals. The PCR amplification was performed in a 2720 PCR machine (ABI, USA) and in a reaction mixture (10 µL) containing 2-10 ng DNA (0.5 µL), 0.5 µL of each forward and reverse primers, 5 µL 2×Es Taq MasterMix and 3.5 µL of double distilled water. PCR was performed as follows: 5 min at 95°C, 30-35 cycles of 30 s at 95 °C, 30 s at 55–61 °C, 40 s at 72 °C, and 10 min at 72 °C. PCR products were detected using capillary electrophoresis (BIOptic's Qsep100 dna-CE, Taiwan), and allele size was estimated using Q-Analyzer Software. Primers that amplified reproducible and score-able peaks of the expected size were further characterized using 32 wild-caught *S. pharaonis* individuals. The PCR products were genotyped using the method mentioned above.

To verify the transferability of the developed microsatellite loci from *S. pharaonis* three closely species of the sepiidae, we tested cross-amplification on the following three sepiidaes: *Sepia lycidas*, *Sepia esculenta,* and *Sepiella japonica*, with 10 individuals from each species.

The number of alleles (N_A), observed (H_O) heterozygosity and expected (H_E) heterozygosity were calculated using ARLEQUIN ver. 3.5.1.3 (Excoffier and Lischer, 2010). The polymorphic information content (PIC) was calculatedaccording to Botstein (1980). GENEPOP ver. 4.0.10 was used to examine conformation to Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium between all pairs of loci (Raymond and Rousset, 1995). Significance values were adjusted for multiple comparisons using Bonferroni corrections where necessary (Rice, 1989). Finally, all loci were assessed using MICRO-CHECKER to check for null alleles andscoring errors (Van-Oosterhout *et al*., 2004).

Results and discussion

In this study, we obtained approximately 98.12 nt bases from *S.pharaonis* the Illumina Hiseq 2000 platform. Morethan 56228 microsatellite loci with at least 4 repeats of mono-nucleotide to hexa-nucleotide motifs were detected (Table I). Among these microsatellites, mono-nucleotide motifs were the most frequent (51.65%), followed by di- (30.51%) and tri-nucleotides (15.56%). Quad-, penta- and hexa-nucleotide SSRs had a much lower frequency (1.98%, 0.22% and 0.08%, respectively) (Table I).

Table I.- Frequency of microsatellite motifs identified from *Sepia lycidas* genome.

Microsatellite motif type	Number of loci observed	Percentage (%)
Mono-nucleotide	29,041	51.65
Di-nucleotide	17153	30.51
Tri-nucleotide	8748	15.56
Quad-nucleotide	1,115	1.98
Penta-nucleotide	122	0.22
Hexa-nucleotide	49	0.08
Total	56,228	100

Of 56228 microsatellite loci, we randomly selected 60 microsatellite loci with polynucleotide-repeat types to test primer pairs. Out of 60 primers pairs examined, 21 microsatellite loci appeared to be polymorphic in the population of *S. pharaonis*. The characteristics of these loci are shown in Table II. The number of alleles per loci ranged from 2 to 9, with an average of 4.81. Observed heterozygosity ranged from 0.000 to 0.531 and expected heterozygosity from 0.031 to 0.751, with an average of 0.200 and 0.422, respectively. Five loci (CL1142, CL1770, CL2683, CL3494, CL3674) significantly departed from Hardy-Weinberg equilibrium after Bonferroni correction (P<0.05), perhaps because of population stratification, genotyping errors, or other confounding factors (Zintzaras and Lau, 2008). The polymorphic information content (PIC) values ranged from 0.030 to 0.717. Of these 21 loci, six loci were highly informative (PIC > 0.5), nine showed as mildly informative (0.25 < PIC < 0.5), and six were lowly informative (PIC < 0.25) (Table II). No significant evidence for null alleles or linkage disequilibrium was detected (P>0.05). The polymorphism rate of polynucleotide SSRs (35%) developed for *S. pharaonis* in this study was similar to that in *P. bengalensis* (34.3%; Eo *et al*., 2016), *Vriesea simplex* (33.3%; Neri *et al*., 2015) but higher than that in *Labeo rohita* (12.2%; Chhotaray *et al*., 2015) and *Artemia parthenogenetica* (11.5%; Nougué *et al*., 2015). This finding indicates that the polynucleotide-repeat microsatellites may also be powerful tools to study population structure and genetic diversity of *S.pharaonis*.

To examine these polymorphic microsatellite markers developed in *S.pharaonis* for utility with other species, cross-amplification of these microsatellite loci was tested on three other species (*S .lycidas*, *S. esculenta* and *S. japonica*) (Table III). The results showed that all 21 loci except CL9851 and UN11117 were effectively amplified, and 10 of 19 loci showed high polymorphisms in *S. lycidas*, indicating a higher transferability of these microsatellite markers in *S. lycidas* (Table III). Fifteen loci amplified and 4 of 15 loci showed polymorphisms in *S. esculenta*. Thirteen of 21 loci were cross-amplified in *S. japonica*, but all were monomorphic. As expected, cross-amplification levers were higher in *S. lycidas* and *S. esculenta* than

Table II.- Characterization of 26 polymorphic microsatellite loci isolated from *Sepia pharaonis.*

Locus	Primer sequence (5'-3')	Repeat motif	T_a (°C)	Size	Na	Ho	He	PHWE	PIC	Accession number
CL1142	F:GAGCACTCTGTATTGGTTTTTGG	TC	55	158-168	6	0.000	0.746	***	0.705	KX264431
	R:TGATTTCCATTTCCATGTTGAAT	(2*6)								
CL1684	F:AGAATCAAAGATCAAAAGCTGGC	TAA	55	111-139	7	0.323	0.387	0.427	0.370	KX264432
	R:AGAGAGAATGGTTTCAAGATCCC	(3*5)								
CL1770	F:AGAATCAAAGATCAAAAGCTGGC	TAA	55	160-178	5	0.094	0.686	***	0.623	KX264433
	R:TGTACTGCAAACAGTTGTTGGAT	(3*5)								
CL2553	F:TTTTCAATTATGCTTTTGATGGAA	AAC	55	142-158	4	0.375	0.377	0.557	0.335	KX264434
	R:TATATGGGGTTAGGGGAAACAGT	(3*6)								
CL2683	F:GCTCAAAATCTGTATGCAGGAAA	AC	55	172-178	4	0.000	0.488	***	0.417	KX264435
	R:ATTCCGGGTAAGCTGTACAAAGT	(2*7)								
CL2709	F:CACTTTCCACTATATCCCACACC	TC	55	144-162	5	0.219	0.456	0.104	0.389	KX264436
	R:CAGGCAAAATGAAATTTGAAAAC	(2*7)								
CL3025	F:CAGCTGACATTACCATCAAAACA	TTA	55	142-166	9	0.406	0.711	0.101	0.684	KX264437
	R:AGGGTAAGATGGGTAATCCTTGA	(3*5)								
CL3105	F:TTAACAAGGTTTGAAGATCACGC	CA	55	165-173	2	0.063	0.061	0.855	0.058	KX264438
	R:CTGAAAACTGTTCTGGTTTGCAT	(2*6)								
CL3494	F:GCCAAGTGATGATAGCTTAGTGG	AC	55	172-180	4	0.031	0.506	***	0.438	KX264439
	R:TTTTATAACTTTCCAGCACCCCT	(2*6)								
CL3674	F:ATAATGTCGCCACTAGTCTTCCA	CTG	55	145-154	4	0.031	0.708	***	0.651	KX264440
	R:GAAAAGAAAGACAGGAGGGAAAA	(3*6)								
CL4541	F:ATCTCTTCTGCAATGTTTCTTGG	TGT	55	169-181	5	0.219	0.347	0.093	0.323	KX264441
	R:AGAGAAAACAAATCTCTGGACCC	(3*7)								
CL4649	F:CGTCTTGGATTCATCTTCAAAAC	AAC	55	140-143	2	0.097	0.092	0.777	0.087	KX264442
	R:TGTCTACCCATTTCGATTTTGTT	(3*5)								
CL5671	F:CAAGAAACTTCAAAATCAGGGAA	TG	55	144-158	6	0.281	0.493	0.105	0.459	KX264443
	R:AAAGAAAGAGCTTTTGTTGGTCA	(2*7)								
CL6545	F:TGGAATTTGTCTACTGCAATCAA	AC	55	144-164	7	0.194	0.402	0.062	0.375	KX264444
	R:TGAAAGTCTTGTCACCCCTACAT	(2*7)								
CL9976	F:AGATCGGATAATGAGTCTGTGGA	GAG	55	160-184	9	0.469	0.751	0.166	0.717	KX264445
	R:CCATGAACTGTTGAAATGACAAA	(3*5)								
CL9851	F:AGCGACTGAATGGTGTATATTGG	CATCTT	55	160-166	2	0.031	0.031	0.928	0.030	KX264446
	R:AAATCCTGAAAGCAATCACTGAA	(6*4)								
UN10287	F:GTGACCCGACAACGAAAAATC	GCC	55	164-182	6	0.516	0.675	0.080	0.705	KX264447
	R:ATAGTTCTTTTTGCCGTCCATTT	(3*7)								
UN11117	F:TTTCTTTGCCTTCTCTTCCTCTT	TTTC	55	164-178	5	0.500	0.556	0.062	0.499	KX264448
	R:TCAATGTTTCCCTTATTGGAGAG	(4*6)								
UN12159	F:CGAGCAAGCAGAGGTAAATAACTT	AC	55	156-162	3	0.065	0.063	0.998	0.061	KX264449
	R:GCAAATTCCTCTCTTTACACTTGG	(2*8)								
UN13157	F:AATTTGCCTTCATCTTTCACCTT	TC	55	156-172	2	0.031	0.031	0.928	0.030	KX264450
	R:AGAGAACAGGCATCTATCTTCCC	(2*6)								
UN13552	F:CAACATCTTGAAAGGACACAACA	TG	55	160-166	3	0.258	0.228	0.884	0.203	KX264451
	R:ATGGTCTTCCTCCTCTCTTTCAC	(2*7)								

in *S. japonica* due to a closer phylogenetic relationship between these three species.

To the best of our knowledge, our study is the first to report the isolation of microsatellite markers in *S. pharaonis* using high-throughput sequencing technology and to test the cross-amplification in related species: *S. lycidas, S. esculenta* and *S. japonica*. These microsatellite loci will be powerful tools to study population structure and genetic diversity, which may provide new information to guide its conservation and management strategies for *S. pharaonis*. More importantly, most of them showed good applicability in three closely related species. The results indicated that the five loci had good transferability at the genus level.

Acknowledgements

This work was financially supported by Chinese National Natural Science Foundation (41406138), Natural Science Foundation of Zhejiang Province (LY130190001), the International Science and Technology Cooperation

Program of China (No. 2014DFT30120).

Table III.- Cross-amplification of developed microsatellite loci in three related species tested with 10 samples each.

Locus	*Sepia lycidas*	*Sepia esculenta*	*Sepiella japonica*
CL1142	+ (1)	+ (1)	–
CL1684	+ (1)	+ (1)	+ (1)
CL1770	+ (4)	+ (3)	+ (1)
CL2553	+ (1)	+ (1)	+ (1)
CL2683	+ (2)	+ (2)	+ (1)
CL2709	+ (1)	+ (1)	+ (1)
CL3025	+ (7)	+ (1)	+ (1)
CL3105	+ (2)	+ (1)	+ (1)
CL3494	+ (1)	–	–
CL3674	+ (2)	+ (2)	+ (1)
CL4541	+ (3)	+ (1)	+ (1)
CL4649	+ (2)	–	–
CL5671	+ (1)	+ (1)	+ (1)
CL6545	+ (1)	–	+ (1)
CL9976	+ (1)	–	–
CL9851	–	–	–
UN10287	+ (5)	+ (3)	+ (1)
UN11117	–	+ (1)	–
UN12159	+ (2)	–	–
UN13157	+ (2)	+ (1)	+ (1)
UN13552	+ (1)	+ (1)	–

+, amplified; –, no amplification; Numbers of alleles are indicated in brackets.

Statement of conflict of interest

Authors have declared no conflict of interest.

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