Conservation Genet Resour (2012) 4:479–481
DOI 10.1007/s12686-011-9579-0

TECHNICAL NOTE

Isolation and characterization of 20 microsatellite loci in *Neverita didyma* (Röding 1798)

Ning Li · Qi Li · Minyu Jin · Lingfeng Kong · Hong Yu

Received: 12 November 2011 / Accepted: 18 November 2011 / Published online: 26 November 2011

Abstract *Neverita didyma* is a commercially important and over-exploited species. In order to investigate its genetic diversity and population structure, 20 polymorphic microsatellite loci were isolated and characterized. The number of alleles per locus ranged from 2 to 20, and the observed and expected heterozygosities varied from 0.276 to 1.000 and from 0.331 to 0.939, respectively. These microsatellites will be a useful tool for fisheries management and conservation programme for this species.

Keywords *Neverita didyma* · Microsatellite · Genetic variation

Neverita didyma is widely distributed along the Indo-West Pacific region. Due to its high nutritive and economic value, *N. didyma* is one of the most commercially important resources in China, Japan and Korea. In recent years, however, the wild stocks of *N. didyma* have experienced dramatic population declines due to over-exploitation and the deterioration of coastal environment. The significant decline of *N. didyma* stocks not only alerts people to pay more attention to this species and its endangered situation, but also attracts concern on genetic variation and population structure. Although many studies of *N. didyma* have been carried out on morphology, and reproductive biology (Liu and Sun 2009), little is known about genetic diversity and structure. Microsatellite markers have codominant, multiallelic and highly polymorphic nature, so they have been widely adopted in population genetics and conservation of biological resources. For this reason, we first developed and characterized 20 polymorphic microsatellite loci in *N. didyma.*

Genomic DNA was isolated with a modified phenol–chloroform protocol (Li et al. 2006) from foot muscle of one live *N. didyma* caught from Qingdao, Shandong Province, China, and subsequently digested by *Mbo*I. DNA fragments were ligated to oligonucleotide adapters (Yuan et al. 2009). Size fractions of 300–1,000 bp were isolated and hybridized to Biotin-labeled dinucleotide repeat sequences $[(CA)_{15}]$ after electrophoresis on a 2% NuSieve GTG agarose gel. Then the hybridization complex was lifted out with streptavidin-coated magnetic spheres (Promega). After washing, the bound enriched DNA was eluted from the magnetic beads.

After amplified by PCR, the selected fragments were ligated to pMD19-T vector (Takara) and then transformed into *Escherichia coli* DH5α competent cells (TOYOBO). The white clones were picked out by blue/white screening, and screened with two vector primers and the non-biotin-labeled $(CA)_{12}$ primer (Yuan et al. 2009). Screening amplifications were performed as follows: 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 56°C for 45 s and 72°C for 45 s, then a final extension at 72°C for 5 min. A total of 360 clones were selected for amplification. The clones that generated two or more bands were chosen and sequenced using BigDye Terminator Cycle sequencing kit and an ABI PRISM 3730 Genetic Analyser (Applied Biosystems). Eighty sequences that contained microsatellites with at least four uninterrupted repeats were screened by the software of SSRHUNTER1.3 (Li and Wan 2005). After abandoning hybrid clones, duplicates and those with short unique regions flanking the microsatellite array, 52 sequences were found suitable for primer design.

PCR primers for each microsatellite locus were designed by using PRIMER 5 (http://www.premierbiosoft.com/),

N. Li · Q. Li (✉) · M. Jin · L. Kong · H. Yu
Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao 266003, China
e-mail: qili66@ouc.edu.cn

Table 1 Levels of variability at 20 polymorphic microsatellite loci in *Neverita didyma*

Locus	Genebank accession no.	Repeat motif	Primer sequence (5′-3′)	T_a (°C)	No. of alleles	Size range (bp)	H_o	H_E	*P*-value
Nd01	JN998361	$(GT)_{11}N_{10}(GT)_5N_{72}(GT)_5$	F:TTTGTGGACACTATCCTACAC R:TTAAGCCAGTCAACTTCATT	50	13	250–310	0.833	0.891	0.0572
Nd02	JN998362	$(CA)_{11}(CG)_4$	F:TGCGTTTATCTTCCACTCACAC R:AGCAACTAATGTCCCCTTTCAC	56	10	206–224	1.000	0.888	0.0182
Nd03	JN998363	$(CA)_5N_{144}(AC)_4$	F:CACTTGCGTCCGAGGCTG R:CCTTGAATGGCGCTTTGC	52	3	216–236	0.407	0.338	0.6453
Nd04	JN998364	$(TG)_7N_{10}(TG)_7N_6(GT)_4$	F:TACCCATTCCCCTTAGTTT R:ACGCACATACCAGCATCTC	42	7	224–244	1.000	0.721	0.0000*
Nd05	JN998365	$(TG)_5$	F:AGAAGCAAAATTGGTGCG R:TTCAGTTTAGGCTTTTGTATCTT	50	12	130–154	0.828	0.892	0.0316
Nd06	JN998366	$(GA)_7N_{21}(GT)_5$	F:ATGAGGAGCCCGTGATAG R:TCTCCCTTCCTATCTAGCTC	52	6	244–260	0.714	0.768	0.1976
Nd07	JN998367	$(AC)_6N_{18}(GC)_4N_{77}(AC)_4$	F:GTCTGAACGCAGGGATGGA R:TTCTCAATTCTCAAAAGGCAGC	48	7	166–194	0.600	0.673	0.0112
Nd08	JN998368	$(GA)_5N_{27}(GA)_4N_{19}(GT)_5$	F:TGGAGCGTGGCTGTTTAC R:TATCGTTCAGGGCTTTCG	48	4	146–152	0.276	0.621	0.0000*
Nd09	JN998369	$(GT)_4N_{20}(TG)_5$	F:AGAAGTGGGGTGGGGAGA R:ACTGTTGCCATCCGTGGT	50	7	186–204	0.379	0.753	0.0000*
Nd10	JN998370	$(CA)_4N_{77}(AC)_4$	F:CGTTTTGCTGTAAAGATT R:GGAGTAGAAGGTGGACTG	52	5	212–226	0.300	0.556	0.0000*
Nd11	JN998371	$(AC)_5N_{41}(AC)_4$	F:CTGCCTCAGTCATCTAACAC R:GAGCACGCTGAAGATTGTA	48	5	236–254	0.448	0.617	0.0093
Nd12	JN998372	$(CA)_{11}N_{22}(AC)_6$	F:TCAATCAGTCCGTTTCTCG R:GAAGCCGAGCACCACTAG	54	20	222–298	0.920	0.939	0.0000*
Nd13	JN998373	$(GT)_9(TG)_8$	F:TCATCCGAACGACAGACT R:CAGGCAGACCATGTATAGAAAC	50	18	264–382	0.778	0.906	0.0553
Nd14	JN998374	$(CA)_6$	F:AGCATCTTTATTGCCTTTG R:TTAAATGAGTAATAATATGTGCG	60	6	274–294	0.828	0.737	0.0000*
Nd15	JN998375	$(CA)_5AACT(AC)_4AA(AC)_4$	F:AAGTTCCCATCCACCTGC R:GATTTGCCTCCCCTTGAT	48	4	216–226	0.414	0.477	0.0404
Nd16	JN998376	$(TG)_4N_{88}(TG)_4$	F:GGTTGAGGACGGAAAGAA R:TGAACACGCCTTGAGATG	48	5	244–252	0.800	0.737	0.0452
Nd17	JN998377	$(GT)_4$	F:TATGTAGGAGCGTGTATGTGC R:CCCCATCCCTTAGACTTCA	50	2	250–260	0.407	0.331	0.5493
Nd18	JN998378	$(TG)_{14}N_{35}(GT)_4N_{20}(GT)_7$	F:GAACGGGTTACAAATGAGG R:GCCATGACGACCACAATAT	50	12	256–294	0.500	0.905	0.0000*
Nd19	JN998379	$(TG)_5N_{51}(GT)_5$	F:AACCGATACCGTTTCTTCCT R:TCTCACCAACCGTTTTATCTG	48	4	222–230	1.000	0.748	0.0000*
Nd20	JN998380	$(GA)_7N_{21}(GT)_5$	F:CGTTGAACAATGAAATGAG R:TCTCCCTTCCTATCTAGCTC	48	7	260–274	0.630	0.798	0.0108

T_a annealing temperature, H_o observed heterozygosity, H_E expected heterozygosity

* Indicates significant deviations from Hardy–Weinberg equilibrium after sequential Bonferroni correction ($P < 0.05/20$)

and tested on 30 natural individuals of *N. didyma* captured from Panjin, Liaoning Province, China. PCR reaction was performed in 10-μl volumes containing 0.25 U *Taq* DNA polymerase (Takara), 1× PCR buffer, 0.2 mM dNTP mix, 1 μM of each primer set, 1.5 mM $MgCl_2$ and about 100 ng template DNA. The conditions were performed as follows: 3 min at 94°C followed by 35 cycles of 45 s at 94°C, 45 s at the annealing temperature listed in Table 1 and 45 s at 72°C, then a final extension of 5 min at 72°C. The PCR products were resolved by 6% denaturing polyacrylamide gel and silver staining. A 10-bp DNA ladder (Invitrogen) was used as a reference marker for allele size determination. The number

of alleles and observed (H_O) and expected (H_E) heterozygosities were estimated by MICROSATELLITE ANALYSER software (Dieringer and Schlötterer 2003). Tests for linkage disequilibrium (LD) and deviations from Hardy–Weinberg equilibrium (HWE) were performed by GENEPOP 4.0 (Rousset 2008).

Among the 52 primer pairs developed, 20 microsatellite loci (38.5%) were found polymorphic (Table 1). The number of alleles per locus ranged from 2 to 20 with an average of 7.85, and the observed and expected heterozygosities ranged from 0.276 to 1.000 and from 0.331 to 0.939, with an average of 0.653 and 0.715, respectively. Tests for linkage disequilibrium showed a nonrandom association ($P < 0.01$) between two pairs of loci (Nd01-Nd02 and Nd06-Nd20). Eight loci (Nd04, Nd08, Nd09, Nd10, Nd12, Nd14, Nd18 and Nd19) deviated significantly from HWE after correction for multiple tests, which may be caused by the presence of null alleles (Pemberton et al. 1995). The high variabilities at these loci indicated that they would be a useful tool for fisheries management and conservation programme of *N. didyma*.

Acknowledgments The study was supported by the grants from National Marine Public Welfare Research Program (200905020), and National Natural Science Foundation of China (31072207).

References

Dieringer D, Schlötterer C (2003) Microsatellite analyser (MSA): a platform independent analysis tool for large microsatellite data sets. Mol Ecol Notes 3:167–169

Li Q, Wan JM (2005) SSRHUNTER: development of a local searching software for SSR sites. Hereditas 27:808–810

Li Q, Yu H, Yu RH (2006) Genetic variability assessed by microsatellites in cultured population of the Pacific oyster (*Crassostrea gigas*) in China. Aquaculture 259:95–102

Liu Q, Sun ZX (2009) Histology of gonad and reproductive cycle of *Neverita didyma*. Trans Oceanol Limnol 2:67–72

Pemberton JM, Slate J, Bancroft DR, Barrett JA (1995) Nonamplifying alleles at microsatellite loci: a caution for parentage and population studies. Mol Ecol 4:249–252

Rousset F (2008) GENEPOP'O007: a complete reimplementation of the GENEPOP software for Windows and Linux. Mol Ecol Resour 8:103–106

Yuan Y, Li Q, Kong L, Pan Y (2009) Isolation and characterization of microsatellite loci in the tuba false fusus (*Hemifusus tuba* Gmelin). Conserv Genet Resour 1:9–11