Molecular Ecology Notes (2005) **5**, 121–123 doi: 10.1111/j.1471-8286.2005.00857.x

PRIMER NOTE

Polymorphic microsatellites for the study of newly established populations of the gastropod *Cyclope neritea*

BENOÎT SIMON-BOUHET,*† CLAIRE DAGUIN,* PASCALE GARCIA-MEUNIER† and FRÉDÉRIQUE VIARD*

**Evolution et Génétique des Populations Marines, UMR 7127 CNRS, Station Biologique de Roscoff, B.P. 74, 29682 Roscoff cedex, France, †Laboratoire de Biologie et Environnement Marins, Institut de la Mer et du Littoral, Avenue Lazaret, Port des Minimes, 17000 La Rochelle, France*

Abstract

Both human-mediated introductions and climatic changes may promote the settlement of species in new areas outside of their natural geographical range. To investigate the settlement of recently established populations of the neogastropod *Cyclope neritea*, we developed eight microsatellite markers. Their usefulness was studied in two native populations previously found to be monomorphic with mitochondrial markers. The eight loci were found to be polymorphic in both populations, with two to 18 alleles per locus. This result shows promise for these loci in studies of recently founded populations of *C. neritea*.

Keywords: biological invasion, *Cyclope neritea*, marine gastropod, microsatellite

Received 30 September 2004; revision accepted 15 October 2004

Over the last few decades, the release of alien marine species has greatly increased (Occhipinti-Ambrogi & Savini 2003). For species found in new areas close to their native range, both human-mediated introductions and natural spread may explain the range expansion (Dukes & Mooney 1999). The origin of recently settled populations of the marine neogastropod *Cyclope neritea* along the French Atlantic coasts is questionable (for a detailed description of its colonization pattern, see Bachelet *et al*. 2004). Two processes could be involved in the settlement of these new populations: (i) a natural spread towards north favoured by environmental changes and/or (ii) a human introduction from distant native populations, related to the release of marine organisms for aquaculture. We aim to investigate fine scale mechanisms involved in the early stages of the settlement of the new *C. neritea* populations in contrast with populations from the recognized native range. We developed eight microsatellite markers and tested their usefulness by analysing two native populations.

Genomic DNA from three individuals from the Thau lagoon (French Mediterranean Sea) was isolated from < 15 mg of foot muscle using DNeasy Tissue Kit (QIAGEN). A genomic library enriched for CA repeated microsatellites was constructed following the protocol described by Billote *et al*. (1999) based on Kijas *et al*. (1994). Briefly, total DNA was digested with *Rsa*I (Promega). After purification on Nucleospin Column (Macherey-Nagel), 1 μg of digested DNA was ligated to *Rsa*I adapters (Rsa21F: 5′-CTCTTGCTTACGCGTGGACTA-3′ and Rsa25R: 5′-TAGTCCACGCGTAAGCAAGAGCACA-3′). For the enrichment procedure, hybridization of DNA fragments to biotinylated $(AC)_{10}$ probes attached to streptavidin-coated magnetic beads (Streptavidin MagneSphere® Paramagnetic Particles; Promega) was carried out. The purified enriched fraction was ligated into pGEM-T easy (Promega) and transformed into *Escherichia coli* JM109 competent cells. Recombinants with appropriate insert sizes were determined by polymerase chain reaction (PCR) (350–800 base pairs; 288 clones), transferred to positively charged nylon membranes and screened by hybridization with digoxigenin-labelled DIG-$(TG)_{10}$ probes. From a total of 144 positive clones sequenced on a 3100 Genetic Analyser using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems), 36 primer pairs were designed using PRIMER 3 software (Rozen & Skaletsky 2000). After preliminary tests for amplification on agarose gels, 24 forward primers were fluorescently labelled with infra-red fluorescent dye IRD700 or IRD800 for screening on a Li-Cor NEN Global IR2 DNA sequencer. The genomic DNA for genotyping was obtained using DNeasy Tissue Kit (QIAGEN). Amplification conditions were optimized for eight loci (Table 1) for

Correspondence: Benoît Simon-Bouhet, Fax: +335 4650 0294; E-mail: bsimonbo@univ-ir.fr

Table 1 Characteristics of the eight microsatellite loci isolated from *Cyclope neritea* (GenBank Accession nos: AY771365–AY771372). T_i and T_a stand for the initial and optimal annealing temperature of the touch-down PCR procedure; Cycle 1 refers to the number of cycles used to reach the optimal annealing temperature whereas Cycle 2 refers to the number of cycles used at the optimal annealing temperature. N_{Tall} and H_T: number of alleles and total heterozygosity over the whole sample ($N = 48$), N_{all} and H_E: number of alleles and expected heterozygosity at the population level, F_{IS}: estimator for deviation to Hardy–Weinberg proportions and associated *P*-values (exact test)

									Thau ($N = 24$)			Faro ($N = 24$)		
Locus	Core sequence	Primer sequences (5′–3′)	T_i-T_a (°C) (Cycle 1)	Cycle 2	$MgCl_2$ (mM)	Allele size range (bp)	N_{Tall}	H_T	N_{all}	H_E	F_{IS}	N_{all}	H_E	F_{IS}
Cn-2C4	$(GT)_{15}$	F: ATCGAGCAGAAAAGACATGAC	65–58	24	1.5	151–172	5	0.66	5	0.61	0.106	2	0.51	0.269
		R: GAAAGTGTGACCCCGTGAG	(8)								($P = 0.305$)			($P = 0.237$)
Cn-2H10b	$(GA)_{16}GG(GA)_5G$ $GAGAGA(ACTG)_4$	F: CCACGGTGGTCCCTACTT	60–52	24	1.5	118–193	9	0.83	6	0.73	0.033	6	0.80	0.134
		R: AATGAGCGGTTAATCTGACAA	(8)								($P = 0.096$)			($P = 0.449$)
Cn-3C4	$(TGTC)_9(TGGC)_2$ $(TGTC)_6$	F: TCTCCCAATCCCAGTATAAAG	58–50	32	2.5	206–239	6	0.72	5	0.69	0.091	4	0.58	−0.247
		R: AACCCAACTTGCCAACTATG	(8)								($P = 0.323$)			($P = 0.787$)
Cn-1H8	$(GT)_{28}$	F: CCTTCTTGGTGCTGTGTTTCTG	62–52	25	1.5	130–142	5	0.47	4	0.43	0.609	3	0.52	0.666
		R: GGAGTAGGTAGAGTTAGTGGAATGTC	(10)								($P = 0.000$)			($P = 0.000$)
Cn-1A10	$(TG)_{10}(AG)_{15}GGG$ $(AG)_{21}(TG)_3$	F: GCGTTACAGGCAAATGC	60–52	24	1.5	152–224	15	0.89	13	0.90	0.246	6	0.74	0.084
		R: CAGAATCCGTGCTTCAGC	(8)								($P = 0.079$)			($P = 0.451$)
Cn-1A8	$(GA)_{36}N_{40}(GT)_3A$ $(TG)_6N_{15}(GT)_4$	F: GACACTGCCCAGAACAGTC	64–54	25	2.5	237–324	18	0.93	16	0.93	0.460	3	0.61	0.702
		R: GAAGTAAACAGTGGAAATAATAATAC	(10)								($P = 0.000$)			($P = 0.006$)
Cn-3B2	$(GT)_{12}GC(GT)_2$ $GC(GT)_3$	F: CTACCACACACCGATGTCCAG	65–55	24	1.5	172–190	4	0.71	3	0.60	0.665	2	0.26	0.324
		R: CACCTATGTTGCGTGCAGC	(10)								($P = 0.001$)			($P = 0.288$)
Cn-2B7	$(CA)_7$	F: CAGTGGCAAGCTGCTTTAGATC	63–55	22	2.5	176–178	2	0.42	2	0.23	0.254	2	0.51	−0.154
		R: CAACAACGACAAAGGCAAAGC	(8)								($P = 0.310$)			($P = 0.680$)
Overall (SD)										0.64 (0.23)	0.290 ($P = 0.000$)		0.57 (0.17)	0.171 ($P = 0.005$)

which preliminary tests showed unambiguous patterns. Each reaction was performed in 20 μL and contained approximately 50 ng of DNA, 0.05 μM of fluorescently labelled forward primer, 0.075 μM of unlabelled forward primer, 0.125 μM of unlabelled reverse primer, 1.5 or 2.5 μM of $MgCl_2$ depending on the locus (Table 1), 0.25 mM of each dNTP, 0.1 g/L of bovine serum albumin, 1X PCR buffer [75 mM Tris-Hcl pH 8.8 at 25 °C, 20 mM $(NH_4)_2SO_4$ and 0.01% Tween 20] and 0.50 U Thermoprime Plus DNA Polymerase (ABgene), using a MJ Research PTC-200 thermocycler (MJ Research Inc.). Thermal cycling conditions followed a locus-specific touch-down PCR procedure: 3 min of initial denaturation step at 95 °C followed by cycles (Cycle 1 in Table 1) of denaturation at 95 °C for 45 s, annealing at temperature decreasing by 1 °C per cycle from T_i to T_a (Table 1) for 45 s and extension at 72 °C for 45 s, then followed by a locus-specific number of cycles (Cycle 2 in Table 1) at 95 °C for 45 s, annealing at final T_a (Table 1) for 45 s and extension at 72 °C for 45 s. A final extension step was carried out for 7 min at 72 °C. PCR products were screened on a 6.5% polyacrylamide gel using a Li-Cor NEN Global IR2 DNA sequencer system. Allele sizes were determined using a known DNA sequence.

Polymorphism at the eight loci was tested in two populations of *C. neritea* from its native range [Thau (French Mediterranean Sea), $N = 24$ and Faro (south Portugal, Atlantic), $N = 24$] that were found to be monomorphic when sequencing a mitochondrial gene fragment (Cytochrome Oxidase I, Bachelet *et al*. 2004). No linkage disequilibria across loci were detected using GENEPOP version 3.4 (Raymond & Rousset 1995). This software was also used to estimate the number of alleles, the expected heterozygosities and adequacy of genotypic proportions to Hardy–Weinberg expectations by an approximate exact test (detailed in Rousset & Raymond 1995; Table 1). Over the 48 individuals analysed, the number of alleles ranged from two to 18 and expected heterozygosities from 0.42 to 0.93, respectively. At the population level, deviations from Hardy–Weinberg equilibrium were variable across loci and populations. For instance, Cn-1A8 showed a large heterozygote deficiency only in Faro that might indicate the presence of null alleles as a result of a phylogeographical break in the species' native range (Bachelet *et al*. 2004). A larger number of individuals and populations should thus be examined to better assess the reliability of this locus. Nonetheless, both populations were found to be polymorphic [e.g. mean number of 6.75 (SE 5.01) and 3.50 (SE 1.69) alleles in Thau and Faro, respectively] and exhibited similar levels of expected heterozygosity. The microsatellite loci developed in the present study are thus considerably more polymorphic than the mitochondrial marker previously used and are of interest to study fine-scale processes involved in the settlement of new populations of *C. neritea*.

Acknowledgements

The authors are grateful to Carolyn Engel for improvements of the manuscript. This project is part of the INVABIO program of the Ministère de l'Ecologie et du Développement Durable (project n°D4E/SRP/01115). F.V. acknowledges support from the European Network of Excellence 'Marine Genomics' (contract n°505403) and the CNRS (ATIP program). This project also benefited from the facilities of the Genomer and Ouest-Genopole sequencing platform. B.S.-B. was supported by a PhD grant from the Ministère de l'Education et de la Recherche.

References

Bachelet G, Simon-Bouhet B, Desclaux C *et al*. (2004) Invasion of the eastern Bay of Biscay by the nassariid gastropod *Cyclope neritea*: origin and effects on the resident fauna. *Marine Ecology Progress Series*, **276**, 147–159.

Billote N, Lagoda P, Risterucci AM, Baurens FC (1999) Microsatellite-enriched libraries: applied methodology for the tech development of SSR markers in tropical crops. *Fruits*, **54**, 277–288.

Dukes JS, Mooney HA (1999) Does global change increase the success of biological invaders? *Trends in Ecology and Evolution*, **14**, 135–139.

Kijas JMH, Fowler JCS, Garbett CA, Thomas MR (1994) Enrichment of microsatellites from the Citrus genome using biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles. *Biotechniques*, **16**, 657–662.

Occhipinti-Ambrogi A, Savini D (2003) Biological invasions as a component of global change in stressed marine ecosystems. *Marine Pollution Bulletin*, **46**, 542–551.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): a population genetics software for exact tests and ecumenicism. *The Journal of Heredity*, **86**, 248–249.

Rousset F, Raymond M (1995) Testing heterozygote excess and deficiency. *Genetics*, **140**, 1413–1419.

Rozen S, Skaletsky H (2000) PRIMER 3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, New Jersey.