Molecular Ecology Notes (2004) **4**, 125–126 doi: 10.1111/j.1471-8286.2004.00591.x

PRIMER NOTE

Microsatellite markers for the cosmopolitan marine diatom *Pseudo-nitzschia pungens*

KATHARINE M. EVANS and PAUL K. HAYES
School of Biological Sciences, University of Bristol, Woodland Road, Bristol BS8 1UG, UK

Abstract

Very little information is available on the genetic structure of populations of phytoplankton species. Here we report the characterization of six polymorphic microsatellite loci in the marine diatom, *Pseudo-nitzschia pungens*, using clones (between 87 and 213 per locus) isolated from spring blooms in the North Sea. The number of alleles per locus ranged from five to 19 and observed heterozygosities ranged from 0.55 to 0.86.

Keywords: algae, diatom, microsatellites, *Pseudo-nitzschia*

Received 6 October 2003; revision received 30 October 2003; accepted 12 December 2003

Although phytoplankton form the base of the food web in marine ecosystems and are implicated in many of the biogeochemical reactions that drive climate change, remarkably little is known about their population biology. In particular, only a few studies have investigated the genetic structures and genetic diversities of their populations (e.g. Gallagher 1980; Skov *et al*. 1997; Rynearson & Armbrust 2000; Hayes *et al*. 2002). Of these, only the study by Rynearson & Armbrust (2000) used microsatellite markers. By developing microsatellite markers for the marine diatom *Pseudo-nitzschia pungens*, we aim to gain further insight into the genetic structure of phytoplankton populations, over both temporal and spatial scales.

Pseudo-nitzschia is a marine, planktonic genus whose members are found in seas worldwide. Interest in this genus has increased since 1987, when a bloom of *P. multiseries* (the closest relative to *P. pungens*) caused an amnesic shellfish poisoning event which led to the death of three people in Canada (Bates *et al*. 1989). *Pseudo-nitzschia pungens*, however, is nontoxic and forms an important component of the annual spring phytoplankton blooms in European waters.

Genomic DNA was extracted from approximately 50 mL of exponentially growing clonal cultures established from Helgoland waters (North Sea) in the spring of 2002 and 2003, following the protocol described in Rynearson & Armbrust (2000). Extracted DNA from a single clone was either singly or doubly enriched for CA and CT repeats following a protocol based on Edwards *et al*. (1996). In brief, the DNA was digested with *Alu*I, ligated to a *Mlu*I adapter, amplified using one of the adapter oligonucleotides and hybridized to biotinylated microsatellite oligonucleotides immobilized on Dynabeads (Dynal Biotech, Inc.). Rinsed, captured DNA was re-amplified as above and cloned into the Original TA cloning system (Invitrogen); double enrichment was achieved by re-capturing post-Dynabead amplification products and by repeating all subsequent steps as above. Cloned DNA fragments were amplified using M13 forward and reverse primers. Sequencing was performed using a sequencing kit (Amersham Biosciences) and carried out on a MegaBACE 1000 sequencer. Sequences containing suitable microsatellites were exported to the PRIMER3 software package (Rozen & Skaletsky 2000).

Of the initial 24 clones sequenced from each library, the singly enriched CA library yielded three microsatellites and the doubly enriched CA library yielded 11, an enrichment of approximately 45%. Neither the singly nor the doubly enriched CT libraries yielded microsatellites. Consequently, further sequencing was only performed on additional clones from the doubly enriched CA library. In total, 415 clones were sequenced, of which 180 (43%) contained microsatellites. Primer pairs were designed to flank 26 cloned CA repeats and their ability to amplify the corresponding microsatellite loci tested using a PTC-200 gradient thermal cycler (M J Research). The 20-μL reactions contained 1 μL of template DNA, 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton®X-100, 2 mM $MgCl_2$, 0.2 mM dNTPs, 75 ng of each primer and 0.5 U of *Taq* polymerase (Promega).

Correspondence: P. K. Hayes. Fax: + 44 (0)117 9257374; E-mail: Paul.Hayes@bristol.ac.uk

Table 1 Attributes of six microsatellite loci for a North Sea isolate of *Pseudo-nitzschia pungens*

Locus	*n*	Core sequence*	Primer sequences (5′–3′)	T_a	bp	N_A	H_O	H_E
PP1 (AY423902)	185	$(GT)_{11}GGAAA(GA)_{10}$	F: CGTCTGTGGAACGAAAATTG R: GTGTCTCTACCGATCGCACA	58	221–250	11	0.67	0.76
PP2 (AY423903)	144	$((GT)_3T)_9N_{16}(GATTGAT)_5 (GAATGAT)_4$	F: TCCAGACTGGTCCTGCTACC R: CCTGTACGGTATGTGCTCGAA	60	171–251	9	0.66	0.65
PP3 (AY423904)	151	$(GT)_4N_{13}(GT)_4N_{37}(GATTT)_7$	F: ACTGCGTTTGCTCTTTCGAG R: GCCAGCAAATGGAACAACTT	58	195–266	19	0.83	0.86
PP4 (AY423905)	87	$(GT)_{12}GGAT(GT)_3GGAT (GTAT)_2(GT)_2AT(GT)_4 (ATGT)_2(GT)_3(ATGT)_2(GT)_9$	F: CGTATCGTGTATCGCATTGTG R: ATGCTGTGACAAACGTTCCA	55	136–207	14	0.86	0.85
PP5 (AY423906)	167	$(GT)_2AT(GT)_8AT(GT)_4$	F: GGGTAGTGCTTCGGTTCCT R: AGAACTCGCAAATCCGACTG	55	189–202	6	0.55	0.58
PP6 (AY423907)	213	$(G(CA)_3C)_2GC(AC)_2 (G(CA)_3C)_2AC(G(CA)_7C)_2$	F: GAGGCGCAACAGTCGTAAAG R: AATCCCGTGCGTGTAGTTTC	55	199–241	5	0.65	0.67

*N indicates bases (usually T or G) that are within the core but that do not form part of a repeat unit.
The number of individuals tested (*n*), annealing temperature (T_a, °C), allele size range (bp), number of alleles (N_A) and observed (H_O) and expected (H_E) heterozygosities are given. GenBank Accession nos are given in parentheses below each locus.

After 3 min at 94 °C, 35 cycles were performed: 1 min at 94 °C, 1 min at 48–65 °C, 30 s at 72 °C and a final extension step at 72 °C for 10 min. For the 13 primer pairs that generated amplification products of the expected size, 5′-fluorescently labelled forward primers (6-FAM or HEX) were used in repeat amplification reactions using a number of *Pseudo-nitzschia* isolates and the products were sized using a MegaBACE 1000. Of these 13 primer pairs, six amplified scorable polymorphic microsatellite loci; primer pairs for these six loci have been further tested on between 87 and 213 clonal isolates (Table 1). The core sequences, primer sequences, optimal annealing temperatures, size range of the alleles, number of alleles observed and heterozygosity values for these loci are listed in Table 1.

The observed number of alleles per locus ranged from five to 19 and the observed heterozygosities from 0.55 to 0.86 (Table 1), suggesting a high degree of diversity within the North Sea *P. pungens* population. GENEPOP 3.3 (Raymond & Rousset 1995) was used to detect deviations from Hardy–Weinberg equilibrium at each locus and to test for linkage disequilibrium between pairs of loci. A significant ($P < 0.05$) deficit of heterozygotes was observed only at locus PP1; none of the six loci were in highly significant linkage disequilibrium ($P > 0.001$).

So far only the primers for loci PP1 and PP6 have been tested for cross-reactivity with congeners *P. multiseries* (four clonal isolates) and *P. seriata* (two clonal isolates). Cross-reactivity was observed only when the primer pair for locus PP6 was used to amplify DNA from the four *P. multiseries* isolates; one allele distinct from those in *P. pungens* was observed.

Acknowledgements

We thank Stefanie Kühn for isolating clones of *P. pungens*, Barbie Handley for help with genotyping, Keith Edwards and Colin Lazarus for technical advice and the NERC for financial support.

References

Bates SS, Bird CJ, de Freitas ASW *et al.* (1989) Pennate diatom *Nitzscha pungens* as the primary source of domoic acid, a toxin in shellfish from eastern Prince Edward Island, Canada. *Canadian Journal of Fisheries and Aquatic Sciences*, **46**, 1203–1215.

Edwards KJ, Barker JHA, Daly A, Jones C, Karp A (1996) Microsatellite libraries enriched for several microsatellite sequences in plants. *Biotechniques*, **20**, 758–760.

Gallagher JC (1980) Population genetics of *Skeletonema costatum* (Bacillariophyceae) in Narragansett Bay. *Journal of Phycology*, **16**, 464–474.

Hayes PK, Barker GLA, Batley J *et al.* (2002) Genetic diversity within populations of cyanobacteria assessed by analysis of single filaments. *Antonie Van Leeuwenhok*, **81**, 197–202.

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

Rozen S, Skaletsky HJ (2000) PRIMER3 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, NJ.

Rynearson TA, Armbrust EV (2000) DNA fingerprinting reveals extensive genetic diversity in a field population of the centric diatom *Ditylum brightwellii*. *Limnology and Oceanography*, **45**, 1329–1340.

Skov J, Lundholm N, Pocklington R, Rosendahl S, Moestrup Ø (1997) Studies on the marine planktonic diatom *Pseudo-nitzschia*. 1. Isozyme variation among isolates of *P. pseudodelicatissima* during a bloom in Danish coastal waters. *Phycologia*, **36**, 374–380.