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Microsatellites isolation and polymorphism in introduced populations of the cultivated seaweed *Undaria pinnatifida* (Phaeophyceae, Laminariales)

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The brown seaweed *Undaria pinnatifida*, native to Asia where it is extensively cultivated, is one of the few pandemic marine invaders that has spread to both hemispheres in the last 30 years, colonizing both artificial and natural habitats (see references in Silva et al. (2002)). In Australasia, this species appears to have been introduced via ship hulls or ballast water, whereas its spread in Europe is postulated to be a consequence of its introduction for aquaculture in Brittany, France. Recent studies have highlighted its potential for expansion and its impact on native communities (Thornber et al. 2004) as well as its capacity to colonize disturbed areas (Valentine and Johnson 2003). To elucidate the reasons for – and to potentially manage and prevent – its successful establishment outside its native range, it is essential to determine the mechanisms and processes behind *U. pinnatifida* introductions, local recruitment and reproduction. Microsatellite markers provide useful tools to trace back such short-term evolutionary processes and dispersal patterns (Davies et al. 1999; Holland 2000; Sakai et al. 2001). Here, we isolated 20 microsatellite markers and carried out a preliminary survey of the polymorphism in three populations of *U. pinnatifida* sampled in the native (Japan) and introduced range (France and New-Zealand).

Genomic DNA of *U. pinnatifida* was extracted from fresh meristematic tissue of two individuals from Perros-Guirec and Brest (France) by a CTAB method including two chloroform extractions, with an addition of 0.6 vol of ethanol between extractions to remove polysaccharides. An enriched library for tandem AC repeats was constructed using the protocol described by Billote et al. (1999), based on Kijas et al. (1994). A total of 7.5 μg of DNA was digested by *Rsa*I, purified using spin columns (Nucleospin extract®, Macherey-Nagel) and ligated to adaptors Rsa21 (5′-CTCTTGCTTACGCGTGGACTA-3′) and Rsa25 (5′-TAGTCCACGCGTAAGCAAGAGC-ACA-3′) at room temperature for 1H30 then overnight at 4 °C. Ligated DNA was purified using spin columns (Nucleospin extract®, Macherey-Nagel; final elution volume of 100 μl) and PCR amplified in a final volume of 50 μl with 10 μl of purified DNA, 0.25 mM of each dNTP, 3 mM MgCl2, 5 U of Taq polymerase (Thermoprime, Abgene), 300 ng of Rsa21. Amplification was done as follows: initial denaturation for 4 min at 95 °C, then 20 cycles of 30 s at 94 °C, 1 min at 60 °C, 1 min 30 s at 72 °C, with a final elongation step of 5 min at 72 °C. Amplified DNA was then denatured at 95 °C for 15 min and incubated with 150 pmol of 5′-biotinylated-(AC)10 in 0.5×SSC for 20 min at room temperature. Hybridized fragments were then isolated with streptavidin-coated magnetic particles (Magnesphere® paramagnetic particles, Promega). After purification, the enriched DNA fragments were amplified in a final volume of 100 μl with 10 U of Taq polymerase (Thermoprime, Abgene), 10 μl of DNA,

Table 1. Characteristics of microsatellite markers in *U. pinnatifida*

Locus	Repeat array	Primer 5′→ 3′	T_i	Allele size range
Up-AC-1B2	$(TC)_5$ N_{99} $(AC)_7$	F: AAAGAGTCTGGCGTGCGG R: AGACTTGATTGGACCTCACCC	60	264–272
Up-AC-1B5	$(GC)_3$ $(GT)_7$ $(T)_9$ G $(T)_8$	F: CCAACCTGTTCGTGAGCCTTTAG R: GGGATCGCTGAACCCTACGC	65	273–283
Up-AC-1C1	$(GT)_7$	F: ACGCCACCCTCCCCAACATC R: AACACGCACTGACGGTCCATACAC	65	212–218
Up-AC-1C9	$(TG)_6$ N_{67} $(GT)_3$ GG $(GT)_2$ N_{58} $(AT)_4$	F: ACAAAGGGTCAGAGCCGC R: CAATCCCACTGTAAAAGTAGCC	64	265–267
Up-AC-1G2	$(AC)_2$ $(CA)_4$ AGC $(CA)_4$ N_{52} $(AC)_6$	F: AGTATGGATATATGATTTTCATAAC R: TGAACAGGGGATGAGGGTGC	55	370–380
Up-AC-1H4	$(CA)_7$	F: CCGACGATTCTGGGTTATGGAGG R: GGCTGGGTGCTATTTTCGATTTC	60	175–181
Up-AC-1H5	$(CA)_5$ A_5 $(CG)_2$ A_6 (AC_7) $(GA)_4$ N_{14} A_9 N_{68} $(A)_6$ C $(A)_8$ GAC A_8 $(AC)_5$	F: GTCGGGTGACAACGGTAACACG R: CGCACCTGAGGCACATTATCC	60	355–365
Up-AC-2A2	$(CA)_3$ AA $(CA)_4$ N_{115} $(AC)_7$	F: AACACAATGCGCACAAAACACG R: CGACGGGCCTTGGCCTACG	60	230–234
Up-AC-2A8	$(CAG)_6N_{14}$ $(CAG)_3$ N_{53} $(CAG)_3(GAG)_2$ CAG	F: TGCATATCGTCCAGGGTGTTTTC R: CCCAGCCAGGGTCTTGTTTC	60	309–316
Up-AC-2B2	$(AC)_7$	F: ATAATGGAAGACGGACAGAATAC R: GTTCCTTCTTCTTTCGTTGGC	60	166–170
Up-AC-2B4	$(GT)_9$	F: CGTCAAAGTGAGGACCACCG R: GGAAGAAGGCACGAGCGATG	60	417–423
Up-AC-2C1	$(GT)_9$	F: GAGAGAAAACACGGTGCCCC R: GAGGACATCCCCAAGATTACGG	60	152–162
Up-AC-2E8	$(GT)_9$	F: GTGTGACAGCTATCACGATTCGC R: GGATGTCGACGAGTAAAGAACGG	60	210–218
Up-AC-3D1	$(GT)_4$	F: CTCTCGTCTTTACCTTTGGCTGATG R: AATACATCCGCACCACCGCTG	65	249–252
Up-AC-3H12	$(CA)_3$ CGG $(CA)_4$ N_{46} $(AC)_4$ AG $(CA)_4$	F: ACCGATGTCGTTGGGTATG R: CGTGGGATTGGTGATGTCAC	60	210–213
Up-AC-4C12	$(GTT)_7$	F: TTATTTGCCCACAGACAGTGAGATG R: GACCACGGACTTGATTATGCGAG	65	170–179
Up-AC-4E9	$(CA)_7$	F: GTTGTTTGTGCTGTAGGGTCATC R: AGATTTCGCTCGTCGGTCC	60	149–157
Up-AC-4G2	$(CA)_7$	F: TTACCTGACGGACGGAGCC R: ACCGCACCATGCTAGTCTATTAAC	65	261–267
Up-AC-4G9	$(TG)_7$	F: ACCATTGTGTCAGGCATCGTTTG R: GACGGGGGTGGGGACAAGC	64	231–239
Up-AC-4H6	$(TG)_6$	F: AGGCAGCAATTCTTCTAACCCC R: CAGGGGCGACCAGCATTTC	60	203–206

Locus name, repeat array, primer sequences, initial temperature (T_i) in touchdown PCR program, allele size range. Clone sequences are available from GenBank under accession numbers AY38593 to AY38596, and AY38598 to AY38613.

3 mM $MgCl_2$, 560 ng of Rsa21 primer. For amplification, an initial denaturation at 95 °C for 1 min was done followed by 20 cycles of 40 s at 94 °C, 1 min at 60 °C, 2 min at 72 °C, with a final elongation step of 5 min at 72 °C. PCR products were then ligated into pGEM®-T easy vector (Promega) and transformed into JM109 competent *E. coli*. Insert PCR products for 384 positive clones were dot-blotted on nylon N+ membranes (Roche) with a BioDot apparatus (BioRad) and screened for microsatellites by overnight hybridization at 56 °C with a digoxygenine-5′ labelled $(TG)_{10}$ probe followed by a chemiluminescent detection procedure (Dig luminescent detection

Table 2. Polymorphism at the population level in *U. pinnatifida*

	N_{Tall}	Japan			New-Zealand			France		
		N_{all}	H_e.	$\hat{f}$	N_{all}	H_e.	$\hat{f}$	N_{all}	H_e.	$\hat{f}$
Up-AC-1B2	5	5	0.270	0.387	2	0.439	0.340	2	0.162	−0.073
Up-AC-1B5	8	5	0.725	−0.082	2	0.403	0.281	3	0.228	−0.100
Up-AC-1C1	4	2	0.093	1	2	0.156	1	2	0.474	0.430
Up-AC-1C9	2	1	0.000	–	2	0.235	0.333	1	0.000	–
Up-AC-1G2	7	5	0.562	−0.115	4	0.600	**0.819**	2	0.274	0.173
Up-AC-1H4	4	4	0.332	0.252	2	0.358	**0.863**	2	0.361	0.428
Up-AC-1H5	6	4	0.161	−0.040	4	0.709	0.181	2	0.046	0
Up-AC-2A2	3	2	0.254	**0.839**	3	0.592	0.015	1	0.000	–
Up-AC-2A8	2	1	0.000	–	1	0	–	2	0.085	1
Up-AC-2B2	3	1	0.000	–	2	0.156	−0.070	1	0.000	–
Up-AC-2B4	4	3	0.330	0.078	2	0.251	**0.626**	2	0.328	0.088
Up-AC-2C1	6	3	0.202	−0.078	5	0.695	**0.548**	3	0.646	0.196
Up-AC-2E8	4	–	–	–	2	0.467	0.201	4	0.646	0.372
Up-AC-3D1	2	1	0.000	–	1	0.000	–	2	0.341	**0.697**
Up-AC-3H12	2	1	0.000	–	2	0.439	0.340	1	0.000	–
Up-AC-4C12	4	1	0.000	–	3	0.324	**0.619**	2	0.511	0.238
Up-AC-4E9	6	2	0.156	1	5	0.753	**0.887**	4	0.574	**0.451**
Up-AC-4G2	4	1	0.000	–	3	0.569	0.171	3	0.489	**0.789**
Up-AC-4G9	5	3	0.196	−0.065	4	0.453	−0.106	1	0.000	–
Up-AC-4H6	3	2	0.120	−0.045	2	0.439	−0.045	2	0.089	1
All loci	4.20	2.47	0.179	0.153	2.65	0.402	**0.389**	2.10	0.263	**0.372**

Number of alleles over the whole study (N_{Tall}) and within populations (N_{all}), gene diversity (H_e) and $\hat{f}$-estimates of F_{is} (Weir and Cockerham al. 1984) for three *U. pinnatifida* populations. Bold values for $\hat{f}$-estimates refer to significantly positive values (permutation test, $n = 2000$ performed with Genetix software (Belkhir et al. 2003) after a Bonferroni adjustment for multiple tests in each population testing against a heterozygote deficiency (Rice 1989)).

kit, Roche). Of the 384 clones, 124 positive clones, chosen according to the insert size (ranging from 400 to 1000 base pairs) and hybridization intensity, were sequenced using an ABI 3100 capillary sequencer. Of a total of 52 designed primer pairs, 20 pairs with forward primers labelled with infra-red fluorescent dye IRD700^{TM} or IRD800^{TM} were used for screening on a Li-Cor NEN Global IR2 DNA sequencer.

We analyzed three *U. pinnatifida* populations (24 individuals in each): (1) Nagasaki (Japan), from the species' native range, (2) Dunedin, from New-Zealand, where introduction and spread were closely correlated with shipping activities with recent proliferation in natural areas and (3) Perros-Guirec, from Brittany, France where wild populations of *U. pinnatifida* became established after deliberate introduction for aquaculture. Genomic DNA was extracted using the DNeasy Plant 96 kit (Qiagen) and diluted to 1:100 prior to PCR. All loci were amplified with the same touchdown PCR procedure using a PTC200 thermocycler (MJ Research): 15 μl final volume, 2.5 μl of DNA template, 0.25 mM of each dNTP, 2.5 mM $MgCl_2$, 0.35 U of EuroTaq polymerase (Euroclone), 0.1 μM fluorescent labelled forward primer, 0.26 μM non-labelled forward primer, 0.33 μM non-labelled reverse primer, and 0.13 mg/ml Bovine Serum Albumin. Touchdown PCR included initial denaturation for 3 min at 95 °C, then 45 s at 95 °C, 45 s at initial temperature (T_i, Table 1) and then decreasing 1 °C per cycle for 10 cycles, 45 s at 72 °C, 20 cycles of 45 s at 95 °C, 45 s at T_i-10 °C, 45 s at 72 °C, with a final elongation step of 7 min at 72 °C. PCR products were screened on 6.5% polyacrylamide gels using the Li-Cor IR2 sequencer. Allele sizes were determined using a known DNA sequence.

No linkage disequilibria across loci were detected (permutation tests in Genetix software v. 4.02, Belkhir et al. (2003)). The number of alleles, expected heterozygosities and deviation from Hardy–Weinberg equilibrium for each locus were computed using the program Genetix. Over the

whole data set ($n = 72$ individuals), the total number of alleles ranged from 2 to 8 according to loci (Table 2). One locus (Up-Ac-2E8) failed to amplify in Nagasaki, indicating possible null alleles. At the population level, the genetic diversity was variable across populations. Perros-Guirec and Nagasaki exhibited the lowest variability with 58 and 60% of polymorphic loci at the 95% level. Conversely, 90% of the loci examined were polymorphic with the same criterion in Dunedin. Dunedin also displayed the highest level of variability ($H_e = 0.40$), which is roughly twice the value observed in Nagasaki ($H_e = 0.18$) or Perros-Guirec ($H_e = 0.26$) populations. The discrepancies in genetic diversity between the two introduced populations may indicate differential introduction and spreading processes, confirming previous mitochondrial data (Voisin et al. 2005). Nevertheless, both introduced populations showed significant heterozygote deficiencies that were twice those observed in Japan. This result might be explained by either a Wahlund effect due to recurrent introductions from genetically differentiated sources or by a shift towards a more inbred mating system in non-native populations. Overall, this preliminary study highlights the usefulness of the microsatellite loci developed herein for the analysis of local population structure and diversity.

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