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PRIMER NOTE

Characterization of microsatellite loci in the dwarf eelgrass *Zostera noltii* (Zosteraceae) and cross-reactivity with *Z. japonica*

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Abstract

***Zostera noltii* is an important species of eelgrass occurring along European, north African, Mediterranean, Black Sea and Azov Sea coasts. Nine microsatellite loci were developed and no linkage disequilibrium was observed. Cross-amplification was observed for all loci (polymorphic) in *Z. japonica*; only four loci amplified (monomorphic) in *Z. marina*.**

Keywords: dwarf eelgrass, microsatellites, seagrasses, Zosteraceae, *Zostera noltii*

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The dwarf eelgrass, *Zostera noltii*, is widely distributed in inter- and subtidal habitats along the coasts of Europe from temperate Norway to tropical Mauritania and also the Mediterranean, Black and Azov Seas (Phillips & Meñez 1988). Populations in some areas have experienced significant declines, while populations in other areas have expanded dramatically (Reise *et al*. 1989; Philippart & Dÿkema 1995). As eelgrass meadows are important habitat modifiers (Bruno & Bertness 2001), development of single-locus genetic markers will further our understanding of genetic differentiation, mating systems and restoration efforts.

For microsatellite library construction, *Z. noltii* was collected from Massholm (Baltic Sea) and DNA was extracted and purified from leaves as previously described (Doyle & Doyle 1987). Species identity was confirmed by comparing rDNA internal transcribed spacer region (ITS1 and ITS2) sequences from collected individuals with published sequences for the species (data not shown). A library was developed commercially using biotin-labelled microsatellite motifs attached to magnetic beads (Olsen *et al*. 2002). Cloning, sequencing and screening procedures were conducted as previously described (Olsen *et al*. 2002) and subsequently identified nine polymorphic microsatellite loci.

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Microsatellite analysis entailed DNA extraction from five to seven 1-cm pieces of new leaves previously dried and stored in silica crystals. Dried leaves were placed into a 2-mL round-bottomed microcentrifuge tube and frozen by partial submergence in liquid N_2. The frozen tissue was powdered in the tube with a mini mortar. DNA was extracted by adding 800 µL 2% cetyltrimethyl ammonium bromide and 2 µL 2-mercaptoethanol to the powdered tissue and incubating at 65 °C for *c*. 45 min, followed by three extractions with equal volumes of chloroform : isoamyl alcohol (Doyle & Doyle 1987). DNA was isolated as described previously (Coyer *et al*. 2002) except that the Sephaglas pellet was resuspended in 1 mL 50% ethanol for *c*. 10 min instead of overnight.

All polymerase chain reactions (10 µL total volume) contained 1 µL of the Sephaglas resuspension (100 µL) as a DNA template, 10 mM Tris HCl, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 or 0.24 µM of each primer [forward primer 5′ labelled with the fluorescent labels NED (Applied Biosystems), 6-FAM or HEX (Sigma)], 0.01% bovine serum albumin and 0.25 U *Taq* polymerase (Promega). The polymerase chain reaction was performed with a PTC-100 thermocycler (MJ Research) (94 °C for 3 min followed by 35 cycles of 94 °C for 40 s, 57.5 °C for 40 s and 72 °C for 10 min).

Amplification products were denatured (90 °C for 2 min) and separated on a 4.5% denaturing polyacrylamide gel

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Table 1 Primer sequences and characteristics of nine polymorphic microsatellite loci in *Zostera noltii*

Locus (GenBank Accession no.)	Core sequence	PCR primers (5′–3′)	No. of alleles	Size range	Sample locations (no. of genets) H_E (H_O)		
					Sylt (40)	Zeeland (44)	Mauritania (36)
ZnB1 (AY566799)	$(GA)_4G(GA)_{20}$	F: TTGACAAAAGTAGGTGGAGTTGA R: GGCAGAGAAGAGCAGTACACG	13	100–124	0.895 (0.807)	0.727 (0.727)	0.743 (0.889)
ZnB3 (AY566800)	$(GA)_{21}$	F: CTTTCGTTCGTTCATAAAGCA R: CCCAATTCTCATCGACAACC	16	188–222	0.886 (0.968)	0.570 (0.591)	0.784 (0.833)
ZnB8 (AY566801)	$(GA)_{14}$	F: AGATGGCGGAAATCAAACC R: CCTGTCACTTGCCACTTGTC	6	129–161	0.183 (0.193)	0.000 (0.000)	0.082 (0.083)
ZnD6 (AY566802)	$(CT)_5TT(CT)_2TT(CT)_{13}$ $(GTCT)_2GT(CT)_{10}$	F: GACCACGTGCAATTCTGAAA R: TTCCCTGCACTTGTAGACCC	10	199–235	0.401 (0.375)	0.434 (0.500)	0.731 (0.806)
ZnE7 (AY566803)	$(GA)_{18}$	F: AAACAGGAACGCGAAGAAGA R: TGGTGACCTTTCTCATTGGA	11	135–171	0.707 (0.742)	0.545 (0.591)	0.758 (0.861)
ZnF8 (AY566804)	$(CT)_{17}$	F: GCCACGACAATGTGAACAAC R: CGTGATGAATGAAAGAGCGA	10	197–221	0.580 (0.613)	0.675 (0.750)	0.577 (0.694)
ZnF11 (AY566805)	$(CT)_1C(CT)_{15}$	F: AGACGCAGACTCGGACTCAT R: GAAGATGCATTATTCATTCACCC	8	273–288	0.712 (0.710)	0.108 (0.114)	0.366 (0.444)
ZnH8 (AY566806)	$(CT)_3(CA)_2T(CT)_{16}$	F: TTCGACGACAGACAGAAACG R: AGAGAGGAAGACGGTGACGA	8	169–183	0.590 (0.548)	0.582 (0.659)	0.716 (0.778)
ZnH10 (AY566807)	$(CT)_{13}$	F: TCTGCCGGTGTGTGAAACT R: CGTCGTTTTAAATTGCCTCTT	7	158–168	0.696 (0.710)	0.570 (0.568)	0.485 (0.444)

H_E, expected heterozygosity; H_O, observed heterozygosity; PCR, polymerase chain reaction.

Table 2 Cross-amplification of *Zostera noltii* microsatellite loci in *Zostera* spp.

Species (populations/ total individuals)	*ZnB1*	*ZnB3*	*ZnB8*	*ZnD6*	*ZnE7*	*ZnF8*	*ZnF11*	*ZnH8*	*ZnH10*
Z. marina (7/24)	x	2	2	x	x	x	x	2	2
Z. japonica (2/12)	3	6*	5	7	4	6	3	4	5*

*One sample could not be amplified. All *Z. marina* genotypes were monomorphic.
The number of alleles is presented for each locus; x, weak or absent signal.

using an ABI 377 autosequencer (Applied Biosystems). Estimators of observed and expected heterozygosities (Nei's gene diversity) and linkage disequilibrium were calculated with GENETIX 4.02 (Belkhir *et al*. 2001) using 36–44 genets from Sylt (Germany), Zeeland (the Netherlands) and Banc d'Arguin (Mauritania) (Table 1). Significance was tested with permutation and sequential Bonferroni corrections (Rice 1989).

No significant linkage disequilibrium or deviation from Hardy–Weinberg equilibrium (HWE) was detected (10 000 and 2000 permutations, respectively). All nine loci were polymorphic in the sister species *Z. japonica* (Les *et al*. 2002) collected from Vancouver Island (Canada) and four were monomorphic (none were polymorphic) in the more distantly related *Z. marina* (Table 2). Two loci (*ZnB*3 and *ZnH*10) could not be amplified in one sample of *Z. japonica* and, thus, null alleles may be present. In contrast, only one of five microsatellite loci developed for *Z. marina* yielded amplification products (monomorphic) in *Z. noltii* and none cross-reacted with *Z. japonica* (Reusch 2000). Cross-amplification of microsatellite primers between *Z. noltii* and *Z. japonica* reflects sister species status.

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