

## PRIMER NOTES

**Polymorphic microsatellite markers in the ornate dragon lizard, *Ctenophorus ornatus***NATASHA R. LEBAS\* and  
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The ornate dragon *Ctenophorus ornatus* is an agamid lizard which lives exclusively on granite outcrops in southern Western Australia. Whilst some studies have characterized the display behaviour of Australian agamids (e.g. Carpenter *et al.* 1970), few have investigated their mating systems (but see Cogger 1978; Olsson 1995). *C. ornatus* is sexually dimorphic, territorial and has a diverse range of display behaviours. A population has been followed over the breeding season to investigate mating behaviour and field reproductive success.

Initial investigations of reproductive success using RAPDs indicated a high degree of relatedness between the adults in the population (N. R. LeBas, unpublished data). Intensive land clearing in the wheatbelt of Western Australia is likely to have limited dispersal between outcrops, possibly generating high levels of inbreeding within populations. Highly polymorphic markers were therefore required to distinguish between potential parents. We have developed five polymorphic microsatellite markers for *C. ornatus*. These markers will also be valuable for investigating population structure in this potentially vulnerable species. To our knowledge these are the first microsatellite markers developed for an agamid species.

Genomic DNA was digested with *Sau3A1* and 200–700 bp fragments were excised. The size-selected fragments were ligated into pGEM32f+ (Promega) and transformed into competent ElectroMAX DH 10B cells (Life Technologies). Transformed cells were grown on LB-agar plates and 1300 recombinants transferred to 96-well microtitre plates. Recombinant colonies were transferred onto Nylon membranes (Hybond-N+ Amersham) by vacuum blotting and hybridized with  $\gamma^{32}\text{P}$ -labelled di- (CA)<sub>10</sub>, (AG)<sub>10</sub>, (AT)<sub>10</sub> and trinucleotide (AAG)<sub>8</sub>, (AAT)<sub>8</sub>, (AAC)<sub>8</sub> oligonucleotides. Fourteen positives were obtained (1% of clones) all from the dinucleotide probes and 12 of these were sequenced. Ten positives had microsatellite sequences with 10 or more repeat units and were sequenced in both directions. Primers were designed for the seven sequences with the greatest number of repeat units. Primers were designed with Primer Express (Applied Biosystems) and Primer3 (<http://www.path.cam.ac.uk/cgi-bin/primer3.cgi>).

Genomic DNA for PCR was extracted from clipped toes using a standard proteinase K phenol–chloroform procedure (Sambrook *et al.* 1989). Microsatellite primers were end-labelled with [ $\gamma^{33}\text{P}$ ]-ATP prior to amplification. PCR reactions of 10  $\mu\text{L}$  contained ~ 25 ng of DNA, 1.5–2.5 mM MgCl<sub>2</sub>, 1 $\times$  reaction buffer (Promega: 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100), 0.5 U *Taq* polymerase (Promega), 400  $\mu\text{M}$  of each dNTP, 3 pmol of each primer and an overlay of mineral oil. Tth Plus DNA Polymerase and reaction buffer (Fisher Biotech) were used for primers Co12E10 and Co7G1. PCR amplifications were in a Hybaid TouchDown Thermocycler and consisted of an initial denaturation at 94 °C for 3 min then 30 cycles of 45 s at 94 °C; 45 s at  $T_a$  °C (see Table 1) and 45 s at 72 °C. The final elongation step was 72 °C for 7 min for primers Co12E10 and Co7G1 and 70 °C for 15 min for all other primers. PCR products were resolved on 6% denaturing polyacrylamide gels and visualized by autoradiography. Size of fragments was determined by comparison with a standard sequencing reaction of pUC18 DNA (Amersham).

**Table 1** Characteristics of microsatellite loci successfully amplified in *Ctenophorus ornatus*. Repeat motif and size are for the sequenced allele. Number of alleles, expected ( $H_E$ ) and observed ( $H_O$ ) heterozygosity were estimated from 79 individuals (Co12E10  $n = 78$ ). GenBank accession numbers: AF159512–AF159516

| Locus   | Repeat motif  | Primer sequence (5'–3')                             | Size (bp) | No. of alleles | $H_O$ | $H_E$ | Annealing temp. (°C) |
|---------|---|---|-----------|----------------|-------|-------|----------------------|
| Co10F11 | (TC) <sub>17</sub>                                      | GCCAGTTACTGTGATTTGGTTCCA<br>GGAACACATGCACAGAAAGCAA  | 104       | 12             | 0.772 | 0.743 | 58                   |
| Co6A6   | (TC) <sub>10</sub> (AC) <sub>9</sub> (AG) <sub>15</sub> | GCCACTATTGGTTTTTAATTTCCC<br>TTCCAACCTGATGTCTGGCATTC | 146       | 8              | 0.646 | 0.674 | 57                   |
| Co9C11  | (AG) <sub>17</sub>                                      | CAATGACCTCCTCCAAAAAG<br>TGTGGCTGATTTTACACCTGTTG     | 169       | 7              | 0.696 | 0.747 | 56                   |
| Co12E10 | (GT) <sub>13</sub>                                      | GCAGGCTCGTATGGAAGAAG<br>ACCGACTCATCGTGCTTAAA        | 151       | 12             | 0.679 | 0.818 | 57                   |
| Co7G1   | (TC) <sub>4</sub> (GT)(TC) <sub>8</sub>                 | TCCACAACCGCAGTAAATCA<br>TGGAATTTTTCTCAGAGGGA        | 187       | 5              | 0.436 | 0.378 | 56                   |

Five loci amplified successfully and were polymorphic (Table 1). Two other loci also amplified products, but persistent stuttering prevented reliable scoring despite optimization and, in one case, primer redesign. Locus Co12E10 showed significant deviation from Hardy–Weinberg equilibrium ( $\chi^2$ -test;  $P < 0.05$ ). Null allele frequency estimates using CERVUS (Marshall *et al.* 1998) indicated possible null alleles at loci Co12E10 (frequency = 0.086) and Co9C11 (frequency = 0.031). At locus Co12E10 a consistently nonamplifying offspring further suggested the presence of a null allele (Pemberton *et al.* 1995). Caution is therefore required for parentage assignment based on exclusion at this locus.

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- of their appearance, flying capabilities or for the sport of pigeon racing. Some sport pigeon breeds including the Viennese Highflier (Wiener Hochflugtaube), which consists of several different lines, are becoming increasingly rare and are considered seriously endangered. Pigeon DNA microsatellites were developed to clarify the breeding origin of the Viennese Highflier and the genetic relationship between different lines. Microsatellite variability was analysed in several lines of the Viennese Highflier and in some breeds believed to be ancestral or to have been crossbred into the Highflier in the past. Furthermore, free-living urban pigeons caught in different districts of Vienna were screened.
- As the abundance of avian microsatellites seems to be much lower than that estimated for other vertebrate genomes (Primmer *et al.* 1997), we used an enrichment method for establishing microsatellite-rich genomic libraries (Armour *et al.* 1994). Partial genomic libraries were constructed with DNA isolated from an 'Old-Austrian Tumbler'. DNA was digested with *MboI*, ligated to linkers (Armour *et al.* 1994) and size-selected (400–700 bp) after agarose gel electrophoresis. Microsatellite sequences were selected by hybridization against 300 ng of denatured di-(CA, CT) or tetranucleotide (GATA, GGAT) repeat sequences (for synthesis see Schlötterer 1998) immobilized on a small piece of nylon membrane. Pre-selected sequences were reamplified using one linker oligonucleotide as primer, digested with *MboI* and cloned into the *BamHI* site of pUC19. Following electroporation into *Escherichia coli* XL1 cells low-density plates (< 200 colonies/plate) were screened for microsatellite-positive clones with radiolabelled simple sequence oligonucleotides. Colony hybridization of enriched libraries suggested that more than 90% of clones contained a microsatellite sequence. Seventy-five di- and 75 tetranucleotide repeat-containing clones were randomly picked. One hundred and two positive clones were sequenced on an ABI 377 sequencer using BigDye terminator chemistry (Perkin-Elmer Applied Biosystems). Primers for seven loci (Table 1) were designed from unique sequence regions flanking microsatellites. Forward primers were labelled with fluorescent dyes. Reverse primers were tailed with a nontarget-specific sequence to overcome allele sizing problems associated with the primer sequence-dependent adenylation of PCR products by most *Taq* polymerases (Brownstein *et al.* 1996).
- Pigeon genomic DNA was isolated from blood-filled quills during molting (adults) or feather growth (squabs) and from blood samples taken from the wing vein, using a salt–chloroform method (Gammel & Akiyama 1996). Standard 15  $\mu$ L PCR reactions included approximately 100 ng of DNA, 7.5 pmol primers (primer pair Cl $\mu$ D32 was amplified with 3.75 pmol), 0.25 mM of each dNTP, 2 mM MgCl<sub>2</sub> and 0.75 U of *Taq* polymerase (Gibco BRL) in 1 $\times$  PCR buffer (67.7 mM Tris-HCl, pH 8.8; 16.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 0.01% Tween 20). Microsatellites were amplified on a PE GeneAmp 2400 cyclor (Perkin-Elmer Applied Biosystems), for 3 min at 95 °C, followed by 30 cycles of 45 s at 95 °C, 40 s at 54 °C and 90 s at 72 °C and finally 10 min at 72 °C. PCR products were pooled and separated on a laser-based capillary electrophoresis instrument, the ABI PRISM 310 Genetic Analyser (Perkin-Elmer Applied Biosystems), applying standard loading and electrophoresis conditions (Wenz *et al.* 1998). Alleles were sized relative to an internal size standard

## Polymorphic DNA microsatellites in the domestic pigeon, *Columba livia* var. *domestica*

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Since the domestication of the wild rock pigeon (*Columba livia*) several hundred different domestic pigeon breeds have been established. Most breeds are kept exclusively because

**Table 1** Characteristics of seven pigeon DNA microsatellite loci

| Locus        | Repeat motif       | Primer sequences (5'–3')                                       | Label | Allele range | Accession no. |
|--------------|--------------------|--|-------|--------------|---------------|
| Cl $\mu$ D17 | GT <sub>17</sub>   | F: TCTTACACACTCTCGACAAG<br>R: <u>GTTTCCACCCAAATGAGCAAG</u>     | FAM   | 114–128      | AF188629      |
| Cl $\mu$ T17 | CATC <sub>13</sub> | F: ATGGGTTTGGAGATGTTTTG<br>R: <u>GTTTGATGGAGTTGCTATTTTGCT</u>  | FAM   | 208–244      | AF188632      |
| Cl $\mu$ D16 | GT <sub>17</sub>   | F: GCAGTGATAAAGTTCTGGAACA<br>R: <u>GTTTGCCTCACCGTGACATCA</u>   | TET   | 85–134       | AF188628      |
| Cl $\mu$ D19 | GT <sub>15</sub>   | F: CCGTTTCTTCTAATGCAC<br>R: <u>GTTTGGATTCTGGGAGTGTATG</u>      | TET   | 171–198      | AF192275      |
| Cl $\mu$ D32 | CA <sub>15</sub>   | F: GAGCCATTTCACTGAGTGACA<br>R: <u>GTTTGCAGGAGCGTGTAGAGAAGT</u> | HEX   | 136–158      | AF188630      |
| Cl $\mu$ T13 | GATA <sub>15</sub> | F: TCCAGAAGACACAGGCTAGT<br>R: <u>GTTTGCAGGAGCGTGTAGAGAAGT</u>  | HEX   | 190–212      | AF188631      |
| Cl $\mu$ D01 | GT <sub>30</sub>   | F: GATTTCTCAAGCTGTAGGACT<br>R: <u>GTTTGGATTGGTTGGCCATC</u>     | HEX   | 68–120       | AF188627      |

F, forward primer; R, reverse primer.

Underlined sequences represent a nontarget specific tail (see text).

**Table 2** Number of alleles (*A*) and observed heterozygosities (*H<sub>O</sub>*) in different pigeon breeds. Deviations from Hardy–Weinberg equilibrium were investigated using an exact test (\**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001) as implemented in GENEPOP (Raymond & Rousset 1995). Numbers of analysed pigeons are in parentheses

| Locus        | Breed                | AT(16) | BH(42)  | DA(41)  | GK(38)  | KI(20) | RG(26)  | SZ(7)   | UP(28) |
|--------------|----------------------|--------|---------|---------|---------|--------|---------|---------|--------|
| Cl $\mu$ D17 | <i>A</i>             | 3      | 4       | 4       | 5       | 3      | 3       | 1       | 6      |
|              | <i>H<sub>O</sub></i> | 0.81   | 0.48**  | 0.54**  | 0.63    | 0.30   | 0.35*   | 0.00    | 0.57   |
| Cl $\mu$ T17 | <i>A</i>             | 5      | 5       | 5       | 6       | 3      | 5       | 5       | 7      |
|              | <i>H<sub>O</sub></i> | 0.94   | 0.26    | 0.66    | 0.37*** | 0.75   | 0.42    | 0.57    | 0.82   |
| Cl $\mu$ D16 | <i>A</i>             | 5      | 6       | 6       | 5       | 3      | 6       | 3       | 11     |
|              | <i>H<sub>O</sub></i> | 0.75   | 0.79    | 0.78    | 0.26*** | 0.55   | 0.46*** | 0.14**  | 0.71   |
| Cl $\mu$ D19 | <i>A</i>             | 2      | 3       | 3       | 4       | 3      | 5       | 2       | 5      |
|              | <i>H<sub>O</sub></i> | 0.00*  | 0.07*** | 0.24*** | 0.18*** | 0.30   | 0.19*** | 0.29*** | 0.32   |
| Cl $\mu$ D01 | <i>A</i>             | 3      | 9       | 5       | 4       | 3      | 4       | 3       | 10     |
|              | <i>H<sub>O</sub></i> | 0.50   | 0.83    | 0.68    | 0.45    | 0.55   | 0.58    | 0.29    | 0.71   |
| Cl $\mu$ D32 | <i>A</i>             | 3      | 7       | 5       | 6       | 3      | 5       | 5       | 8      |
|              | <i>H<sub>O</sub></i> | 0.75   | 0.62    | 0.46    | 0.42*   | 0.65   | 0.65    | 0.57    | 0.82   |
| Cl $\mu$ T13 | <i>A</i>             | 3      | 5       | 5       | 3       | 3      | 4       | 2       | 8      |
|              | <i>H<sub>O</sub></i> | 0.56   | 0.52    | 0.59*   | 0.40**  | 0.60   | 0.58    | 0.29    | 0.64   |

AT, Old-Austrian Tumbler; BH, Budapest Highflier; DA, Danzig Highflier; GK, Viennese Highflier-Gekranzelt; KI, Viennese Kiebitz; RG, Viennese Highflier-Rotgestrich; SZ, Szegedin Tumbler; UP, urban pigeon.

(TAMRA GS 500) using GeneScan 2.0 and Genotyper 2.1 (Perkin-Elmer Applied Biosystems).

Pigeons examined belong to seven different breeds (Table 2). Breed samples were obtained from two to 10 breeders and may consist of related animals, whereas urban pigeons should be a random sample. The number of alleles at each variable locus and their observed heterozygosities in different breeds are detailed in Table 2. In 16 out of 56 locus–breed combinations a significant departure from Hardy–Weinberg equilibrium became evident. The majority of deviations were found within the breed GK (five loci) and across six breeds at locus Cl $\mu$ D19 (Table 2). Population substructuring is the most likely explanation for observed deviations in GK, because the number

of breeding populations is limited with almost no exchange between populations. To our knowledge deviations from Hardy–Weinberg expectation for locus Cl $\mu$ D19 are not caused by a nonamplifying allele or by sex-specific inheritance. Family analysis revealed that alleles for Cl $\mu$ D19 are apparently transmitted in a non-Mendelian way (data not shown), but the reason for the unusual inheritance pattern remains unclear.

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## A suite of highly polymorphic microsatellite markers in turbot (*Scophthalmus maximus* L.) with potential for use across several flatfish species

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The turbot (*Scophthalmus maximus* L.) is a commercially important flatfish and there is considerable interest in this species with respect to fisheries and aquaculture. We report the development and characterization of an entire suite of novel polymorphic microsatellite markers for turbot which add to those that have been reported to date. A study comparing Irish and Norwegian populations using three previously developed microsatellite markers identified a clear need for additional markers (Coughlan *et al.* 1998). Estoup *et al.* (1998) subsequently developed eight microsatellite markers which they have evaluated in parentage assessment studies in hatchery populations. Studies on the population structuring of wild turbot have generally utilized allozyme electrophoresis and reported

very low levels of genetic variability (e.g. Bouza *et al.* 1997). The use of microsatellite markers displaying higher levels of polymorphism might reveal finer details of population structuring.

We have used a combination of two techniques to isolate microsatellite markers from turbot: a random amplified polymorphic DNA (RAPD)-based technique (Ender *et al.* 1996) and an enriched library technique (Kandpal *et al.* 1994). For the RAPD technique, polymerase chain reaction (PCR) profiles from 11 primers were electrophoresed, Southern blotted, hybridized to a <sup>32</sup>P-labelled CA/GT polynucleotide, and bands identified as positive cloned and sequenced (Iyengar *et al.* 2000). A number of microsatellite sequences were selected for primer design and tested in 10 wild turbot (Table 1; nos 1–8). For the enriched library, *Mbo*I-digested genomic DNA was size-fractionated by electrophoresis followed by excision and purification of 200–500 bp fragments through a Qiaquick column (Qiagen). A total of 3–5 µg was used for ligation to 9–15 µg of a *Mbo*I adaptor molecule (see Kandpal *et al.* 1994). A PCR was performed on the ligated DNA using one of the adaptor oligonucleotides as a primer in a 50-µL volume using the following conditions: 1 × 94 °C, 4 min; 35 × 94 °C, 30 s, 55 °C, 30 s, 72 °C, 120 s; 1 × 72 °C, 10 min. PCR product (4 µg) was denatured and hybridized to 2 µg of biotinylated (CA)<sub>15</sub> oligonucleotide overnight at 50 °C in 100 µL containing 0.5 M sodium phosphate, pH 7.4 + 0.5% SDS. Enrichment was carried out using streptavidin-coated magnetic beads (Promega) and a magnetic particle concentrator (Dyna) (see Mundy & Woodruff 1996). DNA from the washes was purified using Qiaquick columns and amplified in PCR reactions as described above. Enrichment was confirmed by electrophoresis of PCR products and standard Southern blotting and hybridization procedures. DNA from the most enriched fraction was cloned into pGEM-T Easy following extended A-tailing by the addition of 25 mM dATP and 1 U *Taq* DNA polymerase upon completion of the PCR reaction, and incubating at 72 °C for 15 min. Eight hundred recombinant clones were screened by gridding onto replica plates, colony blotting and hybridization to a <sup>32</sup>P-labelled (CA)<sub>15</sub> oligonucleotide. Sixty-six clones identified as positive were sequenced using automated sequencing, 40 of which were found to contain microsatellite sequences. Microsatellites containing ≤ 12 perfect CA repeats (19/40) were not used for primer design. Seven clones were found to be too close to the linker and two clones were found to be identical. Primers were designed for 13 microsatellites and tested on 10 wild turbot. Results obtained from 10 of these loci are listed in Table 1 (nos 9–18). Microsatellite PCRs were carried out in 10 µL containing 50 ng of template, 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 75 mM Tris-HCl, pH 8.8, 0.01% Tween 20, MgCl<sub>2</sub> as in Table 1, 7.5 pmoles of each primer, 0.15 µL [α<sup>32</sup>P]-dATP (≥ 2500 Ci/mmol), 0.2 mM dNTPs and 1 U *Taq* DNA polymerase (Advanced Biotechnologies) using a Perkin-Elmer 2400 thermal cycler. The PCR conditions were as follows: 1 × 94 °C, 4 min; 30 × 94 °C, 30 s, annealing temperature in Table 1, 30 s, 72 °C, 30 s; 1 × 72 °C, 10 min. A volume of 10 µL of the reaction was denatured and loaded onto a 6% acrylamide gel along with a sequencing reaction of M13mp18 for allele size estimation. The gel was run for 1.5–3 h and the products were visualized by autoradiography.

**Table 1** Microsatellite markers isolated in turbot with primer sequences, PCR conditions, observed heterozygosity ( $H_O$ ) and expected heterozygosity ( $H_E$ ) in 10 wild-caught individuals

| Locus           | Repeat sequence  | Primer sequences (5'–3')                       | Annealing temp. (°C) | MgCl <sub>2</sub> (mM) | No. of alleles (sizes) | $H_O$ | $H_E$ |
|-----------------|--|--|----------------------|------------------------|------------------------|-------|-------|
| 1 B11-I 12/6/3  | (CA) <sub>12</sub> CG(CA) <sub>6</sub> CG(CA) <sub>3</sub>   | GTGTGTTACTGCTGATCTAGC<br>ATGTTCCATCTCATTCTGTC  | 55                   | 1.0                    | 5<br>(175–183)         | 0.50  | 0.66  |
| 2 B12-I GT14    | (GT) <sub>14</sub>   | GTGATGGAAGATTGTACCAG<br>CACAATAAAGGATAGACAGG   | 58                   | 1.5                    | 4<br>(113–119)         | 0.70  | 0.69  |
| 3 B18-II CA70   | (CA) <sub>70</sub>   | CAGAGGGATAAATTCTGTGC<br>TACTGGCATCATGGTCAAC    | 56                   | 1.0                    | 11+<br>(267–333)       | 0.88  | 0.90  |
| 4 F1-OCA19      | (CA) <sub>19</sub>   | AGTTACACCAGTGCACAGAG<br>CCAGGCCATCCACATTTAAC   | 56                   | 1.5                    | 6<br>(99–113)          | 0.90  | 0.76  |
| 5 F8-ICA26†     | (CA) <sub>26</sub> (GA) <sub>3</sub>   | ATTCTACGAGTCAGGCTCG<br>TTCGATGTCAGTCGAGGACG    | 61                   | 1.5                    | 7+<br>(233–295)        | 0.77  | 0.81  |
| 6 F8-I 11/8/17‡ | (CA) <sub>11</sub> T(CA) <sub>8</sub> T(CA) <sub>17</sub>  | CACTAATTACACGGCAGACG<br>AATCGGATGAGACGGCGAAC   | 62                   | 1.5                    | 7<br>(195–233)         | 0.80  | 0.80  |
| 7 F12-I TG16‡   | (TG) <sub>16</sub> (CG) <sub>4</sub>   | CAGTCATATCAGCAGATGCC<br>AATTCCAGCCGACAACTCTC   | 56                   | 1.0                    | 7<br>(111–123)         | 0.70  | 0.78  |
| 8 F12-I AG18‡   | (AG) <sub>18</sub> G(GA) <sub>5</sub>  | GGCAGGACTGTACATTTTGG<br>AGTCAGCCAGTTTACTGCAC   | 60                   | 2.5                    | 6*<br>(217–259)        | 0.57  | 0.74  |
| 9 1/4AC18       | (AC) <sub>18</sub>   | AGAAAGGCTCGACCAGCTCC<br>TGATGGCTCATAGTGGCTAC   | 62                   | 0.8                    | 5*<br>(207–223)        | 0.57  | 0.62  |
| 10 2/5TG14      | (TG) <sub>14</sub>   | GAGGATTGGTGACCTTGGC<br>CCTCAGACTACTGAAGTCAC    | 62                   | 1.0                    | 8<br>(94–124)          | 0.60  | 0.80  |
| 11 3/3GT        | (GT) <sub>14</sub> GC(GT) <sub>8</sub> (TG) <sub>4</sub> T(TG) <sub>3</sub>                              | GATCTAGTTGTCTAACAGG<br>ATAGCAGGTCCTGCTAAAG     | 58                   | 0.8                    | 7+<br>(165–197)        | 0.66  | 0.79  |
| 12 3/9CA15      | (CA) <sub>15</sub>   | AGAGTGAAGAACGTACCTGC<br>CAATGGAGAGGCAGTATCGG   | 60                   | 1.0                    | 6<br>(226–245)         | 0.80  | 0.71  |
| 13 3/20CA17     | (CA) <sub>17</sub>   | AAGCACCCATACGCAGG<br>AAATTGGAAACTGGACCC        | 62                   | 1.5                    | 8<br>(80–96)           | 1.00  | 0.84  |
| 14 4/3COMP      | (A) <sub>5</sub> CA(AC) <sub>4</sub> CA(AC) <sub>4</sub> G(AC) <sub>5</sub> G(AC) <sub>7</sub>           | GTACTTAAGCAGCTGTTCCGC<br>GAAACGCAGTTAGTTCCTGTC | 58                   | 2.5                    | 5+<br>(181–243)        | 0.66  | 0.75  |
| 15 4/4CA4/13    | ATG(AC) <sub>20</sub> AAT(CA) <sub>13</sub> AT(CA) <sub>3</sub><br>(AC) <sub>4</sub> G(CA) <sub>13</sub> | GCACCTCATGCTGACATACAG<br>GACTCAGTGACTGATGAATC  | 64                   | 1.5                    | 4<br>(125–131)         | 0.60  | 0.66  |
| 16 4/5CA22/6/2  | (CA) <sub>22</sub> CT(CA) <sub>6</sub> CT(CA) <sub>2</sub>   | ACTCTGAGGGCAGAGGAAC<br>GCTGTGGTAATCAGAAAGC     | 60                   | 0.8                    | 10+<br>(153–183)       | 0.88  | 0.87  |
| 17 5/4CA20      | (CA) <sub>20</sub> A(AC) <sub>2</sub>  | GACGGAAGCTCGGAGATTC<br>GATTGGTGACCTTGGCAAC     | 63                   | 1.5                    | 7<br>(166–196)         | 0.40  | 0.78  |
| 18 7/1TC18      | (TC) <sub>18</sub>   | GAGAACTGAGCTGAGTCAC<br>AATGATTATCACCGCTGCAC    | 60                   | 1.5                    | 5<br>(160–172)         | 0.60  | 0.66  |

\*Only seven individuals assayed.

†Only nine individuals assayed.

‡Both markers within F8-I and F12-I situated within 2.3 kb.

Loci numbered 1–8 obtained using the RAPD technique (GenBank accession nos AF182080, AF182082–182087, AF182100).

Loci numbered 9–18 obtained using the enriched library method (GenBank accession nos AF182088–AF182097).

Other sequences also submitted (GenBank accession nos AF182081, AF182098, AF182099).

**Table 2** Results of screening of primers developed for turbot across several flatfish species

| Locus        | Scophthalmidae                                  |   | Bothidae   | Pleuronectidae                                 |                                   |   |   |   |  | Soleidae                                |  |   |
|--------------|---|---|--|--|-----------------------------------|---|---|---|--|---|--|---|
|              | Megrim<br><i>Lepidorhombus<br/>whittiaionis</i> | Brill<br><i>Scophthalmus<br/>laevis</i> | Imperial<br>scaldfish<br><i>Arnoglossus<br/>imperialis</i> | Witch<br><i>Glyptocephalus<br/>cynoglossus</i> | Dab<br><i>Limanda<br/>limanda</i> | Long rough dab<br><i>Hippoglossoides<br/>platessoides</i> | Halibut<br><i>Hippoglossus<br/>hippoglossus</i> | Lemon sole<br><i>Microstomus<br/>kitt</i> | Plaice<br><i>Pleuronectes<br/>platessa</i> | Dover<br>sole<br><i>Solea<br/>solea</i> | Thick back sole<br><i>Microchirus<br/>variegatus</i> | Sand<br>sole<br><i>Solea<br/>lascaris</i> |
| B11-I 12/6/3 | -   | -                                       | -  | -  | -                                 | -   | -   | -   | -  | -                                       | -  | -   |
| B12-I GT14   | +   | +                                       | -  | -  | +                                 | -   | +   | -   | -  | +                                       | -  | -   |
| B18-II CA70  | ++*   | ++                                      | -  | ++*  | ++*                               | -   | +   | ++*                                       | ++*  | ++*                                     | -  | +   |
| F1-OCA19     | +   | +                                       | -  | -  | -                                 | +   | -   | -   | -  | -                                       | -  | -   |
| F8-ICA26     | -   | -                                       | -  | -  | -                                 | -   | -   | -   | -  | -                                       | -  | -   |
| F8-I 11/8/17 | -   | -                                       | -  | -  | -                                 | -   | -   | -   | -  | -                                       | -  | -   |
| F12-I TG16   | +   | +                                       | +  | -  | +                                 | +   | -   | -   | +  | -                                       | +  | -   |
| F12-I AG18   | -   | ++                                      | -  | -  | -                                 | -   | -   | -   | -  | ++                                      | -  | -   |
| 1/4AC18      | -   | -                                       | -  | -  | -                                 | -   | -   | -   | -  | -                                       | -  | -   |
| 2/5TG14      | +   | +                                       | -  | -  | +                                 | -   | -   | +   | -  | +                                       | -  | -   |
| 3/3GT        | +   | +                                       | -  | +  | -                                 | -   | -   | +   | -  | -                                       | +  | -   |
| 3/9CA15      | -   | -                                       | -  | -  | -                                 | -   | -   | -   | -  | -                                       | -  | -   |
| 3/20CA17     | -   | -                                       | -  | -  | -                                 | -   | -   | -   | -  | -                                       | -  | -   |
| 4/3COMP      | ++  | ++                                      | +  | +  | +                                 | +   | +   | +   | +  | ++                                      | +  | +   |
| 4/4CA4/13    | +   | ++                                      | -  | +  | +                                 | +   | -   | +   | +  | +                                       | +  | +   |
| 4/5CA22/6/2  | +   | ++                                      | -  | +  | -                                 | -   | -   | -   | -  | +                                       | -  | +   |
| 5/4CA20      | +   | ++                                      | +  | -  | -                                 | -   | -   | -   | -  | +                                       | -  | +   |
| 7/1TC18      | ++*   | ++                                      | +  | -  | +                                 | +   | +   | -   | -  | +                                       | -  | -   |

-, no band obtained.

+, band present at expected size but weak and/or smeary — may improve with optimization of PCR conditions.

++, strong band present at expected size.

\*Band smaller than expected; †band larger than expected.

The primers were also tested in single individuals from a range of flatfish using the conditions detailed for turbot. The results, summarized in Table 2, highlight the possibility of some of these being used in studies on several flatfish species.

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- advanced our understanding of the population genetics of seagrasses (Procaccini & Mazella 1998; Reusch *et al.* 1999b), a polyphyletic assemblage of marine angiosperms displaying low variability in traditional codominant markers (e.g. allozymes). Here, I present five (CT)<sub>n</sub>/(GA)<sub>n</sub> microsatellite loci for eelgrass *Zostera marina* L., the most widely distributed seagrass species of the northern hemisphere, and a test of cross-species amplification in the congeneric *Z. japonica* and *Z. noltii*. The microsatellite loci were isolated and identified from an enriched genomic library described in Reusch *et al.* (1999a). Eighty-four positive bacterial clones were sequenced on an ABI-377 automated sequencer using the BigDye-terminator cycle sequencing kit (Perkin-Elmer). Primers were designed using Primer 3.0 (Rozen & Skaletsky 1996). For polymorphism testing, *Zostera* spp. genomic DNA was extracted from dried leaf material using CTAB (Doyle & Doyle 1987) and amplified by PCR (polymerase chain reaction). A 10- $\mu$ L reaction contained 0.2–1 ng of crude DNA extract, 10 mM Tris-HCl (pH 9), 50 mM KCl, 200  $\mu$ M of each dNTP, 2.0 mM MgCl<sub>2</sub>, 0.3–1.2  $\mu$ M of each primer, 0.1% (w/v) bovine serum albumin and 0.5 units of *Taq* polymerase (Promega). Products were visualized on a Perkin-Elmer ABI-377 automated sequencer using forward primers labelled with fluorescent dyes. Allele sizes were scored against an internal lane standard. PCR conditions were: 3 min denaturation at 94 °C, followed by 33–40 cycles of 1 min at 94 °C, 1 min annealing at 57 °C and 1 min at 72 °C (extension), followed by a terminal extension step of 20 min.
- Five polymorphic loci with uninterrupted repeat motifs were identified in which alleles always differed by one repeat unit (Table 1). The PCR conditions were optimized by varying the primer concentrations such that the five loci can be amplified in two multiplex PCR reactions, each combining two and three loci, respectively (Table 1).
- Allele numbers, and expected/observed heterozygosities of 30 individuals in each of three populations (Maasholm, Baltic Sea, 54–41°N 10–00°E; Sylt, North Sea, 54–58°N 8–22°E; and Halifax, Northeast Atlantic, 44–42°N 63–11°W) are given in Table 2. A high number of alleles was detected. In particular, the loci ZosmarCT-17H and ZosmarCT-35 displayed between 11 and 18 alleles within populations and are among the most polymorphic microsatellites identified for plant species thus far. Given an expected/observed heterozygosity which is frequently > 0.8 these loci will be very useful for paternity analyses in eelgrass, especially when combined with the already existing seven loci (accession nos AJ009898–AJ009904, Reusch *et al.* 1999a).
- No linkage disequilibrium could be detected between any locus pair including the previously reported seven loci using the software Arlequin 3.1. (Schneider *et al.* 1997) ( $P > 0.01$  in all 72 comparisons for each of the three populations, Bonferroni correction not applied).
- I also tested whether all 12 microsatellite primer pairs currently available for *Z. marina* would yield amplification products in two other species of the genus *Zostera*, *Z. noltii* and *Z. japonica*. Only the locus ZosmarCT-35 amplified *Z. noltii* isolates from the German Wadden Sea and yielded monomorphic amplification products of 90 bp among eight individuals. None of the primers amplified isolates of *Z.*

## Five microsatellite loci in eelgrass *Zostera marina* and a test of cross-species amplification in *Z. noltii* and *Z. japonica*

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The application of DNA microsatellites has considerably

**Table 1** Description of five polymorphic microsatellites in eelgrass *Zostera marina*. Both multiplex PCR reactions can be run in a single lane on an automated sequencer (ABI 377, Perkin-Elmer) after pooling the products in the volumetric ratio 1:1 (A:B). The sequences have been submitted to the EMBL nucleotide database (accession numbers AJ249303–AJ249307)

| Locus          | Primer sequences (5'–3')          | Repeat array       | Label | Multi PCR | C Prim ( $\mu\text{M}$ ) | Size of alleles (bp)  |
|----------------|-----------------------------------|--------------------|-------|-----------|--------------------------|---|
| ZosmarCT-12-f  | CGT TCA TCT TGT CCT CGT CC        | (CT) <sub>13</sub> | 6-Fam | A         | 0.3                      | 126, 128, 130, 132, 134, 136  |
| CT-12-r        | TTT CAT TTC CAT TTC CCA CC        |                    |       |           |                          |   |
| ZosmarCT-19-f  | CCC AAG AAA TAT AAA ATC GGG G     | (CT) <sub>11</sub> | Hex   | A         | 0.3                      | 140, 150, 152   |
| CT-19-r        | CTT CTC CTT CCG CCG CTA C         |                    |       |           |                          |   |
| ZosmarCT-35-f  | TCT TGG GCT TTT AAT TAG CG        | (CT) <sub>15</sub> | Ned   | A         | 0.3                      | 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 122 |
| CT-35-r        | AAA GAG AGA CCT AAA GAT ATG GGC   |                    |       |           |                          |   |
| ZosmarCT-20-f  | TGG AAG GAG TTT CGA TGT ATC C     | (CT) <sub>13</sub> | 6-Fam | B         | 0.5                      | 150, 152, 154, 156, 160, 162, 164, 166  |
| CT-20-r        | GGG AGA TTT GCA GTG TAG AAT TTA G |                    |       |           |                          |   |
| ZosmarCT-17H-f | TCT TTA CCA ACC GAT CTC CG        | (CT) <sub>26</sub> | Ned   | B         | 1.2                      | 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 149, 157               |
| CT-17H-r       | AAA CAC AAG CAA AAC AGT TAG TCA G |                    |       |           |                          |   |

**Table 2** Allele number (*A*), and observed/expected heterozygosity ( $H_O/H_E$ ) in three eelgrass *Zostera marina* populations ( $N = 30$  individuals)

| Locus Zosmar | Sylt     |       |       | Halifax  |       |       | Maasholm |       |       |
|--------------|----------|-------|-------|----------|-------|-------|----------|-------|-------|
|              | <i>A</i> | $H_O$ | $H_E$ | <i>A</i> | $H_O$ | $H_E$ | <i>A</i> | $H_O$ | $H_E$ |
| CT-12        | 6        | 0.250 | 0.289 | 2        | 0.147 | 0.138 | 2        | 0.10  | 0.096 |
| CT-19        | 2        | 0.307 | 0.477 | 2        | 0.424 | 0.451 | 2        | 0.344 | 0.344 |
| CT-35        | 18       | 0.730 | 0.891 | 17       | 0.848 | 0.921 | 12       | 0.897 | 0.867 |
| CT-20        | 5        | 0.307 | 0.365 | 7        | 0.424 | 0.424 | 1        | —     | —     |
| CT-17H       | 11       | 0.923 | 0.890 | 13       | 0.935 | 0.903 | 12       | 0.690 | 0.856 |

*japonica* ( $N = 8$ ) collected in Willapa Bay, Washington State, USA. In other plant groups, primers developed for a single species are often applicable in other species of the same genus or even family (e.g. van Treuren *et al.* 1997; White & Powell 1997; Streiff *et al.* 1998). The lack of cross-species amplification in the genus *Zostera* possibly reflects a long taxonomic separation between *Z. marina*, *Z. noltii* and *Z. japonica* despite their morphological similarity.

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## Isolation and characterization of microsatellite loci in the Japanese marsh warbler *Locustella pryeri*

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The Japanese marsh warbler *Locustella pryeri*, formerly referred to as *Megalurus pryeri*, breeds in marshes with reed and sedge beds in limited areas at the northern and central parts of Honshu, the main island of Japan (Fujita & Nagata 1997). Because of a decrease in the number of breeding pairs, due to habitat destruction, this species has been listed as threatened in Japan (The Environment Agency of Japan 1998). Molecular markers for analysing genetic variability within and among populations are a promising tool for conservation (Avise & Hamrick 1996; Smith & Wayne 1996). Here, we report five microsatellite loci from the genome of the Japanese marsh warbler, and examine their allelic variation in a natural population and cross-species amplification of these loci in other related species of warblers.

A partial genomic library was constructed from *Sau3AI*-digested genomic DNA extracted from whole blood of an unknown-sex individual, in which size-selected DNA fragments ranging from 600 to 2000 bp were ligated into pBluescript II. At first, approximately 4000 transformed colonies were screened using a mixture of  $^{32}\text{P}$ -5' end-labelled (CA)<sub>8</sub> and (CT)<sub>8</sub> probes. After a second screening, 43 positive clones were obtained and sequenced using DNA Sequencer SQ5500-L (Hitachi). Then, five primer sets for PCR amplification were designed based on the nucleotide sequences of clones containing CA/GT or CT/GA-repeats (Table 1). For genotyping on the same DNA sequencer, forward primers (upper row in Table 1) were labelled with Texas Red using 5' oligonucleotide Texas Red labelling kit (Amersham Pharmacia Biotech).

Genetic variability was examined in 32 individuals captured at Hotoke-numa in Aomori Prefecture, Japan. Genomic DNA

**Table 1** Characterization of five microsatellite loci in the Japanese marsh warbler *Locustella pryeri* (N = 32)

| Locus | Repeat structure*  | Primers (5'-3')                               | Annealing temp. (°C) | Size (bp)* | No. of alleles | Heterozygosity |          | DDBJ Accession no. |
|-------|--|---|----------------------|------------|----------------|----------------|----------|--------------------|
|       |  |   |                      |            |                | Observed       | Expected |                    |
| MSLP1 | (CT) <sub>7</sub>  | TGCAGTTAAACCAGCCCGAA<br>TAGCAGGAGCGTAGACAAAG  | 55                   | 83         | 2              | 0.31           | 0.31     | AB031373           |
| MSLP2 | (CA) <sub>14</sub>   | TAACTACAGCCAGTTAGAAG<br>TGAAGTTACTGGTAGCCTTTG | 50                   | 100        | 3              | 0.16           | 0.18     | AB031374           |
| MSLP3 | (CT) <sub>6</sub>  | TCAGAAATGAAATCTCCCGC<br>TAATTTGGATACTTCCACCC  | 50                   | 164        | 3              | 0.44           | 0.49     | AB031375           |
| MSLP4 | (CA) <sub>13</sub> CG-<br>(CA) <sub>2</sub> C <sub>3</sub> (CA) <sub>2</sub> | TGCCATGTCCCTGCCTATCC<br>TTGGCTCTGCCGCACCTCCC  | 55                   | 133        | 3              | 0.53           | 0.60     | AB031376           |
| MSLP5 | (CA) <sub>8</sub> GAGC-<br>(CA) <sub>3</sub>                                 | TCATCACCATGGCAACAAAT<br>TAATCCCTGAGTTTGAACATA | 50                   | 157        | 7              | 0.72           | 0.79     | AB031378           |

\*Cloned sequence.

| Species                          | Primer set |       |       |       |       |
|----------------------------------|------------|-------|-------|-------|-------|
|                                  | MSLP1      | MSLP2 | MSLP3 | MSLP4 | MSLP5 |
| <i>Locustella fasciolata</i>     | +          | +     | +     | +     | -     |
| <i>L. ochotensis</i>             | +          | +     | +     | +     | -     |
| <i>L. pleskei</i>                | +          | +     | +     | +     | -     |
| <i>Acrocephalus bistrigiceps</i> | -          | +     | -     | +     | -     |
| <i>A. arundinaceus</i>           | -          | +     | -     | +     | -     |
| <i>Cettia diphone</i>            | +          | +     | -     | +     | -     |
| <i>Cisticola juncidis</i>        | -          | -     | -     | -     | -     |
| <i>Phylloscopus borealis</i>     | -          | +     | -     | +     | -     |
| <i>P. tenellipes</i>             | -          | +     | -     | +     | -     |
| <i>Urosphena squameiceps</i>     | +          | +     | -     | +     | -     |

+, distinct product of expected size; -, no amplification.

was extracted from a micro amount of whole blood using a standard phenol-chloroform extraction method (Sambrook *et al.* 1989). PCR amplification was carried out using ABI GeneAmp system 9700 (PE Biosystems) in an 8–10- $\mu$ L reaction mixture containing 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl (pH 8.3), 0.2 mM dNTP, 0.2  $\mu$ M of forward primer, 0.05  $\mu$ M of Texas Red-labelled forward primer, 0.25  $\mu$ M of reverse primer (lower row in Table 1), 0.25 U *rTaq* DNA polymerase (TaKaRa), and 10–50 ng of genomic DNA. After denaturation at 95 °C for 2 min, cycling was performed for 32–35 cycles under the following conditions: 30 s at 95 °C, 20 s at the locus-specific annealing temperature (see Table 1) and 30 s at 72 °C. Cross-species amplification using whole blood DNA of other warblers was also performed under the same PCR conditions.

The five loci showed a low but distinct allelic variation ranging from 2 to 7 alleles in the individuals examined. The observed heterozygosities at these loci ranged from 0.16 to 0.72. All the polymorphic loci conformed to Hardy–Weinberg expectations when tested using the program GENEPOP 3.1d (Raymond & Rousset 1995). The low allelic variation might be ascribed to the examined samples derived from a small, isolated population, although genetic variability in other populations also needs to be investigated.

Some, if not all, microsatellite loci were cross-amplified in the 10 related warbler species examined (Table 2). Interestingly,

the MSLP5 locus, that showed the highest allelic variation in the Japanese marsh warbler, failed in cross-amplification in all the related warbler species examined. The other four loci were amplified successfully among the three congeners of the Japanese marsh warbler. The present microsatellites will be useful for assessing genetic variability within and among populations of the Japanese marsh warbler and, to some extent, the other species examined here.

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## Development of primer sets to amplify fragments of conserved genes for use in population studies of the fungus *Daldinia loculata*

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*Daldinia loculata* (Lév.) Sacc. is a xylariaceous fungal species that frequently produces stromata on deciduous trees killed by fire. It has a circumpolar distribution, and was, in Europe, until recently erroneously referred to as *D. concentrica* in the literature (Johannesson *et al.* 2000). Both the life cycle of *D. loculata* and the mechanism of its establishment in the host after a forest fire are obscure. The aim of this study was to develop codominant and highly reproducible molecular markers

for this species, by using published sequence data from conserved nuclear genes of closely related species. This approach has previously been used for creating specific markers to study population genetics and speciation of several ascomycetous species (e.g. Koufopanou *et al.* 1997; Carbone & Kohn 1999). Here we present eight primer sets, derived from conserved parts of genes from mainly pyrenomycetous fungi, which successfully amplify the corresponding genes in *D. loculata*. Seven of the amplification targets contain variable loci within a small sample set of *D. loculata* and will be used as molecular markers for strain identification and studies of the population structure of the fungus.

Oligonucleotide primers were designed from conserved protein-coding regions of 16 genes reported from *Neurospora crassa* and at least one additional pyrenomycetous or, as in one case, plectomycetous, species. The design was performed manually following standard primer analysis methods (Innis *et al.* 1990).

Six single-ascospore isolates, originating from three geographically distant Swedish populations of *D. loculata*, were used in the initial screening of the primer sets. Mycelia for DNA extraction were grown for 2 weeks in 2% malt extract liquid medium at 21 °C in darkness, and the DNA was extracted as described by Högberg *et al.* (1995).

PCR amplifications were performed using a Perkin-Elmer Cetus DNA thermal cycler (GeneAmp 480) under the following conditions: initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 1.5 min, annealing at 50–55 °C for 1.5 min and extension at 72 °C for 1.5 min, with a final extension at 72 °C for 10 min. The total 50-µL reaction volume contained approximately 1 ng of template DNA, 10 mM Tris-HCl, 50 mM KCl, pH 8.3, 3.5 pmol of each primer, 1.25 U of AmpliTaq Gold™ (Perkin-Elmer), 200 µM dNTP and 1.5–3.0 mM MgCl<sub>2</sub>. PCR products were separated on 1.0% agarose gels (LE-agarose, FMC BioProducts, Rockland, USA), stained with ethidium bromide and visualized under UV light. Fragments of eight genes were successfully amplified as single PCR products in the initial screening of the primer sets, and their primer sequences are shown in Table 1.

**Table 1** Primer sets used to amplify conserved genes of *Daldinia loculata*, their origin and corresponding amplification targets

| Protein                                  | Primers*  | Acc. no† | Position‡              | T <sub>a</sub> (°C) |
|--|---|----------|------------------------|---------------------|
| Actin                                    | TATGTGCAAGGCCGGTTTCGCCGGT<br>TAGCAGAGCTTCTCCTTGATGTCACG     | U78026   | 492–516<br>1272–1297   | 52                  |
| Calmodulin                               | AGGTCTCYGAGTTC AAGGAGGCCCTTC<br>TTYTGCAATCATGAGCTGGACGAATC  | X70923   | 37–62<br>432–457       | 52                  |
| Chitin synthase 1                        | CGCGATATYGTMAA CCGTGAAGAAGTC<br>TACGATCTTCGGCCAAGAACATGTTTC | M73437   | 1457–1482<br>2204–2229 | 50                  |
| Cyclophilin                              | TAGYTTCTTCGAYMYGAGTGGGAGG<br>CGAACTTCTCGCCGTAGATGGTCTT      | X17692   | 1502–1527<br>2092–2116 | 52                  |
| Elongation factor 1-α                    | RGACAAGRCTCACATCAACGTSGT<br>CCAGTRATCATGTTCTTGATGAART       | D45837   | 604–627<br>1187–1211   | 52                  |
| Glyceraldehyde 3-phosphate dehydrogenase | YGGTGTCTTCAACCACACYGASAA<br>RTANCCCCAYTCRTTTCRTACCA         | U67457   | 1211–1234<br>1851–1874 | 50                  |
| Heat stress protein 80–1                 | GAGTGGCTSRGCTTCRTCAAGGGTGT<br>GTACTCATCRATGGGGTTCRACMAGGA   | S58539   | 27–52<br>448–473       | 52                  |
| Protein kinase C                         | AAGATCGAGCGCAAAGGCTCTGATC<br>TATCTCTTSARTGCCTGCTTCAAGAG     | Y12002   | 406–432<br>1077–1102   | 50                  |

\*From 5' to 3', when degenerated Y = C/T; M = A/C; R = A/G; S = C/G; N = A/C/G/T.

†GenBank accession numbers of *Neurospora crassa* sequences used for primer construction.

‡Position in the *N. crassa* nucleotide sequence used for construction of each primer.

**Table 2** Distribution of nucleotide polymorphism in seven nuclear gene loci of *Daldinia loculata*

| Locus   | Ac (761 bp)                                     | Cam (835)   | Cph (544)   | Efa (702)                   | Gpd (723)                     | Hsp (447)   | Pkc (749)         |
|---------|---|---|---|-----------------------------|-------------------------------|-------------|-------------------|
| Site*   | 3 3 4 4 4 5 5<br>2 3 2 5 7 2 3<br>2 7 1 3 7 5 1 | 1 1 4 6 6 7 7 7 7 7<br>6 7 9 7 9 7 0 8 0 3 3 5 6<br>2 8 6 7 0 1 5 8 3 0 9 9 2 | 1 1 1 1 1 1 1 1 1 1 2 2 2 2 4 4<br>6 8 8 9 1 2 2 4 4 7 7 9 9 2 3 6 9 4 6<br>3 6 9 6 8 1 4 2 5 5 6 3 8 8 0 4 5 6 8 | 2 2 2<br>4 0 0 1<br>9 5 8 5 | 4 5 5 5<br>7 3 5 6<br>8 5 8 9 | 3<br>3<br>3 | 4 5<br>9 8<br>1 8 |
| Con.†   | C C T C T C G                                   | A C T A T G C C C C C C T   | T A C A C T C A C C G A T T A A G T T   | G T C T                     | C C A T                       | T           | A C               |
| Strain‡ |   |   |   |                             |                               |             |                   |
| A10     | . . . . C T .                                   | . T . . C . T T . . . . .   | . . . . . . . T . . . . . T T C C   | A . . .                     | T . G .                       | .           | . T               |
| A36     | . T C T . . .                                   | . . . . C A . . . T . . C   | . . . . . . . T . . . . . T . C .   | . A T C                     | . . . A                       | .           | .                 |
| T13A    | . . . T . . .                                   | . T . G . . . T . . T T C   | . . . G . C . . . . . G . . . . C   | . . . .                     | . . G .                       | .           | T .               |
| T15     | T . . . C T C                                   | . . C . . . T . T . . . .   | . . . . T . G . . . A . . G . . . .   | . . . .                     | . G G .                       | C           | T .               |
| U49-5a  | T . . . C T C                                   | C . . . . . . . . . . .   | . . A . . . . T . . . . C . . . . .   | . A T C                     | T . . .                       | .           | T .               |
| U49-4a  | T . . . . . .                                   | . . . . . . T . T . . . .   | C C . G . . . . . T . G . G G . . . .   | . . . .                     | . . . .                       | .           | . .               |

\*Site numbers are written vertically and indicate polymorphic positions in the sequences.

†Consensus sequence.

‡A10 and A36 origin from the county of Dalarna; T13A and T15, county of Södermanland; U49-4a and 5a, county of Lappland. All isolates were collected by Hanna Johannesson & Anders Dahlberg 1996.

PCR products were purified using the QiaQuick PCR purification kit (QIAGEN Inc.), and sequences were determined with an Applied Biosystems 310 sequencer using the *Taq* DyeDeoxi Terminator™ cycle system (Perkin-Elmer). Sequence analyses and alignments were performed manually or using the MacVector 6.0 software. The identities of the sequences were verified by BLAST searches in the GenBank database. All successfully amplified genes, with the exception of chitin-synthase 1, were polymorphic among the six *D. loculata* isolates initially screened. All polymorphic sites were biallelic. The size of the fragment and the distribution of the nucleotide polymorphism for each locus is shown in Table 2. A search for restriction enzyme recognition sites to separate the alleles was made manually and was successful in five of the genes. Alleles of actin and calmodulin can be separated with *Hae*III, cyclophilin with *Taq*I, elongation factor 1- $\alpha$  and glyceraldehyde-3-phosphate dehydrogenase with *Ssp*I.

To determine the segregation ratios of the alleles, at least 30 single-spore isolates from stromata heterozygous for each locus were investigated. The results show that alleles at all loci segregated according to a pattern that would be expected for a 1:1 ratio ( $\chi^2$  statistics,  $P < 0.9$ ; data not shown), with the exception of the alleles at the elongation factor 1- $\alpha$  locus that segregated 2:1.

The primer pairs used in this study successfully amplify their target region of four other *Daldinia* species (*D. concentrica*, *D. cf. fissa*, *D. cf. grandis* and *D. petriniae*; data not shown).

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### Novel microsatellite loci isolated from the northern krill, *Meganyctiphanes norvegica* (Crustacea, Euphausiacea)

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*Meganyctiphanes norvegica*, the Northern krill, is a pelagic crustacean which shows a wide geographical range of distribution, being present on both sides of the North Atlantic Ocean, in the Mediterranean and other marginal seas. This euphausiid plays an ecological key role in the marine habitat, and it constitutes the main food resources for many species of whales and other marine vertebrates (Mauchline 1980). Its ecological relevance, the large geographical distribution and the pelagic habit make *M. norvegica* a very interesting species for population genetics studies. Here we report a set of novel highly variable microsatellites suitable to investigate the genetic structure of *M. norvegica*.

Genomic DNA for marker isolation was extracted with a standard phenol–chloroform protocol (Sambrook *et al.* 1989). DNA for routine genotyping was released by a Chelex 100 protocol (Walsh *et al.* 1991) from ethanol-fixed specimens sampled in 1996. All the polymerase chain reactions (PCRs) were performed on a GeneAmp 9700 thermal cycler (Perkin-Elmer).

A partial genomic library was constructed from DNA of a single specimen following standard protocols (Ausubel *et al.* 1990). Phenol-extracted DNA was digested to completion with *Mbo*I. Selected fragments (between 200 and 800 bp) were isolated from a 1% agarose gel by electroelution into dialysis bags (Sambrook *et al.* 1989), and desalted using Microcon 100 columns (Amicon). Size-selected fragments were ligated into the dephosphorylated *Bam*HI site of pUC18 (Pharmacia). Ligation products were cloned into MOS-blue competent cells (Amersham). Five thousand recombinant clones were blotted on nylon filters (Hybond N+, Amersham) and hybridized overnight, in 5 $\times$  SSC, 0.1% SDS, 5 $\times$  Denhardt's solution. Three cocktails of  $\gamma^{33}$ P end-labelled oligonucleotide probes containing different repeats [(GGAT)<sub>6</sub> (AGCT)<sub>6</sub> (AGAC)<sub>6</sub> (TACT)<sub>7</sub> (TAGT)<sub>7</sub> (ATCT)<sub>7</sub> (AAAG)<sub>7</sub> (AAAC)<sub>7</sub>], or [(AAAT)<sub>8</sub> (TGT)<sub>8</sub> (TCT)<sub>8</sub> (TAG)<sub>8</sub> (TAT)<sub>11</sub>]. or [(TAG)<sub>8</sub> (TAT)<sub>11</sub> (AC)<sub>12</sub> (AG)<sub>12</sub>], were hybridized overnight at 58 °C, 60 °C, and 62 °C, respectively. Twelve positive clones were isolated, and plasmid DNA was purified using a commercial kit (QIAprep Spin, Qiagen). Purified plasmid DNA (approximately 500 ng) was sequenced using a commercial cycle-sequencing kit (ThermoSequenase, Amersham), according to the manufacturer's instructions. Cycle-sequencing conditions were as follows: 1 min at 94 °C, 1 min at 48 °C, 1 min at 72 °C for 30 cycles. Sequencing primers (pUC18 universal primers: M13–40 and M13 reverse) were 5' end-labelled with [ $\gamma^{33}$ P]-dATP using T4 polynucleotide kinase (New England Biolabs). One pmol of labelled oligo was used in the sequencing reaction. Sequencing products were electrophoresed on 6% denaturing polyacrylamide gels on a vertical apparatus (Gibco-BRL S2). Sequencing gels were dried using a Bio-Rad gel dryer and visualized by autoradiography (24–72 h).

**Table 1** Primer sequence, melting temperature ( $T_m$ ), size (bp), and degree of polymorphism of five *Meganyctiphanes norvegica* microsatellites

| Locus<br>(GenBank of<br>accession no.) | Repeat motif                          | Primer sequence (5' to 3')                             | $T_m$ (°C) | Size (bp) | No. of alleles<br>(No. of<br>specimens) | $H_E$ | $H_O$ |
|--|---------------------------------------|--|------------|-----------|---|-------|-------|
| MnO8<br>(AF150625)                     | (CA) <sub>25</sub>                    | AAATTTCAACCAATGTCACC<br>TGTAAATGTTGCCCATTAAC           | 50         | 266       | 24(48)                                  | 0.95  | 0.84  |
| MnC7<br>(AF150624)                     | (CA) <sub>11</sub>                    | TTGCAACAAAACGATACTATAA<br>GGCCTAACGACAAAAACAA          | 45         | 266       | 24(47)                                  | 0.93  | 0.59  |
| MnG3<br>(AF150626)                     | (AT) <sub>9</sub> (GACA) <sub>5</sub> | CGAACCATGTCCCTAAAGTGACAC<br>TCCAACATCATATTTTCATAATTGGC | 55         | 204       | 11(51)                                  | 0.84  | 0.61  |
| MnD17<br>(AF170799)                    | (AGT) <sub>31</sub>                   | TCCCGTCAATGTTGGTCTCTCTG<br>TCCAACCTTCGGGTTTATTC        | 64         | 219       | 17(30)                                  | 0.90  | 0.93  |
| MnC6<br>(AF170800)                     | (AGT) <sub>24</sub>                   | TCTCATGGCTCACATTATC<br>TCCAACCTCACATTAAGTAGTAG         | 53         | 123       | 12(30)                                  | 0.81  | 0.71  |

All clones contained microsatellite motifs. Primers for eight loci were designed, which amplified fragments ranging from 123 to 266 bp. Five primer pairs produced polymorphic profiles and are reported in Table 1. PCR amplifications were performed in 10 µL volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM MgCl<sub>2</sub> (for primer pairs MnO8 and MnC7) or 1.25 mM MgCl<sub>2</sub> (for MnG3, MnD17, and MnC6), 100 µM of each dNTP, 0.5 units of AmpliTaq Gold (Perkin-Elmer), and 500 nM of each primer. One of the two primers was either <sup>32</sup>P end-labelled using T4 polynucleotide kinase (New England Biolabs) or labelled with the fluorescent dye Texas Red (as provided by the commercial supplier, Genset). The thermal profile was 93 °C for 10 min, then 35 cycles of denaturation at 94 °C (40 s), annealing as in Table 1 (40 s), and extension at 72 °C (40 s). Amplified products were resolved on 6% denaturing polyacrylamide gels. Depending on the kind of labelling used, microsatellites were scored either by autoradiography following gel drying, or visualized on an automated sequencer (Vistra 725, Amersham).

All five loci showed a high level of polymorphism with up to 29 alleles in 48 individuals scored (Table 1). All loci also showed the occurrence of alleles differing in size by a single nucleotide. Four out of five microsatellites (MnO8, MnC7, MnG3, MnC6) showed significant heterozygosity deficit ( $P < 0.001$ ) when Hardy-Weinberg equilibrium was tested (Rousset & Raymond 1995). Observed heterozygosity

ranged between 0.61 (MnG3) and 0.95 (MnO8), indicating that these markers can be very useful for microscale genetic analysis.

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