

Development of new microsatellite loci and evaluation of loci from other pinniped species for the Galápagos sea lion (*Zalophus californianus wollebaeki*)

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Received 29 June 2005; accepted 28 July 2005

Key words: (AC) motif enrichment, cross-species amplification of microsatellite loci, Galápagos sea lion, microsatellite loci, *Zalophus californianus wollebaeki*

The Galápagos sea lion is common across the whole Galápagos archipelago. Together with sharks, whales and dolphins it plays a central role as a key predator in the Galápagos marine ecosystem. The taxonomic status of the Galápagos sea lion is still controversial, since some authors considered it a conspecific of the Californian sea lion (*Zalophus c. californianus*), (Scheffer 1958, Trillmich 1979, Eibl-Eibesfeldt 1984, Perrin et al. 2002, Brunner 2004), while others (Eibl-Eibesfeldt 1955, Rice 1998, Salazar and Bustamante 2003) view it as a different species and follow the taxonomic classification by Sivertsen (1953), a decision bolstered by distinct behavioural traits (Eibl-Eibesfeldt 1984).

Regardless of its taxonomic rank, however, the Galápagos sea lion deserves special attention from a conservation viewpoint, since, as other marine organisms in the Galápagos, they are subject to extreme changes in food abundance due to climatic fluctuations caused by El Niño Southern Oscillation (ENSO) events. During such an event all age classes experience extreme mortality rates (Trillmich and Dellinger 1991). Population recovery from these recurring natural events is hampered by human activities, especially by the increasing exploitation of marine resources, often

in disrespect of existing regulations (see Danulat and Edgar 2002).

Knowledge of the patterns and levels of genetic differentiation between populations is highly needed to develop conservation plans for this species. Moreover, an understanding of the patterns of relatedness between individuals in a given population will contribute enormously to further our understanding of the behavioural ecology of this highly gregarious marine organism (Wolf et al. 2005).

We pursued these two general aims by screening for variation at multiple microsatellite loci, since this type of genetic marker is a powerful tool to determine both patterns of spatial differentiation and relative relatedness amongst individuals within a population. Here, we describe the cloning and characterization of 8 new microsatellite loci for the Galápagos sea lion. In addition, we tested amplification success of 23 published microsatellite loci developed for 7 closely related species in the order Pinnipedia.

All sea lion samples which have been used for this study were collected on the Galápagos island Caamaño (0°45' S, 90°16' W) as part of an ongoing behavioural study. DNA was extracted from freshly preserved tissue samples (100% ethanol)

using the DNeasy® tissue kit from Qiagen™. Genomic DNA was stored in Tris–EDTA buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0) and used for all subsequent reactions. The microsatellite library was based on the genomic DNA from 5 different individuals and enriched for (AC)₁₂ nucleotide stretches, since the (AC) dinucleotide repeat motif is most abundant in mammalian genomes (Toth et al. 2003). The enrichment protocol followed the FIASCO method (Zane et al. 2002) with the following modifications: (i) a 450–1000 bp fraction of the AFLP digestion-ligation reaction were cut from an agarose gel. This fraction was rerun and isolated again; (ii) PCR cycles to amplify *Mse*I adaptor flanking fragments were reduced to 20 cycles; (iii) 500 ng DNA from this enrichment step was mixed with 100 pmol of a 5′-biotinylated (AC)₁₂ oligo-nucleotide probe in a total volume of 100 μl (4.2× SSC, 0.07% SDS) at 67 °C for 2 h; (iv) 200 μl of Dynabeads M-280 Streptavidin beads solution (DYNAL) were washed with 1×PBS, 0.1% BSA and subsequently resuspended at 67 °C in 400 μl (4.2 SSC, 0.07% SDS solution). 200 μl of bead solution were added to the fragment-probe mix and incubated for 30 min at 67 °C; (v) low stringency washes (5 min each) were performed four times with 1×SSC, 0.01% SDS at 38 °C. Four high stringency washes close to the dissociation equilibrium of the DNA fragment/(AC)₁₂ complex were carried out in a 0.06× SSC and 0.01% SDS solution at 38 °C (10 min each). Further steps followed the standard FIASCO protocol (Zane et al. 2002). Fragments enriched for (AC) repetitive stretches were cloned using the TOPO TA Cloning® Kit from Invitrogen™. Ninety-six clones were randomly picked from an agar plate and sequenced with the T3 primer (5′-ATTAACCTCACTAAAGGGA-3′) using and ABI3100 automated DNA sequencer following the manufacturer protocols. Sixty-five out of these clones carried the (AC) repetitive motif, and for 27 clones primers could be designed in the flanking region of the microsatellite using the program Primer3 (Rozen and Skaletsky 2000).

First, polymorphism and utility was tested for the 27 new and 23 cross species amplified microsatellite loci on an A.L.F.™ DNA sequencer (Pharmacia Biotech). All but one of the 27 new microsatellite loci amplified. Of the remaining 26 loci, 24 were polymorphic. So far we have analysed 8 of the most promising new and 7 of the success-

fully cross species amplified microsatellite loci on a Megabace™ 1000 sequencer (Amersham), as this fragment analysis system was considerably more accurate than the A.L.F.™ sequencer. As being part of an ongoing behavioural study number of individuals analysed for the different loci ranged between 7 and 287. All PCR reactions were performed in a 25 μl scale: template concentration ranged from 50–100 ng of genomic DNA, 1× Promega® PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 μM of each primer (fluorescently labelled with Flu, FAM, HEX or TET from MWG Biotech) and 1 U of Promega® TaqDNA polymerase. Except for the annealing temperature the PCR profile was the same for all loci: (i) initial denaturation at 94 °C for 3 min; (ii) 1 min respective annealing temperature for the extension step; (iii) 72 °C for 1 min; (iv) 94 °C for 30 s; (v) 30 repeats of step ii–iv; (vi) 72 °C for 5 min.

Table 1 summarizes the characterization of 8 new and 7 successfully cross species amplified microsatellite loci in *Zalophus californianus wollebaeki*. For reasons of accuracy (see above) expected (H_E) and observed (H_O) heterozygosity were only calculated for the loci which were run on the Megabace™ 1000. Departure from Hardy–Weinberg equilibrium (HWE) was calculated using Arlequin 2.00 software (Schneider et al. 2000). Table 2 provides an overview on the cross species amplification success of microsatellite loci for *Zalophus californianus wollebaeki* analysed on the A.L.F. sequencer. For all loci that have been analysed for the same set of at least 20 individuals we calculated also deviation from linkage equilibrium using the Arlequin 2.00 software (Schneider et al. 2000). Here, a highly significant ($\alpha < 0.01$; deviation from HW not involved) deviation from linkage equilibrium was found for Hg6.1/OrrFCB1 ($P = 0.0007$) and for ZcwD02/ZcwC11 ($P = 0.0004$).

From the eight new species-specific microsatellites, six loci are good candidates for use in evolutionary studies having both high variability levels and not showing evidence of deviations from HWE proportions. Of the 23 loci developed on seven distinct pinniped species we found only three did not amplify (OrrFCB21, Lw4, Lc6) in the Galápagos sea lion samples, three loci produced strong stutter bands (OrrFCB7, OrrFCB10, Lw10), three were monomorphic (Hgdii, Aa4, OrrFCB16), and one locus (Hg6.3) showed a

Table 2. Results of the cross-species amplification of 16 microsatellite loci from six closely related species of the order pinnipedia (*Halichoerus grypus* (Hg), *Phoca vitulina* (SGPV), *Pv*, *Pvc*), *Arctocephalus australis* (Aa), *Odobenus rosmarus rosmarus* (OrrFCB), *Leptonychotes weddelli* (Lw), *Lobodon carcinophagus* (Lc)) in *Zalophus californianus wollebaeki* analysed on an A.L.F. sequencer: Locus designation and EMBL/GenBank accession numbers, forward (F) and reverse (R) primer plus the used dye-labelling, repeat motif, size range of alleles [bp], number of alleles per locus, annealing temperature (T_a [°C]), and number of analysed individuals per locus (N) are provided for every locus. Further abbreviations: na = repeat motif not available in original citation; N/A = not amplified; M/A = multiple bands amplified. Original citations of pinniped microsatellite loci: ⁽¹⁾Allen et al. (1995), ⁽²⁾Goodman (1997), ⁽³⁾Coltman et al. (1996), ⁽⁴⁾Gemell et al. (1997), ⁽⁵⁾Buchanan et al. (1998), ⁽⁶⁾Davis et al. (2002)

Locus	Primer sequences (5'-3'), fluorescence labelling	Repeat motif	Size range	No. of alleles	T_a	N
Hgdii ⁽¹⁾	F: Flu-ACCTGCCATAGTGCTCATC R: GAGCCAACTAAGACAAGCC	na	128	1	55	7
G02095	F: Flu-AATCGAAATGCTGAGCCTCC R: TGATTTGACTTCCCTTCCCTG	na	155-159	3	60	25
G02090	F: Flu-GTCTGGTGAATTAGCCATTATAAG R: CAGAGTAAGCACCCAAAGGAGCAG	(AC) ₂₀	178-182	3	57	21
U65444	F: Flu-AGCTAGTGTAAATGATGGTGTG R: TCTGAGAGATTCAGAGTAACCTTC	(AC) ₁₇	118-120	2	52	10
U65445	F: Flu-GAGTATACCTCCATACTACAC R: AGTTGTTCTCCTGACCCAAAG	(AC) ₁₅	156-162	4	52	28
Pvc78 ⁽²⁾	F: Flu-GGTTAAATGTTGTTTACATCT R: AACCAGAAGAATAAGAAATAGCAT	(AO) ₃ AG(AC) ₇	137-139	2	57	10
L40983	F: Flu-CTACTTCTTGGCATTATTCAAG R: CATCCAAACATAATTATATAAACC	na	213	1	49	7
Pvc29 ⁽³⁾	F: Flu-TGTTCAATGACTGATGAATGGATAG R: CTCTAGCCACATCAATGCAAATGG	Perfect	112-120	2	60	18
L40987	F: TET-GAACCAGGGAGGAAGACAGAGTG R: CAGACTGTATCAGGAGGCTTGG	Imperfect	M/A	M/A	60	99
Aa4 ⁽⁴⁾	F: Flu-ATTTCTTACCTTACCCAGCCAG R: CTGGGCTTGTGTTGGGCATAG	Perfect	191-199	5	60	21
na	F: Flu-CAAACCTGGTATCAGTAGTAGCTG R: GAGAGCTCCCTTCCCTACCATG	Imperfect	M/A	M/A	55	11
OrrFCB4 ⁽⁵⁾	F: Flu-ATCACCTCAATGAGAGTTTCATAATC R: CTCCTAACGTAAGTCTACATCTGTA	Perfect	185	1	60	7
G34927	F: ATGCTAGTAGGGACTTCCACTTC R: TTGCGCAATGTGGAGAGATACTTG	Perfect	N/A	N/A	Gradient 52-61	9
G34928	F: TCCCAGAAGACCTACTCC R: ATTCCTTCTCGGTATC	(GT) ₁₇	N/A	N/A	Gradient 46-55	10
OrrFCB8 ⁽⁵⁾	F: FAM-AAACACTAGCCCTGACTTC R: TTACAGAGCAGGAGTTCA	(GT) ₂₅	M/A	M/A	Gradient 45-54	36
G34929	F: GATCTTCACACAGACAC R: GCTATGCTTCTCTCCA	(GT) ₁₆ C(GT) ₇	N/A	N/A	Gradient 44-53	10
OrrFCB10 ⁽⁵⁾						
G34935						
OrrFCB16 ⁽⁵⁾						
G34936						
OrrFCB21 ⁽⁵⁾						
G34938						
Lw4 ⁽⁶⁾						
AF140590						
Lw10 ⁽⁶⁾						
AF140592						
Lc6 ⁽⁶⁾						
AF140580						

significant deviation from HWE. Accordingly, we did not find evidence that loci which have been cloned from other pinniped species yielded worse results than specifically cloned microsatellite loci in *Zalophus californianus wollebaeki*. The combination of newly developed and previously published microsatellite markers add new useful markers that, together with the recently published microsatellite loci for the Californian sea lion (*Zalophus californianus californianus*) (Hernandez-Velazquez et al. 2005), will enable to address any kind of genetically based questions for sea lions of the Galápagos archipelago.

Acknowledgements

The authors thank Elke Hippauf for performing an excellent job in the laboratory, Arne Nolte and Till Bayer for helpful discussions, Fritz Trillmich for supporting the project, the *Servicio Parque Nacional Galápagos* and the *Charles Darwin Research Station* for granting the sampling permits and for providing the necessary infrastructure. This work is part of a major study on social and genetic structure of the Galápagos sea lion funded by the *Volkswagen Stiftung* to Fritz Trillmich and Jochen B.W. Wolf. Sebastian Steinfartz has been supported by a fellowship (STE 1130/2-2) of the German Research Community (DFG). The Yale Institute for Biospheric studies supported this project through funds to Adalgisa Caccone.

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