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Abstract

We isolated and tested six novel microsatellite loci in sanderling (*Calidris alba*) from Greenland for paternity analyses. In addition, we tested 11 already published microsatellite markers which were originally developed for the congeneric species *Calidris melanotos*, the pectoral sandpiper. All loci were polymorphic, but five of the cross-species loci were not scorable due to suboptimal amplification patterns. The 12 successful loci were tested on 87 individuals, yielding an average of 9.0 (range 4-19) alleles per locus and mean expected heterozygosity of 0.70. Because this data set contained families, tests for Hardy-Weinberg equilibrium, linkage disequilibrium and probability of identity were done on a subset of the data containing 25 adults caught in the same year. The overall probability of identity was $1.0 \cdot 10^{-13}$. Only one locus displayed significant homozygote excess and all loci were unlinked. On the basis of female heterozygotes, all loci are assumed to be autosomal.

Keywords: microsatellite markers; parentage analysis; breeding system; population genetics

Zusammenfassung

Neue, Art-übergreifende Mikrosatellitenmarker für Herkunftsanalysen beim Sanderling (*Calidris alba*)

Wir isolierten und testeten sechs neue Mikrosatelliten-Loci auf Eignung für Verwandtschaftsanalysen beim grönländischen Sanderling. Zusätzlich dazu testeten wir 11 bereits publizierte Mikrosatellitenmarker, die ursprünglich für den nahe verwandten Graubruststrandläufer (*Calidris melanotos*) entwickelt worden waren. Alle Genorte waren polymorph, aber fünf der artübergreifenden loci konnten wegen ungenügender Vervielfältigungsmuster nicht ausgewertet werden. Die 12 verbliebenen Genorte wurden für 87 Individuen getestet und ergaben einen Durchschnitt von 9,0 Allelen pro Genort (Bereich: 4 -19) sowie eine mittlere zu erwartende Heterozygosität von 0,70. Weil dieses Set Daten von Familien enthielt, wurden für einen Teil des Sets mit Daten von fünf adulten, im gleichen Jahr gefangenen Vögeln auch statistische Tests für das Hardy-Weinberg-Äquilibrium, das Kopplungs-Ungleichgewicht, und die Identitäts-Wahrscheinlichkeit durchgeführt. Insgesamt betrug die Identitäts-Wahrscheinlichkeit 1,0*10-13. Nur ein einziger locus zeigte einen signifikanten Homozygoten-Überschuß, und alle loci waren ungekoppelt. Aufgrund der weiblichen Heterozygoten wurde angenommen, dass alle loci autosomal waren.

Introduction

Sanderlings *Calidris alba* are small sandpipers that migrate annually between their High Arctic breeding grounds and the non-breeding grounds along temperate and tropical shorelines (Reneerkens et al. 2009). Based on 24 h nest observations in the Canadian Arctic and on the dissection of two incubating females with scars in their oviducts that suggested the laying of two complete clutches, Parmelee (1970) and Parmelee and Payne (1973) concluded that sanderling females lay two clutches in rapid succession. The first clutch was suggested to be incubated by the male and the second by the female. As a result of this breeding system, called 'double-clutching', uniparental males and females would take care of incubation and the subsequent young. Double-clutching has been suggested to also occur in a few other sandpiper species (Breiehagen 1989, Hildén, 1975, 1988).

In contrast to the findings of Parmelee and Payne (1973), seven clutches in northeast Greenland were incubated by both a male and a female (Pienkowski and Green 1976). The existence of both uniparental and biparental clutches next to each other was confirmed in another area in northeast Greenland based on behavioural observations and data of nest attendance of individually recognised birds (Reneerkens et al. 2009; Reneerkens et al. submitted MS) as well as in northern Taimyr, Siberia (Tomkovich and Soloviev 2001). These more recent data demonstrate that 'double-clutching' certainly is not the norm, although it may indeed occur in sanderling. Yet, the existence of clutches incubated by a single parent is not necessarily the result of double-clutching. Clearly, we need more work to unravel the breeding system in sanderling.

Here, we report the development of seven novel microsatelite markers and the cross-amplification of seven already existing, microsatelite loci to allow parentage analyses to test for paternity to verify earlier suggestions based on behavioural observations on 'double-clutching'. Furthermore, it will shed light on poorly understood aspects of the breeding biology of the sanderling, such as the occurrence of extra-pair fertilisations, and possible geographical variations herein.

Methods

Genomic DNA was extracted from tissue of sanderling 2SAN7b (Southhampton Island, Nunavut, Canada) using a standard phenol extraction. A DNA library was constructed following Hamilton et al. (1999). DNA was digested using three blunt-end restriction enzymes (NheI, RsaI, HaeIII; New England Biolabs), and ligated to SNX linkers to enable PCR amplification of inserts. Amplification was followed by hybridization to biotinylated $(GT)_{15}$ and $(CT)_{15}$ di-repeats. Biotinylated DNA fragments were captured using Dynabeads (Dynal). Di-repeat enriched DNA was recovered using PCR. Amplified enriched DNA was ligated into a PCR 2.1 cloning vector, and plasmids were transformed to competent cells

using TA Cloning kit (Invitrogen). A total of 490 clones with inserts were isolated on Hybond Nylon membranes (Amersham) and a second hybridization with biotinylated oligonucleotides using NRET Phototope Star Detection (New England Biolabs) detected 81 clones with di-repeats.

Positive clones were sequenced with forward and reverse M13 primers using BigDye Terminator version 3.1 (Applied Biosystems) and an ABI 3100 automated capillary sequencer (Applied Biosystems). Sequences were assembled and edited using Chromas-Pro 1.33 and MEGA 3.1 (Kumar et al. 2004). Nineteen sequenced clones had motif repeats with unique flanking sequences, for which primers were designed using OLIGO and Jellyfish software. Primers were tested on DNA extracts of blood samples of 26 sanderlings from geographically distinct locations. Products were analysed to screen for polymorphic loci. PCR was carried out on a Mastercycler epgradient S (Eppendorf). The volume of PCR reaction mixture was 12.5 µL, which contained 1X PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin), 200 µM dNTP (Invitrogen), 0.2 µM forward primer (Invitrogen), 0.2 µM reverse primer (Invitrogen), and 0.20 µM universal dye-labelled M13 (TGTAAAACGACGGCCAGT) tail (6-FAM, HEX; Thermo Scientific), 0.25 U Taq (Invitrogen) and 1 µL DNA template. PCR conditions included an initial denaturation step at 94 °C for 4 min, 36 cycles of denaturation at 94 °C for 30 s, primer annealing for 30 s at respectively 50 °C for primer pair gt22; 55 °C for gt24, an3 and m11; 58 °C for m18; 59 °C for m14; followed by primer extension at 72 °C for 30 s. A final step at 72 °C for 5 min was used to complete primer extension. Fragment analysis was run on an ABI 310. Alleles were sized using ROX size standard (GENEMARK350; Northern Biotech); allele sizes were assigned with GelCompar II software (Applied Maths). Cross-species amplifications were done with eleven primer pairs (Cme1 through Cme10 and Cme12) developed for the pectoral sandpiper *Calidris melanotos* (Carter and Kempenaers 2007).

Blood samples (10-50 microliters) of adults, chicks and near-fledged juveniles were collected during the breeding season near our study site at Zackenberg, northeast Greenland (74º30'N 21º00'W). Adults and near-fledglings were bled from the brachial vein, and chicks from the leg vein. Samples were stored in 96% ethanol at -80°C. DNA was extracted using the Sigma mammalian DNA kit.

We analysed the 12 loci for a total of 87 sanderlings caught at Zackenberg which were also molecularly sexed following Fridolfsson and Ellegren (1999) with primers 2602F and 2718R adjusted for calidrid waders (OH, unpublished data). This data set was expected to show linkage disequilibrium (LD) and deviations from Hardy Weinberg equilibrium (HWE) because it contains numerous parent-offspring and sibling relationships. Analyses were therefore also done on a subset of the data, consisting of the adults only (N=25).

The number of alleles and observed and expected heterozygosities were assessed using Arlequin, version 3.11 (Excoffier et al. 2005). Conformity to HWE and LD were evaluated using Genepop, version 4.0 (Rousset 2008). Micro-checker, version 2.2.3 (Van Oosterhout et al. 2004) was used to test for the presence of null alleles. The probability of observing identical multilocus genotypes between two individuals sampled from the same population ('probabiilty of identity', P_{ID}) was estimated from the data set containing the adults only, following Waits et al. (2001) and using the program GIMLET (Valière 2002). Exclusion probabilities (P_{E1} and P_{E2}) were calculated using CERVUS 3.0 (Kalinowski et al. 2007).

Results and Discussion

The six newly developed microsatellite markers (Genbank accession numbers HQ259972-HQ259977) could be amplified consistently (i.e., produced PCR product in all individuals). They were all polymorphic. Of 11 cross-species loci, six gave consistent PCR results. All these 12 loci (six new plus six cross-species) were highly polymorphic (average number of alleles per locus N_A=9.0 and 7.8; average expected heterozygosity H_E=0.699 and 0.708; for full data set and adults only, respectively). On the basis of female heterozygotes, all loci are assumed to be autosomal.

In the full dataset, as expected, excess homozygosity and linkage disequilibrium were observed. Three loci displayed significant deviations from Hardy-Weinberg equilibrium (after Bonferroni correction, an3: P<0.0001; m18: P<0.0001; Cme9: P=0.0023; Table 1) and linkage disequilibrium was significant for 17 out of 66 pairwise comparisons (at Bonferroni corrected P-level of 0.0008). In the partial data set, there was no evidence for linkage between any locus pair and only one locus had an excess of homozygotes (m18: P=0.0022; Bonferroni corrected P-level of 0.0042; Table 1). Micro-checker detected evidence for possible null alleles in the loci an3, m18 (both data sets) and Cme9 (only the partial data set).

The probabilities of identity per locus are shown in Table 1. For all loci together, P_{ID} was estimated to be $1.0 \cdot 10^{-13}$ (Table 1). The average probability that the loci will exclude a random unrelated candidate parent from parentage of an arbitrary offspring when the genotype of the other parent is unknown (P_{E1}) is 0.9969; when one of the parents is known, the exclusion probability (P_{E2}) increases to 0.9999.

Homozygote excess and linkage disequilibrium in the full data set can be attributed to the relatedness among the birds sampled. While pairwise difference (in number of alleles) among the 87 adult and juvenile birds averaged 15.06, the difference between one particular male (z013) and his four presumed offspring (z123-z126), as an example, averaged 9.25. We suspect that the remaining locus (m18) not in Hardy-Weinberg equilibrium among the 25 adults in the partial data set may be attributable to further relatedness, rather than to null

alleles. This is because sanderlings, and males in particular, are faithful to their breeding sites and will return to the same breeding grounds in subsequent years (Reneerkens and Grond 2009). The most extreme known example consists of a brother and sister that both nested during at least two successive breeding seasons less than 500 m from the nest location where they were born; the likelihood of capturing brothers or fathers and sons may be substantial. We conclude that this set of 12 microsatellite markers will be a valuable tool for assessing parentage in sanderling.

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Table 1 - Microsatellite characterisation in *Calidris alba* on the basis of novel loci (above line) and cross-species amplification from Carter and Kempenaers (2007; below line).

			full data set				adults only				
Locus	**Primer sequence (5'-3')**	**Size range (bp)**	**H_O[B]**	**H_E**	**N_A**	**N_T**	**H_O****	**H_E**	**N_A**	**N_T**	**P_{ID}**
an3	F:TTTCTTGAACAAGGAAATC R:GGATGTAAGAACTGATTTGA	264-288	**0.674**	0.818	13	172	0.600	0.812	10	50	$4.5 \cdot 10^{-2}$
gt22	F:GTTTGCTTTGGGTTTGGAGA R:TATGCTCCACCCATCTCCTC	325-333	0.612	0.709	5	170	0.520	0.703	5	50	$1.4 \cdot 10^{-1}$
gt24[A]	F:ATCAGCAGTTTTCTCATTTA R:CGAGAGTGTCAGAAAGGAAG	232-249	0.747	0.716	7	174	0.680	0.733	6	50	$8.4 \cdot 10^{-2}$
m11	F:ATGGGCTGTAAATCCTGTGC R:TGTGAGAAAGGGTGTGGTTG	226-232	0.655	0.575	4	174	0.520	0.562	4	50	$2.8 \cdot 10^{-1}$
m18	F:CTCAGCTTCCTACCGACTGCAC R:CAAGCTTTCCTTGAGGCTGT	260-274	**0.381**	0.730	8	168	**0.375**	0.730	7	48	$1.3 \cdot 10^{-1}$
m14[A]	F:TGCCTCTACTCAGGTGTTCCA R:AGGACCAAGGGTCTCCAACT	297-315	0.552	0.530	9	174	0.520	0.573	8	50	$1.7 \cdot 10^{-1}$
Cme1		103-133	0.828	0.847	11	174	0.760	0.868	10	50	$2.3 \cdot 10^{-2}$
Cme3[A]		156-192	0.871	0.902	19	170	0.960	0.911	16	50	$7.2 \cdot 10^{-3}$
Cme6		200-222	0.807	0.798	10	166	0.880	0.842	10	50	$3.4 \cdot 10^{-2}$
Cme7		90-102	0.744	0.704	7	164	0.760	0.750	6	50	$8.7 \cdot 10^{-2}$
Cme9[A]		138-200	**0.679**	0.752	11	168	0.565	0.749	8	46	$1.1 \cdot 10^{-1}$
Cme12		188-196	0.291	0.307	4	170	0.292	0.267	4	48	$5.8 \cdot 10^{-1}$
overall											$1.0 \cdot 10^{-13}$

[A]) interrupted repeat; [B]) bold values indicate significant homozygote excess (Bonferroni corrected). F = forward primer; R = reverse primer; bp = basepairs; H_O = observed heterozygosity; H_E = expected heterozygosity; N_A = number of alleles; N_T = total number of alleles observed; P_{ID} = probability of identity.