TECHNICAL NOTE

## Eleven new microsatellite loci in the globally threatened Aquatic Warbler (*Acrocephalus paludicola*)

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Received: 1 September 2011/Accepted: 5 September 2011 © Springer Science+Business Media B.V. 2011

**Abstract** We report the isolation and characterization of eleven microsatellite markers from the Aquatic Warbler (Acrocephalus paludicola) the only globally threatened passerine species in Europe. We tested the markers in 23 samples of the species collected in the Biebrza marshes, Poland, between 1990 and 1997. All markers were polymorphic, with 2-16 alleles per locus, and no differences were found between the observed and expected heterozygosity when applying Bonferroni correction and a tablewide significance level of 0.05. We found no evidence for linkage disequilibrium between the markers. The frequency of null alleles was 0.00-0.43. The new markers will allow further insight in the population genetics and population structure of the Aquatic Warbler. Assessing the potential connectivity between breeding populations and wintering areas can guide further conservation efforts.

**Keywords** Acrocephalus paludicola · Aquatic Warbler · Aves · Conservation · Microsatellites

The Aquatic Warbler Acrocephalus paludicola is the only globally threatened passerine bird species in continental

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Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, INF 364, 69120 Heidelberg, Germany Europe and its global population has declined by >90%during the 20th century (Flade and Lachmann 2008). Once widespread in fen mires and sedge meadows in Central and Eastern Europe the breeding area is now reduced to scattered, isolated patches because of widespread habitat destruction (Flade and Malashevich in prep.). Hence, there is the thread of inbreeding depression with all its predicted negative effects, especially in small populations at the periphery of the species range (O'Grady et al. 2006). The Aquatic Warbler is a long-distance migrant to sub-Saharan Africa, but the exact wintering areas have been unknown until recently when a major wintering area was discovered in northern Senegal (Salewski et al. 2009). Habitat destruction in the European breeding areas may be paralleled on the African wintering grounds (Zwarts et al. 2009; Flade et al. 2011). Therefore, knowledge about the connectivity between breeding populations and non-breeding areas has implications for the implementation of conservation strategies that take the entire annual cycle of the species into account.

A first analysis to characterize the genetic structure of eight Aquatic Warbler populations with respect to genetic diversity within—and gene flow between these populations was performed by Gießing (2002) using six cross-species microsatellite markers. The differentiation between populations was found to be low, but this may have been an artefact due to the low numbers of microsatellite loci used. The same markers were used in an attempt to assign 59 Aquatic Warblers wintering in the Djoudj area, Senegal, to one of eleven breeding populations (Vogel 2009). The low assignment rate was partially explained by the low number of microsatellite markers available. In order to reanalyse the genetic structure of the breeding population, to investigate gene flow between populations, potential inbreeding within populations and to be able to reveal connectivity

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Locus	Motif	Primer sequence 5-3'	Size (bp)	$T_{a}$	z	Allelic size range (bp)	Null allele freq.	$\mathrm{H}_{\mathrm{O}}$	$\mathrm{H_{E}}$	EMBL accession no.
AW-01	$(CA)_4TACATGAA(CA)_2GA(CA)_4GA(CA)_4GA(CA)_5$	F: TAGCCTTTTCTGCTAAGTGG R: ACATACCTGGTGCTGAATTT	207	55	2	205–237	0.00	0.04	0.04	HE583272
AW-02	(TG) <sub>13</sub>	F: GAAGGAGCTGAATTTGACAG R: TGTCCTGTGAAGACGTGAT	243	55	16	234–271	0.00	0.87	0.89	HE583273
AW-03	$(GT)_{10}$	F: CTACTAGCAAAGAGGCCTGA R: TTGACTCAGTCTTGTCACCA	204	55	S	200–206	0.00	0.43	0.50	HE583274
AW-04	(GA) <sub>13</sub>	F: TGGCTAACAACACATTTCAG R: CTGCAGCTTGGAGAAGAA	178	55	ю	171–181	0.00	0.48	0.43	HE583275
AW-05	$(CA)_2 TG(CA)_6 CT(CA)_7 CG(CA)_2 TG(CA)_2 CG(CA)_2$	F: TTGTGAAGGTATCCCAAATC R: CACCAGTGAGCCAAGAAG	232	55	7	227–229	0.00	0.09	0.09	HE583276
4W-06	(CT) <sub>14</sub>	F: GCTCCCTCTCCTCCTTACT R: TGGAGCTTGTGGGGGGTGTAG	124	55	0	117–117	0.43*	0.00	0.16	HE583277
AW-07	$ACAT(AC)_7AA(AC)_4$	F: AGAAGTGGTTGATGAATTGC R: CATTGCACAGAGAAAATTCC	166	55	S	162–167	0.11*	0.52	0.71	HE583278
AW-08	$(GA)_2(GT)_8$	F: ACAATGACCAACTCTCCAAG R: ATACATCAGGCCAAAGAGAA	246	55	9	246–258	0.00	0.78	0.76	HE583279
AW-09	(AC) <sub>2</sub> TC(AC) <sub>7</sub> TC(AC) <sub>2</sub>	F: CATTATGCAAGACCCCAATA R: ATCTGTCAGGTTCACTGAGG	153	55	б	149–152	0.17*	0.09	0.61	HE583280
AW-10	(CA) <sub>2</sub> TA(CA) <sub>9</sub> CTCACC(CA) <sub>2</sub> TA(CA) <sub>2</sub>	F: AAGGGAGGAGACCAAATATC R: CCTTGTTCAGCTTCCTGTAT	190	55	8	159–194	0.00	0.52	0.67	HE583281
AW-11	$(AC)_6(AT)_4$	F: TGAAGTCAGTTCCCACATCT R: TCCTTTTTCTTAGGTCATCG	152	55	$\infty$	149–159	$0.11^{*}$	0.52	0.80	HE583282
F forward the gener	1 primer, R reverse primer, $T_a$ annealing Temperature [°C], al excess of homozygotes for most allele size classes	${\cal N}$ number of alleles, ${\cal H}_O$ observed heteroz	sygosity,	$H_E \exp$	ected	heterozygosity	,* null alleles	may be ]	present a	s suggested by

Table 1 Characterization of 11 microsatellite loci isolated from Aquatic Warblers and genotypes of 23 samples of this species

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between breeding areas and wintering sites we developed eleven new species-specific microsatellite markers for the Aquatic Warbler.

The DNA samples used in this study were collected in 2000 in Belarus (n = 1) and in the Ukraine (n = 1) by B. Gießing and in the Biebrza region, Poland, in 1991 by A. Dyrcz (n = 23). Total DNA was isolated from blood samples using standard proteinase K (Merck, Darmstadt) digestion and phenol/chloroform protocols (Sambrok and Russell 2001). Until analyses, the DNA was stored at  $-20^{\circ}$ C.

Quality and quantity of the extracted DNA was assessed with a NanoDrop ND-1000 Spectrophotometer (PQLab Biotechnology, Erlangen, Germany). We constructed and screened a DNA library enriched for microsatellite sequences from the DNA of two Aquatic Warblers following Glenn and Schable (2005) using the restriction enzymes RsaI and BstUI for genomic digestion and applying the specifications given by Niehuis and Korb (2010). The DNA library sequences were assembled and searched for dinucleotide repeats with BioEdit 7.0.9.0 (Hall 1999). DNA fragments with dinucleotide repeats were additionally checked for internal RsaI and BstUI restriction sites because they could indicate an artificial ligation of different DNA fragments. Finally we used the program Primer3 0.4 (Rozen and Skaletsky 2000) to design locusspecific oligonucleotide primers.

To assess polymorphism of the microsatellite loci, we genotyped samples from 23 Aquatic Warblers from a single population (Biebrza area, Poland). The PCR amplifications were conducted in 20  $\mu$ l volumes with 1  $\times$  Taq incubation buffer, 0.2 mM of each dNTP, 0.5 µM each of forward (5' labelled with the dye TAM, HEX or FAM) and reverse primer, 1.5 mM MgCL<sub>2</sub>, 0.25 U Taq polymerase (MP Qbiogene, California, USA) and 50-100 ng DNA. The PCR temperature profile started with an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and ended with a final extension step of 72°C for 10 min. All PCR products were separated with an ABI 3500 automated sequencer (Applied Biosystems, Foster City, USA) on a 5.25% Page Plus gel (Amresco, Solon, USA) with a 36 cm well-to-read distance. GeneScan 500 ROX Size Standard (Applied Biosystems) was applied as reference to assess the size of the PCR products with the aid of the Peak Scanner Software 1.0 (Applied Biosystems).

Of the twelve microsatellite loci that we tested, eleven proved to amplify reliably and were polymorphic, with 2-16 alleles per locus (Table 1). Using Micro-Checker 2.2.3 (van Oosterhout et al. 2004), we estimated a null allele frequency of 0.00–0.43 (Table 1). We found evidence for the occurrence of null alleles for four loci. The observed (H<sub>o</sub>: 0.00–0.87) and expected (H<sub>E</sub>: 0.04–0.89) heterozygosity (Exofficer et al. 2005) did not differ significantly in the eleven markers when applying a sequential Bonferroni correction and a table-wide significance level of 0.05 (Rice 1989). We did not find evidence for linkage disequilibrium between the eleven markers (Exofficer et al. 2005; sequential Bonferroni correction at a table-wide significance level of 0.05).

The new microsatellite markers will help to understand the population genetics of the Aquatic Warblers and can be used to identify conservation problems and to guide further conservation efforts (Salewski in prep.).

Acknowledgments We thank A. Dyrcz and B. Gießing for collecting the samples, E. Limpinsel and A. Ritz for assistance in data analysis.

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