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TECHNICAL NOTE

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

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

Locus	Motif	Primer sequence 5–3′	Size (bp)	T_a	N	Allelic size range (bp)	Null allele freq.	H_O	H_E	EMBL accession no.
AW-01	$(CA)_4TACATGAA(CA)_2GA(CA)_4GA(CA)_4GA(CA)_5$	F: TAGCCTTTTCTGCTAAGTGG	207	55	2	205–237	0.00	0.04	0.04	HE583272
		R: ACATACCTGGTGCTGAATTT								
AW-02	$(TG)_{13}$	F: GAAGGAGCTGAATTTGACAG	243	55	16	234–271	0.00	0.87	0.89	HE583273
		R: TGTCCTGTGAAGACGTGAT								
AW-03	$(GT)_{10}$	F: CTACTAGCAAAGAGGCCTGA	204	55	5	200–206	0.00	0.43	0.50	HE583274
		R: TTGACTCAGTCTTGTCACCA								
AW-04	$(GA)_{13}$	F: TGGCTAACAACACATTTCAG	178	55	3	171–181	0.00	0.48	0.43	HE583275
		R: CTGCAGCTTGGAGAAGAA								
AW-05	$(CA)_2TG(CA)_6CT(CA)_7CG(CA)_2TG(CA)_2CG(CA)_2$	F: TTGTGAAGGTATCCCAAATC	232	55	2	227–229	0.00	0.09	0.09	HE583276
		R: CACCAGTGAGCCAAGAAG								
AW-06	$(CT)_{14}$	F: GCTCCCTCTCTCCTCTTACT	124	55	2	117–117	0.43*	0.00	0.16	HE583277
		R: TGGAGCTTGTGGGTGTAG								
AW-07	$ACAT(AC)_7AA(AC)_4$	F: AGAAGTGGTTGATGAATTGC	166	55	5	162–167	0.11*	0.52	0.71	HE583278
		R: CATTGCACAGAGAAAATTCC								
AW-08	$(GA)_2(GT)_8$	F: ACAATGACCAACTCTCCAAG	246	55	6	246–258	0.00	0.78	0.76	HE583279
		R: ATACATCAGGCCAAAGAGAA								
AW-09	$(AC)_2TC(AC)_7TC(AC)_2$	F: CATTATGCAAGACCCCAATA	153	55	3	149–152	0.17*	0.09	0.61	HE583280
		R: ATCTGTCAGGTTCACTGAGG								
AW-10	$(CA)_2TA(CA)_9CTCACC(CA)_2TA(CA)_2$	F: AAGGGAGGAGACCAAATATC	190	55	8	159–194	0.00	0.52	0.67	HE583281
		R: CCTTGTTCAGCTTCCTGTAT								
AW-11	$(AC)_6(AT)_4$	F: TGAAGTCAGTTCCCACATCT	152	55	8	149–159	0.11*	0.52	0.80	HE583282
		R: TCCTTTTTCTTAGGTCATCG								

F forward primer, *R* reverse primer, T_a annealing Temperature [°C], *N* number of alleles, H_O observed heterozygosity, H_E expected heterozygosity, * null alleles may be present as suggested by the general excess of homozygotes for most allele size classes

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°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 locus-specific 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 µl volumes with 1 × *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 $MgCL_2$, 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_O: 0.00–0.87) and expected (H_E: 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.).

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