Microsatellite markers for two stifftail ducks: the white-headed duck, *Oxyura leucocephala*, and the ruddy duck, *O. jamaicensis*

VIOLETA MUÑOZ-FUENTES,* NICLAS GYLLENSTRAND,† JUAN J. NEGRO,* ANDY J. GREEN* and CARLES VILÀ‡

*Estación Biológica de Doñana (C.S.I.C.), Avda. María Luisa s/n, 41013 Sevilla, Spain, †Department of Conservation Biology and Genetics, Uppsala University, Norbyvägen 18 D, SE-75236 Uppsala, Sweden, ‡Department of Evolutionary Biology, Uppsala University, Norbyvägen 18 D, SE-75236 Uppsala, Sweden

Abstract

Hybridization with a close relative, the North American ruddy duck (*Oxyura jamaicensis*), is a major problem for the conservation of the endangered white-headed duck (*Oxyura leucocephala*). We report the development of 11 microsatellite markers that can facilitate the identification of hybrids as well as the study of the population structure of both species across their distributions. These markers were tested in 63 white-headed ducks and 50 ruddy ducks and show a larger diversity in the latter species.

Keywords: hybridization, microsatellite primers, ruddy duck, white-headed duck

The white-headed duck, *Oxyura leucocephala*, has a fragmented distribution in the western Palaearctic and is classified as endangered by the World Conservation Union (IUCN) (Green & Hughes 2001). In Spain, the population was reduced to a few dozen individuals in the 1970s. Population recovery since then has been marred by the introduction of the congeneric North American ruddy duck, *Oxyura jamaicensis*. Hybridization and genetic introgression with this species is considered a major threat to the white-headed duck (Green & Hughes 2001). The ruddy duck was introduced in Great Britain in the 1950s, and later spread to other European countries. It was first recorded in Spain in 1983. We developed nuclear microsatellite markers to assess the genetic structure and variability of the white-headed duck and that of the ruddy duck in both its original and introduced ranges, and also to identify hybrids between the two species.

We developed separate microsatellite libraries for each species. DNA for library construction was extracted from muscle tissue of one female white-headed duck and one female ruddy duck using DNeasy Tissue Kit (QIAGEN). Approximately 3 μg of extracted DNA was digested using *Mbo*I (Fermentas) and enriched for CA and CATC repeats following the protocol of Fleischer & Loew (1995) with modifications (available upon request). Modifications included biotinylating the 3′ end of the oligonucleotides (Koblízková *et al*. 1998) and adding spacers (Kandpal *et al*. 1994). Positive colonies were amplified through polymerase chain reaction (PCR) using the modified UNI primer (5′-CGACGTTG-TAAAACGAGGCCAGT-3′) and the OMNI primer (5′-ACAGGAAACAGCTATGACCATGAT-3′). Amplified products were sequenced on a MegaBACE capillary sequencer (Amersham) using DYEnamic ET Terminator Cycle Sequencing Kit (Amersham).

Sequences were visualized using autoassembler 2.1 (Applied Biosystems) and PCR primers were designed using primer3 (Rozen & Skaletsky 2000). To avoid labelling individual primers, we added an M13Reverse or CAG tag to the 5′ end of the forward primer, and added a labelled M13Reverse or CAG tag in the amplification reactions (Hauswaldt & Glenn 2003). We added a tail GTT, GTTT or GTTTCT to the 5′ end of the reverse primer to promote adenylation and therefore decrease stuttering and background noise (Brownstein *et al*. 1996). Modified primers were evaluated using netprimer (PREMIER Biosoft International).

Primers were designed for 16 loci, nine corresponding to white-headed duck clones and seven to ruddy duck clones. PCR was carried out in 10-μL reactions containing 1 × Gold Buffer (15 mm Tris-HCl, pH 8.0, 50 mm KCl; Applied

Correspondence: Violeta Muñoz-Fuentes, Fax: +34 954 62 11 25; E-mail: vio@ebd.csic.es

Table 1 Characterization of 11 white-headed duck (*Oxyura leucocephala*, *leu*) and ruddy duck (*Oxyura jamaicensis*, *jam*) microsatellite loci. Species indicates the species from which the microsatellite was isolated

Locus	Species	Repeat motif	Primer secquence (5′–3′)	Third primer	T_a (°C)	Size range (bp)	*O. leucocephala* (n = 63)			*O. jamaicensis* (n = 50)			Total no. different alleles
							N_a	H_O	H_E	N_a	H_O	H_E	
Oxy3	*leu*	$(CA)_{15}$	F: CAGTCGGGCGTCATCACTGCTGGAGGGTAAC R: GTTTAACAAATGGCCCAGCAC	CAG tag	55	181–193	3	0.27	0.28	2	0.12	0.11	5
Oxy4	*leu*	$(TG)_{10}$	F: GGAAACAGCTATGACCATCCCGTCTTACAGGAGA R: GTTAGGCATTTGCACCCTATCAG	M13	57	236–250	3	0.49	0.45	8	0.29	0.69	10
Oxy6	*leu*	$(CA)_{10}$	F: CAGTCGGGCGTCATCAAGATTCTGGGATTCAAA R: GTTAAAAATGGGCTCTTGGAAGG	CAG tag	57	245 –249	2	0.53	0.43	2	0.45	0.48	3
Oxy10	*jam*	$(CA)_{13}$	F: GGAAACAGCTATGACCATCACCAAGGGGAAGAGTCA R: GTTTGTCTGAGGCATTTGCAC	M13	57	158 –172	3	0.49	0.46	11	0.72	0.79	11
Oxy11	*jam*	$(CA)_{11}$	F: CAGTCGGGCGTCATCATGCAGTGAAGTCTGG R: GTTTAGCTCTGCATGGAATGGTG	CAG tag	57	188 –200	3	0.41	0.45	5	0.24	0.25	7
Oxy13	*jam*	$(ATGG)_{11}$	F: CAGTCGGGCGTCATCAGGAATCAATGAGATTAG R: GTTTATGGGGTGCTGCTTCTGAG	CAG tag	57	193–228	1	0.00	0.00	17	0.86	0.87	18
Oxy15	*leu*	$(AC)_{12}$	F: CAGTCGGGCGTCATCACAGAGGTCTCCTTGGTCC R: GTTCAAGCCAGACCAGACGATTTC	CAG tag	55	227–235	1	0.00	0.00	4	0.20	0.19	5
Oxy17	*jam*	$(CA)_{12}$	F: CAGTCGGGCGTCATCAATTTAAGGCCATCCTC R: GTTGGACTGAAAACAGCCACTTC	CAG tag	57	209 –221	1	0.00	0.00	6	0.72	0.72	6
Oxy19	*jam*	$(GT)_{10}$	F: GGAAACAGCTATGACCATACGGTGTAGTTCCCTTC R: GTTGATCCCATGGGCTAGTGAAC	M13	55	218 –222	1	0.00	0.00	3	0.51	0.52	3
Oxy1	*leu*	$(TGGA)_5$ TAGA $(TGGA)_3$	F: CAGTCGGGCGTCATCAGTGGGTTAGATGGATG R: GTTTCCTGCCACATCCCCTCAT	CAG tag	55	134 –154	2	0.00	0.03	3	0.28	0.25	4
Oxy14	*leu*	$(TG)_{15}TT$ $(TG)_6$	F: GGAAACAGCTATGACCATCCACTACATGGGCATC R: GTTATGGCTCATGGGGAAAAAC	M13	55	131–147	2	0.15	0.14	8	0.50	0.71	9

T_a, annealing temperature; bp, base pairs; N_a, number of alleles; H_O, observed heterozygosity, H_E, expected heterozygosity; n, total number of individuals typed.
A third primer fluorescently labelled and complementary to the beginning of the forward primer was included in the PCR amplification (see text).
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Biosystems), 2.5 mm of $MgCl_2$, 1 mm of dNTPs (0.25 mm each), 0.5 μm of reverse primer, 0.45 μm of fluorescently labelled primer, 0.05 μm of tag-labelled primer, 25–100 ng of white-headed duck or ruddy duck genomic DNA and 0.35 U of Ampli*Taq* Gold (Applied Biosystems). PCRs were performed in a PTC-225 Tetrad Thermal Cycler (MJ Research) using the following conditions: 94 °C for 6 min; 35 cycles of 94 °C for 40 s, 57 °C or 55 °C depending on primers (see Table 1) for 20 s, and 72 °C for 30 s; and a final extension at 72 °C for 10 min. PCR products were scored for amplification in agarose gels and then electrophoresed on a MegaBACE sequencer (Amersham). Fragment sizes were determined using genetic profiler version 2.0 (Amersham) by comparison to a size standard. These primers were tested for amplification and poly- morphism in 12 white-headed ducks and 11 ruddy ducks from widespread localities across their ranges. Eleven loci were polymorphic for at least one species (Table 1), one was monomorphic, its size being the same for both species (Oxy2), one locus amplified only in ruddy duck and was monomorphic (Oxy20), and three failed to amplify in both species. Redesigning the latter primers failed to amplify these loci. Six of the 11 polymorphic loci had been isolated from white-headed duck DNA clones and five from ruddy duck DNA clones. The 11 polymorphic loci were then used to screen a total of 57 Spanish white-headed ducks, six Greek white-headed ducks and 50 North American ruddy ducks. We calculated observed and expected heterozygosities (Table 1) and performed Hardy–Weinberg and linkage disequilibrium tests using microsatellite toolkit (Park 2001) and genepop on the web (Raymond & Rousset 1995).

Table 1 summarizes the characteristics of these markers. The mean number of alleles per locus was 1.6 for Spanish white- headed ducks, 1.8 for Greek white-headed ducks, and 6.3 for ruddy ducks. When all loci were considered, the observed heterozygosity (±SD) was 0.216 ± 0.017 for Spanish white- headed ducks, 0.161 ± 0.046 for Greek white-headed ducks and 0.445 ± 0.022 for ruddy ducks. All these measures consistently suggest that the genetic diversity is larger for ruddy ducks than for white-headed ducks. For loci Oxy4, Oxy10 and Oxy13 in ruddy ducks, we identified some alle- les differing by just 1 bp from each other, which indicates additional variability besides the number of tandem repeats in the microsatellite. After applying Bonferroni's sequen- tial correction, Oxy1 in the case of white-headed ducks and Oxy4 and Oxy14 in the case of ruddy ducks did not con- form to Hardy–Weinberg expectations. However, because the samples may include several populations, we cannot evaluate if these deviations imply presence of null alleles, mating biases or just population fragmentation. Evidence for linkage disequilibrium between Oxy4 and Oxy10 was found in both white-headed ducks and ruddy ducks. However, this linkage could also derive from the pooling of individuals from different populations and needs further investigation. No scoring errors associated with

large allele dropout or stuttering were detected using the program micro-checker (Van Oosterhout *et al*. 2004).

These microsatellite markers can be used for genetic population studies and paternity analyses particularly in ruddy ducks. Because most of the alleles are unique for each species (Table 1), the use of these microsatellites has the potential to unambiguously distinguish hybrids from pure individuals and to assess to what degree natural popu- lations have been affected by hybridization.

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