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TECHNICAL NOTE

A marker suitable for sex-typing birds from degraded samples

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Abstract A new primer set was developed for sex-typing birds, *Z37B*. This primer set was designed to amplify alleles of small size to render it suitable for sex-typing degraded samples, including shed feathers. This marker successfully sex-typed 50 % of the species tested, including passerines, shorebirds, rails, seabirds, eagles and the brown kiwi *Apteryx australis* (allele size range =81–103 bp), and is therefore expected to be suitable for sex-typing a wide range of species. *Z37B* sex-typed non-degraded samples (blood), degraded tissue (dead unhatched embryos, dead nestlings and museum specimens) and samples of low quantity DNA (plucked feathers and buccal swabs). The small amplicon sizes in birds suggest that this marker will be of utility for sex-typing feathers, swabs and degraded samples from a wide range of avian species.

Keywords AVES · Birds · Degraded samples · Feathers · Sex-typing · Non-invasive samples

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Introduction

In most bird species the sexes are morphologically indistinguishable, *ca* 50 % of adults (60 % of passerines) and the majority of nestlings (Price and Birch 1996). Blood samples are often used for sex-typing birds; however, taking blood can cause stress, discomfort and may, on occasion, damage wings and/or introduce infection (Joint Working Group on Refinement 2001). It also requires training and appropriate permits. Therefore, less invasive techniques are preferred, especially when studying endangered species—for example, using shed feathers, museum specimens and swabs. Furthermore, studies of fertility and sex ratio require the ability to sex-type degraded tissue (e.g. unhatched eggs, Brekke et al. 2010). However, allelic dropout can occur when attempting to PCR-amplify large products from degraded samples (Toouli et al. 2000) and dropout causes errors in sex-typing (Robertson and Gemmell 2006). We therefore attempted to develop a primer set that amplifies small PCR products (<150 bp) on both the W and Z chromosomes to enable sex-typing of degraded samples.

Methods

Following Dawson et al. (2010), a zebra finch *Taeniopygia guttata* EST microsatellite sequence DV945670 (Replogle et al. 2008) was identified with strong homology to the chicken *Gallus gallus* Z chromosome. We created a consensus sequence from these homologous sequences using MEGA3 (Kumar et al. 2004) and designed a primer set using PRIMER3 v0.4.0 (Rozen and Skaletsky 2000). Both the forward and reverse primers were 100 % identical to both the zebra finch Z and chicken Z chromosomes (no

homologous W chromosome sequence was available). In order to create a primer set suitable for amplifying degraded samples, we designed the primer set to amplify a small product (<150 bp) whilst avoiding the use of degenerate bases (Table 1). Primer sequences, melting temperatures and the expected and observed allele sizes in zebra finch and chicken are provided (Table 1). The locus (DV945670) was homologous to mRNA sequences of the guanine nucleotide binding protein (G protein), q polypeptide (GNAQ) present in many taxa. Both primer sequences were 100 % identical to 9/10 birds assessed, including passerines, penguins and other seabirds, eagle, duck and chicken (details provided in the footnotes of Table 1).

Genomic DNA was extracted from non-degraded samples (bird blood, blood slides), samples of low quantity (shed and plucked feathers, buccal swabs), degraded tissue [dead embryos from unhatched eggs, dead nestlings, museum (toe pad) samples] and crocodile skin using an ammonium acetate protocol (Richardson et al. 2001) or commercial kits (for the museum specimens and mouth swabs). Full details of the samples and extraction methods are provided in Supplementary Table 1. The primer set was tested by sex-typing individuals of 42 avian species including one ratite, the brown kiwi *Apteryx australis* (25 families and 15 orders; Table 2), and the saltwater crocodile *Crocodylus porosus*. Individuals of known sex (both females and males) were included for 40 of the bird species (for two species known females were available but no known males; Table 2). Sexes were previously identified based on morphology, behaviour and/or sex-typing markers (Table 2). PCR reactions were performed in 2-µl (10-µl for museum samples) volumes containing *ca* 10 ng of lyophilised genomic DNA, 1 or 5 µl of QIAGEN Multiplex PCR Master Mix and 0.2 µM of each primer (with the forward primer fluorescently labelled with HEX). PCR amplification was performed using a DNA Engine Tetrad thermal cycler. PCR amplification conditions were 95 °C for 15 min; followed by 35 cycles of 94 °C for 30 s, 56 °C for 90 s, 72 °C for 1 min, and finally 60 °C for 30 min. PCR products were loaded on a 48-capillary ABI 3730 DNA Analyzer and genotypes assigned using GeneMapper software (Applied Biosystems).

Results

All 42 bird species tested amplified, as did the saltwater crocodile (amplicons = 81–110 bp, saltwater crocodile = 110 bp; Table 2). Twenty-one species (50 %) were successfully sexed: including passerines, shorebirds, rails, seabirds, eagles and the brown kiwi (Table 2). In all species sexed, the diagnostic W allele (81–92 bp) was smaller than the Z allele (92–100 bp; Table 2). The difference in size between the W and Z alleles within a species was small (2–19 bp, Table 2) and resolving this difference required an ABI DNA Analyzer.

Individuals were successfully sex-typed from the degraded tissues including unhatched embryos, dead nestlings and museum toe-pads (of the hihi *Notiomystis cincta*), and from samples of low-quantity DNA, i.e. plucked feathers (hihi, northern fulmar *Fulmarus glacialis*) and buccal swabs (corncrake *Crex crex*; Table 2). Individuals whose DNA was extracted from non-degraded blood samples were also successfully sex-typed (Table 2). *Z37B* successfully sex-typed individuals when included as part of a microsatellite multiplex set (hihi and corncrake; PB unpublished data).

Many species displayed a single allele (i.e. of same size) in both sexes and could therefore not be sexed (43 %; Table 2), probably due to failure of the W locus to amplify (or possibly a lack of difference in size between the Z and W amplicons). Eight species (19 %) displayed polymorphism in the Z locus and for three of these species (7 %) all females were homozygous—suggesting that the W locus failed to amplify (probably due to primer–W chromosome base mismatches; Table 2). In some species, the W locus might require a lower PCR annealing temperature to amplify, such as 50 °C. We recommend the use of Qiagen Multiplex Master Mix for PCR sex-typing because it more often enables amplification even when there are some target–primer base mismatches (DAD unpublished data). Although not causing error here, Z (and/or W) polymorphism can lead to error when interpreting sexes, unless a second sex-typing marker and/or known sexes are included (Dawson et al. 2001, Robertson and Gemmell 2006).

Marker *Z37B* is of utility for sex-typing degraded samples. It provides an alternative marker to validate sex-typing data. Most of the passerine species tested could be sex-typed with this marker, suggesting it will be of utility for sex-typing many of the *ca* 5,000 species in this order. In addition, the successful sex-typing of non-passerines including shorebirds, rails, seabirds, eagles and kiwi, suggests *Z37B* will be of utility in a wide range of species.

Table 1 A new sex-typing marker for birds (*Z37B*), designed from the zebra finch *Taeniopygia guttata* Z and chicken *Gallus gallus* Z chromosome

Locus	Primer sequence 5′ – 3′ (and fluoro-label)[a]	Primer T_m (°C)	PCR T_a (°C)	Repeat motif	Expected allele size based on sequences used to design primers (bp)
Primer sequences					
Z37B	(F) [HEX] AACTGGTTGTAGGTATAGTGCAATTATG	60.04	56	$(AT)_n$	94–104
	(R) GATTACAAAGCCAATATGGATGC	59.73			

Sequence details: species origin, sequence type	Sequence accession number/source	Chr. associated with sequence[b]	Repeat motif	Expected allele size based on sequence (bp)	Observed allele sizes (in 6 ZF or 1 CH male(s) (ZZ); bp)[c,d]
Sequences used to design the primer set					
Zebra finch EST	DV945670	Unknown	$(AT)_{12}$	102	99, 103 (ZF)
Zebra finch genome	ENSEMBL	Z	$(AT)_{13}$	104	99, 103 (ZF)
Chicken BAC	AC186343	Z	$(AT)_6$	94	92 (CH)
Chicken genome	ENSEMBL	Z	$(AT)_6$	94	92 (CH)

T_m, melting temperature obtained from PRIMER3 v0.4.0

T_a, annealing temperature used for PCR

[a] Both primer sequences were 100 % identical to all birds assessed except turkey (*Meleagris gallopavo*; no hit). Those 100 % identical included passerines (*Taeniopygia guttata*, *Serinus canaria*, *Zonotrichia albicollis*, *Ficedula albicollis*), seabirds (*Fulmarus glacialis*) including penguins (*Pygoscelis adeliae*, *Aptenodytes forsteri*), eagles (*Haliaeetus albicilla*), ducks (*Anas platyrhynchos*) and chicken (*Gallus gallus*; ENSEMBL and NCBI databases assessed). The chromosome locations in the above species are unknown (except chicken and zebra finch), since the sequences have not been assigned to named chromosomes

[b] Location of locus (DV945670) in the chicken genome: Z chr., 37,645,106 bp and zebra finch genome Z chr., 54,801,985 (as of 7th November 2014)

[c] Known sexes based on morphology of adult birds

[d] See Table 2 for number of individuals of each sex tested, details of the other species tested and identities of those species successfully sexed

EST, expressed sequence tag; *BAC*, bacterial artificial chromosome; *ZF*, zebra finch; *CH*, chicken

Table 2 Assessment of the *Z37B* marker for sex-typing 42 species of birds belonging to 25 families in 15 orders using various tissue types including non-degraded blood, degraded tissue (dead unhatched embryos, dead nestlings and museum specimens) and samples of low quantity DNA (plucked/shed feathers and mouth swabs)

Order (sub-order)[a]	NCBI taxonomic classification	Species, binomial name	Tissue[b]	n	Known females	Known males	Z37B W allele size (bp)	Z37B Z allele size(s) (bp)	Sexed with Z37B?
AVES; Neognathae	NON-RATITES								
Anseriformes	Anatidae	Mallard	Dead embryos	5	2	3		92	N
(Ducks)		*Anas platyrhynchos*							
		Muscovy duck	Blood	2	1	1		92	N
		Cairina moschata							
		Blue duck	Blood	3	2	0		92	N
		Hymenolaimus malacorhynchos							
Bucerotiformes	Bucerotidae	Monteiro's hornbill	Blood	2	1	1		92	N
		Tockus monteiri							
Charadriiformes	Scolopacidae	Ruff	Blood	11	7	4	90	92	Y
(Shorebirds)		*Philomachus pugnax*							
		Curlew sandpiper	Blood	4	2	2	90	92	Y
		Calidris ferruginea							
		Broadbilled sandpiper	Blood	3	1	2	90	92	Y
		Limicola falcinellus							
		Dunlin	Blood	3	1	2	90	92	Y
		Calidris alpina							
		Redshank	Blood	4	2	2	90	92	Y
		Tringa totanus							
		Terek sandpiper	Blood	4	3	1	90	92, 94	Y
		Xenus cinereu							
		Turnstone	Blood	4	2	2	90	92	Y
		Arenaria interpres							
	Charadriidae	Snowy plover	Blood	3	1	2	90	92	Y
		Charadrius nivosus							
		Ringed plover	Blood	4	2	2		92	N
		Charadrius hiaticula							
		"	Plucked Feather	4	2	2		92	N
Columbiformes	Columbidae	Seychelles turtle dove	Blood	2	1	1		92	N
		Streptopelia picturata rostrata							
Coraciiformes	Meropidae	European bee-eater	Blood	2	1	1		94	N
		Merops apiaster							
Falconiformes	Accipitridae	Golden eagle	Blood	19	10	9	90	94	Y

Table 2 continued

Order (sub-order)[a]	NCBI taxonomic classification	Species, binomial name	Tissue[b]	n	Known females	Known males	Z37B W allele size (bp)	Z37B Z allele size(s) (bp)	Sexed with Z37B?
(Eagles and Falcons)	(Accipitrinae)	*Aquila chrysaetos*							
		Spanish Imperial eagle	Blood	7	2	5	92	94	Y
		Aquila adalberti							
		White tailed sea eagle	Blood	10	6	4	90	94	Y
		Haliaeetus albicilla							
		Bonelli's eagle	Blood	9	6	3	90	94	Y
		Hieraaetus fasciatus							
		Common buzzard	Blood	10	7	3	88	92	N
		Buteo buteo					(n = 1)		
	Pandioninae	Western osprey	Blood	6	5	1		92	N
		Pandion haliaetus haliaetus							
		"	Shed feather	1	1	0		92	N
	Falconidae	Saker	Blood	5	2	1		93	N
		Falco cherrug							
		Eleonora's falcon	Blood	4	4	0		93	N
		Falco eleonorae							
Galliformes	Megapodiidae	Australian brush-turkey	Blood	2	1	1		94	N
		Alectura lathami							
"	Phasianidae	Chicken (Crittenden breed)	Blood	2	1	1		92	N
		Gallus gallus							
Gruiformes	Gruidae	Blue crane	Blood	2	1	1		92	N
(Cranes and Rails)		*Anthropoides paradiseus*							
	Rallidae	Corncrake	Blood	80	60	20	90	92	Y
		Crex crex	Blood slide	17	9	8	90	92	Y
		"	Buccal swab	8	2	6	90	92	Y
		"	Tissue (Dead adults)	73	44	29	90	92	Y
Passeriformes	Paridae	Blue tit	Blood	24	11	13		97, 99, 103	N
(Songbirds)		*Parus caeruleus*						(F = homozygous)	(W fail?)[c]
"	Estrildidae	Zebra finch	Blood	14	8	6		99, 103	N
	(Passeridae)	*Taeniopygia guttata*						(all homozygous)	
"		Gouldian finch	Blood	15	9	6	81	96, 98, 100	Y
		Erythrura gouldiae							
"	"	Sociable weaver	Blood	15	8	7	81	96, 98, 100	Y
		Philetairus socius							
	Parulidae	Seychelles warbler	Blood	4	2	2	83	96	Y

Table 2 continued

Order (sub-order)[a]	NCBI taxonomic classification	Species, binomial name	Tissue[b]	n	Known females	Known males	Z37B W allele size (bp)	Z37B Z allele size(s) (bp)	Sexed with Z37B?
		Acrocephalus sechellensis							
"	Meliphagidae	Hihi (Stitchbird)	Blood	182	88	94	82	94	Y
"	"	*Notiomystis cincta*	Dead embryos	56	24	32	82	94	Y
"	"	"	Dead nestlings	113	59	54	82	94	Y
"	"	"	Feathers (plucked)	55	36	19	82	94	Y
"	"	"	Museum specimen (toe-pads)	12	4	8	82	95, 97	Y
"	Formicariidae (Thamnophilidae)	Dusky antbird *Cercomacra tyrannina*	Blood	2	1	1		96	N
Piciformes (Woodpeckers)	Picidae	Acorn woodpecker *Melanerpes formicivorus*	Blood	8	4	3		96, 98, 100, 102 (F = homozygous)	N (W fail?)[c]
Procellariiformes (Seabirds)	Hydrobatidae	Leach's storm petrel *Oceanodroma leucorhoa*	Blood	24	10	14	90	92	Y
"	Procellariidae	Round Island petrel *Pterodroma arminjoniana*	Blood	10	4	6	90	92	Y
		Northern fulmar *Fulmarus glacialis*	Plucked feathers	6	3	3	90	92	Y
Psittaciformes (Parrots)	Psittacidae	Peachy-faced lovebird *Agapornis roseicollis*	Blood	2	2	0		93, 95 (F = homozygous)	N (W fail?)[c]
Sphenisciformes (Penguins)	Spheniscidae	Macaroni penguin *Eudyptes sclateri*	Blood	22	8	14		92	N
Strigiformes (Owls)	Tytonidae	Barn owl (Hungarian) *Tyto alba guttata*	Blood	2	1	1		96	N
AVES; Palaeognathae	RATITES								
Apterygiformes (Kiwi)	Apterygidae	Brown kiwi *Apteryx australis*	Blood	14	8	6	92	96-8	Y

n, number of individuals genotyped; Known F, Known M, numbers of known females or males, respectively, genotyped (sex as based on morphology, behaviour and/or PCR-sexed with P2-P8 (Griffiths et al. 1998) and/or 2550F-2718R (Fridolfsson and Ellegren 1999) and/or Z-002 (Dawson 2007))

[a] Details of those providing the samples are listed in Supplementary Table 1

[b] "blood" refers to non-degraded blood, freshly collected and stored in absolute ethanol

Y yes, N no, F female, M male

[c] A complete lack of any heterozygotes in females suggests the W allele failed to amplify

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References

Brekke P, Bennett PM, Wang J, Pettorelli N, Ewen JG (2010) Sensitive males: inbreeding depression in an endangered bird. Proc R Soc Lond B 277:3677–3684

Dawson DA (2007) Genomic analysis of passerine birds using conserved microsatellite loci. PhD Thesis, University of Sheffield, UK

Dawson DA, Darby S, Hunter FM, Krupa AP, Jones IL, Burke T (2001) A critique of CHD-based molecular sexing protocols illustrated by a Z-chromosome polymorphism detected in auklets. Mol Ecol Notes 1:201–204

Dawson DA, Horsburgh GJ, Küpper C, Stewart IRK, Ball AD, Durrant KL, Hansson B, Bacon I, Bird S, Klein Á, Lee J-W, Martín-Gálvez D, Simeoni M, Smith G, Spurgin LG, Burke T (2010) New methods to identify conserved microsatellite loci and develop primer sets of high utility—as demonstrated for birds. Mol Ecol Resour 10:475–494

Fridolfsson AK, Ellegren H (1999) A simple and universal method for molecular sexing of non–ratite birds. J Avian Biol 30:116–121

Griffiths R, Double MC, Orr K, Dawson RJG (1998) A DNA test to sex most birds. Mol Ecol 7:1071–1075

Joint Working Group on Refinement (2001) Laboratory birds: refinements in husbandry and procedures. Fifth report of BVAAWF/FRAME/RSPCA/UFAW. Lab Anim 35:1–163

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform 5:150–163

Price T, Birch GL (1996) Repeated evolution of sexual color dimorphism in passerine birds. Auk 113:842–848

Replogle K, Arnold AP, Ball GF et al (2008) The Songbird Neurogenomics (SoNG) initiative: community-based tools and strategies for study of brain gene function and evolution. BMC Genom 9:131

Richardson DS, Jury FL, Blaakmeer K, Komdeur J, Burke T (2001) Parentage assignment and extra-group paternity in a cooperative breeder: the Seychelles warbler (*Acrocephalus sechellensis*). Mol Ecol 10:2263–2273

Robertson BC, Gemmell NJ (2006) PCR-based sexing in conservation biology: wrong answers from an accurate methodology? Conserv Genet 7:267–271

Rozen S, Skaletsky HJ (2000) In: Krawetz S, Misener S (eds) Bioinformatics methods and protocols methods in molecular biology. Humana Press, Totowa, pp 365–386

Toouli CD, Turner DR, Grist SA, Morley AA (2000) The effect of cycle number and target size on polymerase chain reaction amplification of polymorphic repetitive sequences. Anal Biochem 280:324–326