RESEARCH ARTICLE

A New Marker Based on the Avian *Spindlin* Gene That Is Able to Sex Most Birds, Including Species Problematic to Sex With *CHD* Markers

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We have developed a new marker (Z43B) that can be successfully used to identify the sex of most birds (69%), including species difficult or impossible to sex with other markers. We utilized the zebra finch *Taeniopygia guttata* EST microsatellite sequence (CK309496) which displays sequence homology to the 5′ untranslated region (UTR) of the avian *spindlin* gene. This gene is known to be present on the Z and W chromosomes. To maximize cross-species utility, the primer set was designed from a consensus sequence created from homologs of CK309496 that were isolated from multiple distantly related species. Both the forward and reverse primer sequences were 100% identical to 14 avian species, including the Z chromosome of eight species and the chicken *Gallus gallus* W chromosome, as well as the saltwater crocodile *Crocodylus porosus*. The Z43B primer set was assessed by genotyping individuals of known sex belonging to 61 non-ratite species and a single ratite. The Z and W amplicons differed in size making it possible to distinguish between males (ZZ) and females (ZW) for the majority (69%) of non-ratite species tested, comprising 10 orders of birds. We predict that this marker will be useful for obtaining sex-typing data for ca 6,869 species of birds (69% of non-ratites but not galliforms). A wide range of species could be sex-typed including passerines, shorebirds, eagles, falcons, bee-eaters, cranes, shags, parrots, penguins, ducks, and a ratite species, the brown kiwi, *Apteryx australis*. Those species sexed include species impossible or problematic to sex-type with other markers (magpie, albatross, petrel, eagle, falcon, crane, and penguin species). Zoo Biol. XX:XX–XX, 2016.

Keywords: AVES; bird; data validation; sex typing; *spindlin* gene; W and Z chromosomes

INTRODUCTION

In at least 50% of all bird species, the sexes of adults are morphologically indistinguishable and for the majority of species, nestlings cannot be sexed. We developed a new marker for sex-typing birds that can be used to identify sex in most species, including those that are impossible or problematic to sex-type with other published markers. This marker, *Z43B*, can also be used as a second marker to confirm the accuracy of sex-typing data.

Species Which Are Impossible or Problematic to Sex-Type With Currently Available Markers

One of the most commonly used bird sex-typing primer sets is *P2–P8* which distinguishes between sex based on a difference in size between amplicons of CHD-Z and CHD-W genes (Chromodomain-Helicase-DNA-binding gene; Griffiths et al. [1998]; see the BIRD SEX-TYPING webpage: http://www.shef.ac.uk/nbaf-s/databases/birdsexing). This set is able to sex approximately 80% of non-ratite species [Dawson, 2007]. Species that cannot be sex-typed with *P2–P8* include eagles, falcons, and vultures, Pelecaniformes, Piciformes, geese, owls, petrels, albatrosses, pigeons, and doves (Table 1).

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TABLE 1. Examples of species that cannot be sexed with the P2–P8 *CHD* primer set [Griffiths et al., 1998] or for which optimization was required

Order	Common name	Latin name	P2–P8 sexing result	References
Anseriformes	Bar-headed goose	*Anser indicus*	Sexing not possible	Vucicevic et al. [2013]
Anseriformes	Hawaiian goose	*Branta sandvicensis*	Sexing not possible	Vucicevic et al. [2013]
Columbiformes	Multiple pigeon species		Sexing not possible	Filipa Martins and Susana Lopes, pers. comm.
Columbiformes	Multiple dove species		Sexing not possible	Filipa Martins and Susana Lopes, pers. comm.
Columbiformes	Seychelles turtle dove	*Streptopelia picturata rostrata*	Sexing not possible	Andrew Krupa, pers. comm.
Falconiformes	Eurasian Griffon vulture	*Gyps fulvus*	Sexing not possible	Kocijan et al. [2011]
Falconiformes	Eagles and Old World vultures		Sexing not possible	Itoh et al. [2001], Sacchin et al. [2004], and Reddy et al. [2007]
Galliformes	Indian peafowl	*Pavo cristatus*	Required optimization	Andrew Krupa, pers. comm.
Gruiformes	Blue crane	*Anthropoides paradiseus*	Required optimization	Kate Carstens, pers. comm.
Passeriformes	Black-billed magpie	*Pica pica*	Required optimization	Juan-Gabriel Martinez, pers. comm.
Passeriformes	Brazilian tanager	*Ramphocelus (carbo) bresilius*	Required optimization	Denise Nogueira, pers. comm.
Passeriformes	Pied flycatcher	*Ficedula hypoleuca*	Required optimization	Nicola Goodship, pers. comm.
Passeriformes	Fairy martin	*Hirundo ariel*	Required optimization	Ian Stewart, pers. comm.
Pelecaniformes	European shag	*Phalacrocorax aristotelis*	Sexing not possible	Kocijan et al. [2011]
Pelecaniformes	Scarlet ibis	*Eudocimus ruber*	Sexing not possible	Vucicevic et al. [2013]
Piciformes	White-throated toucan	Ramphastos cuvieri	Sexing not possible	Vucicevic et al. [2013]
Procellariiformes	White-chinned petrel	*Procellaria (aequinoctialis) aequinoctialis*	Sexing not possible	Douglas Ross, pers. comm.
Procellariiformes	Multiple albatross species		Sexing not possible	Douglas Ross, pers. comm.
Spheniscifformes	Gentoo penguin	*Pygoscelis papua*	Required optimization	Douglas Ross, pers. comm.
Spheniscifformes	Macaroni penguin	*Eudyptes (pachyrhnchus) sclateri*	Required optimization	Douglas Ross, pers. comm.
Strigiformes	Eagle owl	*Bubo bubo*	Sexing not possible	Vucicevic et al. [2013]
Strigiformes	Barn owl	*Tyto alba*	Required optimization	Akos Klein, pers. comm.
Ten orders of birds	(47 species)		Required optimization	Jensen et al. [2003]

For those species that can be sexed with *P2–P8*, amplification usually requires a low annealing temperature (48°C) and a touchdown PCR program. Many species require species-specific PCR optimization; for example, testing a range of magnesium chloride concentrations and/or different annealing temperatures, and, in some cases, extending PCR step time lengths and cycle numbers: examples include passerines, cranes, penguins, owls, and other birds of prey (Table 1).

Difficulties in the Genetic Sex-Typing of Ratites

Most ratites can only be sex-typed using species-specific markers and cannot be sex-typed with the *P2–P8* primer set (e.g., ostrich *Struthio camelus*, Griffiths et al. [1998]; Southern cassowary *Casuarius casuarius*, emu *Dromaius novae-hollandiae* and greater rhea *Rhea americana*; Vucicevic et al. [2013]). We therefore tested the utility of the *Z43B* marker in five ratite species.

Factors Affecting the Accuracy of Sex-Typing Data

Several factors can lead to errors in the sexing of individuals when using PCR based methods [Dawson et al., 2001; Robertson and Gemmell, 2006; Casey et al., 2009]. The most commonly observed of these is error due to dropout of the W allele which makes true females (ZW) appear male (ZZ; DAD pers. obs., BIRD SEX-TYPING webpage: http://www.shef.ac.uk/nbaf-s/databases/birdsexing). Allelic dropout [Toouli et al., 2000] is possible for any autosomal/sex locus but is more likely for sex-typing markers, and is caused by base differences between the primer bind regions on the W and Z chromosome homologs (as opposed to difference in the primer bind regions between two alleles of a single autosomal locus). By selecting (Z-W) homologous sequences that are highly conserved between multiple species (ideally species that are distantly related), it is possible to reduce the likelihood of allelic dropout [Dawson et al., 2010]. A second source of error when performing genetic sex-typing is associated with the occurrence of Z polymorphism, as, for example, has been observed for *P2–P8* in auklets [Dawson et al., 2001]. Z-polymorphism leads to some males possessing two differently sized (Z) alleles. It is common to assume that when two differently sized amplicons are observed, it indicates the individual is female, whereas observation of a single amplicon indicates a male, and because of these assumptions, unrecognized Z-polymorphism leads to the incorrect classification of true males as females. A third error source is polymorphism of

the W allele which is rare but is also possible and can lead to error when interpreting sex-typing data. If any of the W alleles are identical or very similar in size to the Z allele they will remain undetected and true females will be mistaken for males. The fourth, and perhaps rarest source of error, is the potential for heteroduplexes, where extra non-specific products are amplified, leading to true males to being mistaken as females, when two alleles are incorrectly assumed to indicate a female [Casey et al., 2009]. These potential sources of error highlight that it is important when interpreting sex-typing data to identify which alleles are specific to the Z chromosome and which are the W-linked alleles, and this is achieved by comparing the sizes of the different alleles amplified in each sex. The allele that is only present in females is predicted to be the W allele and the allele observed in both sexes is expected to be the Z-linked allele. Sex-typing error can be easily recognized by including several individuals of known sex for both sexes, however, sex can be distinguished based on morphology for only *ca* 50% of bird species. Therefore to ensure accurate sex-typing, the only validation method for these sexually monomorphic species, would be to amplify each individual with a combination of two (or more) genetic sex-typing markers (ideally from different loci) and then compare the data from each marker.

We have identified a new marker capable of sex-typing species that *P2–P8* cannot and that can be used as a second marker to confirm the accuracy of bird sex-typing data. We achieved this by identifying a locus with a Z and W homolog for which it was possible to design a marker whose primer sequences are highly conserved among multiple genetically distant bird species. The locus identified was homologous to the avian *spindlin* gene, which is known to be present on the Z and W chromosomes [Itoh et al., 2001; de Kloet and de Kloet, 2003].

METHODS

The zebra finch *Taeniopygia guttata* EST microsatellite sequence CK309496 [Replogle et al., 2008] was obtained from the NCBI EST (EST_others) database. This sequence was found to possess homology to the avian *spindlin* gene which is known to be present on the Z and W chromosomes [Itoh et al., 2001; de Kloet and de Kloet, 2003]. To date, few W homologs of sequence CK309496 exist, however, there are Z homologs as a result of bird genome assembly projects, for which males have typically been sequenced. A sequence alignment was created to compare the sequence (CK309496) to 19 homologous Z/W sequences from 16 bird species, including the Z homologs of nine species, unmapped homologs of seven other species, and the W homologs of chicken *Gallus gallus*, mallard *Anas platyrhynchos*, and turkey *Meleagris gallopavo*, and finally also including the saltwater crocodile *Crocodylus porosus*. Sequences were extracted from online databases (the ENSEMBL genome browser, National Center for Biotechnology Information [NCBI] "nr/nt" Nucleotide collection database, GenBank, and the European Bioinformatics Institute [EBI] including the European Molecular Biology Laboratory [EMBL] and the European Nucleotide Archive [ENA]). The Z and W sequences aligned included those from distantly related species, such as the chicken (Z and W) and the zebra finch (Z) and these displayed variation in the repeat region. The sequence of the zebra finch W paralogue was not available because the zebra finch W chromosome has not yet been sequenced (as of September 30, 2014). We used an approach similar to that of Dawson et al. [2010]. We created a consensus sequence from these multiple homologous sequences using MEGA3 [Kumar et al., 2004] and designed a primer set (*Z43B*) from this consensus sequence using PRIMER3 v0.4.0 [Rozen and Skaletsky, 2000]. After including a single degenerate base in the reverse primer, both primer sequences are an exact (100%) match to homologs in 14 avian species, including eight Z chromosome homologs (including the zebra finch and chicken), the chicken W chromosome and the saltwater crocodile (Table 2A and B). We calculated the expected product sizes in these species using the sequence homologs of the (CK309496) sequence extracted from the NCBI "nr" nucleotide database and the ENSEMBL genome database (Table 2B).

The primer set was tested for its ability to sex 61 non-ratite species of birds belonging to 30 families and 15 orders and also tested in one ratite species, the brown kiwi, *Apteryx australis* (all species tested included both sexes, females and males). We genotyped individuals belonging to four additional ratite species because most ratites require sex-typing using species-specific markers. However, known sexes were not available for the additional ratite species tested. This brought the total number of bird species genotyped to 66 (including 62 species with known sexes). Several species that had been previously found to be difficult to sex-type using the *P2–P8* primer set (see Introduction) were tested with the *Z43B* primer set, including magpie, albatross, petrel, eagles, falcons, crane, owl, penguin, and dove species. Finally, we genotyped saltwater crocodile individuals (unknown sexes) and checked for PCR amplification. When sequence data were available for a species genotyped, we checked that the observed (genotyped) allele size matched the size predicted based on the sequence (i.e., for the saltwater crocodile, and those bird species for which known Z and/or W homologous sequences were available).

Genomic DNA was extracted from blood or feathers using an ammonium acetate protocol [Nicholls et al., 2000; Richardson et al., 2001]. PCR reactions were performed in 2-μl volumes [Kenta et al. 2008], containing *ca* 10 ng of lyophilized genomic DNA, 1 μl of QIAGEN Multiplex PCR Master Mix (QIAGEN, Manchester, UK) and 0.2 μM of each primer (with the forward primer fluorescently labeled with 6-FAM). We recommend the use of QIAGEN Multiplex Master Mix for PCR sex-typing (in both singleplex and multiplex PCRs) because it enables amplification even when

TABLE 2. Details of a new marker (*Z43B*) for sex-typing a wide range of birds (A) Primer sequences and PCR details; (B) A comparison of similarity of the Z43B sex-typing primer sequences to their homologs in various bird and reptiles

A

Locus	Primer sequence 5′–3′ (and fluoro-label)	T_m (°C)	T_a (°C)	Repeat motif amplified in birds	Observed allele size range in 60 bird species (bp) (see Table 3)
Z43B	(F) [6-FAM]-CTTGAGACTAATTCCACTCC (R) TTTACATGGCAGCyTGA	51.37 51.64 or 54.36 (Ψ)	50₮	$(AT)_n$ $(GT)_n$	260–282

B

Order	Family	Species	Chromosome location if stated in seq. record or identified based on seq. homology or observed allele size	% Forward primer sequence similarity (20 bp)	% Reverse primer sequence similarity (17 bp)	Identity of base at the site of reverse primer degen. base[a]	Exp. W allele size (bp)	Exp. Z allele size (bp)	Exp. allele size (unknown chr) (bp)	Obs. W allele (bp) €	Obs. Z allele (bp) €	Sequence accession number and source/type and description
Passeriformes	Estrildidae	Zebra finch *Taeniopygia guttata*	Z (homology and allele size)	100	100	C	Unk.*	n/a	271	261	272	CK309496 EST (Expressed sequence tag)
”	”	”	Z (male = ZZ)	100	100	C	n/a	271	n/a	261	272	Zebra Finch (taeGut3.2.4), Male sequenced ENSEMBL Genome assembly
”	”	”	Z (homology and allele size)	100	100	C	n/a	n/a	271	261	272	XM_002193358.2 spindlin-Z-like (LOC100218023), mRNA
”	Fringillidae	Medium ground-finch *Geospiza fortis*	Unknown[a] (degen C = Z?)	100	100	C	n/a	n/a	272	NT	NT	XM_005423567.1 spindlin-Z-like (LOC102039252), mRNA
”	”	Common canary *Serinus canaria*	Unknown[a] (degen C = Z?)	100	100	C	n/a	n/a	271	(271)	271	XM_009096098.1 spindlin 1 (SPIN1), mRNA
”	”	White-throated sparrow *Zonotrichia albicollis*	Unknown[a] (degen C = Z?)	100	100	C	n/a	n/a	272	NT	NT	XM_005486195.1spindlin-Z-like (LOC102067781), mRNA
”	Muscicapidae	Collared flycatcher *Ficedula albicollis*	Z (homology)	100	100	C	n/a	n/a	269	NT	NT	XM_005060965.1spindlin-Z-like (LOC101819398), mRNA
”	”	”	(Z)[b] (male = ZZ)	100	100	C	n/a	269	n/a	NT	NT	Flycatcher (FicAlb_1.4), *Male sequenced ENSEMBL genome scaffolds
”	Pipridae	Golden-collared manakin *Manacus vitellinus*	Unknown[a] (degen C = Z?)	100	100	C	n/a	n/a	271	NT	NT	XM_008925981.1 spindlin 1 (SPIN1), mRNA
”	Corvidae	American crow *Corvus brachyrhynchos*	Unknown[a] (degen C = Z?)	100	100	C	n/a	n/a	271	NT	NT	XM_008640174.1spindlin 1 (SPIN1), mRNA
”	Acanthisittidae	Rifleman *Acanthisitta chloris*	Z (allele size)	100	100	C	n/a	n/a	271	261	272	XM_009073419.1 spindlin 1 (SPIN1), mRNA
Falconiformes	Falconidae	Saker falcon *Falco cherrug*	Z (allele size)	100	100	C	n/a	n/a	272	266	273	XM_005435916.1 spindlin-Z-like (LOC102053800), mRNA
”	”	Peregrine falcon *Falco peregrinus*	Z (allele size)	100	100	C	n/a	n/a	272	266	273	XM_005239829.1 spindlin-Z-like (LOC101912754), mRNA

TABLE 2. (Continued)

B

Order	Family	Species	Chromosome location if stated in seq. record or identified based on seq. homology or observed allele size	% Forward primer sequence similarity (20 bp)	% Reverse primer sequence similarity (17 bp)	Identity of base at the site of reverse primer degen. base[a]	Exp. W allele size (bp)	Exp. Z allele size (bp)	Exp. allele size (unknown chr) (bp)	Obs. W allele (bp) €	Obs. Z allele (bp) €	Sequence accession number and source/type and description
Psittaciformes	Psittacidae	Budgerigar *Melopsittacus undulatus*	(Z)[b] (male = ZZ)	100	100	C	n/a	271	n/a	NT	NT	Budgerigar MelUnd6.3[b] (Male sequenced) ENSEMBL Genome scaffold
Sphenisciformes	Spheniscidae	Emperor penguin *Aptenodytes forsteri*	Unknown (Poor seq?/poor homology)	75	n/a	n/a	n/a	n/a	n/a	NT	NT	XM_009280978.1 REST corepressor 1 (RCOR1), mRNA
Columbiformes	Columbidae	Rock pigeon *Columba livia*	Unknown[a] (degen C=Z?)	100	100	C	n/a	n/a	271	NT	NT	XM_005512106.1spindlin-Z-like (LOC102091955), mRNA
Galliformes	Phasianidae	Chicken ¥ *Gallus gallus*	W (homology)	100	100	T	265	n/a	n/a	(266) ₸	266	Chicken (Galgal4), Female sequenced. ENSEMBL Genome assembly
”	”	”	Z (homology)	100	100	C	n/a	266	n/a	(266) ₸	266	Chicken (Galgal4), Female sequenced. ENSEMBL Genome assembly
”	”	”	W	100	100	T	265	n/a	n/a	(266) ₸	266	AC175832.2 BAC clone CH261-75N4 from chromosome W, complete sequence
”	”	”	Z	100	100	C	n/a	266	n/a	(266) ₸	266	AC186546.3 BAC clone CH261-9B3 from chromosome Z, complete sequence
”	”	”	W (homology)	100	100	T	265	n/a	n/a	(266) ₸	266	CR391335.1 finished cDNA, clone ChEST679i11
”	”	”	Z	100	100	C	n/a	266	n/a	(266) ₸	266	NM_204633.1 spindlin 1 (SPINZ), mRNA
”	”	”	W	100	100	T	265	n/a	n/a	(266) ₸	266	NM_204191.1 spindlin 1 (SPINW), mRNA
”	”	Turkey *Meleagris gallopavo*	Z (allele size)	90	100	C	n/a	271	n/a	NT	272	Turkey (UMD2), female sequenced ENSEMBL genome assembly
”	”	”	Suspected W (Z = 272 bp and degen T=W?)[a]	90	88	T	261	n/a	n/a	NT	272	Turkey (UMD2), female sequenced ENSEMBL genome assembly
Anseriformes	Anatidae	Mallard *Anas platyrhynchos*	Z (allele size)	100	100	C	n/a	271	n/a	263	271	Duck (BGI_duck_1.0), female sequenced ENSEMBL genome scaffolds
”	”	”	W (allele size)	100	88	T	262	n/a	n/a	263	271	Duck (BGI_duck_1.0), female sequenced ENSEMBL genome scaffolds

TABLE 2. (Continued)

B

Order	Family	Species	Chromosome location if stated in seq. record or identified based on seq. homology or observed allele size	% Forward primer sequence similarity (20 bp)	% Reverse primer sequence similarity (17 bp)	Identity of base at the site of reverse primer degen. base[a]	Exp. W allele size (bp)	Exp. Z allele size (bp)	Exp. allele size (unknown chr) (bp)	Obs. W allele (bp) €	Obs. Z allele (bp) €	Sequence accession number and source/type and description
Reptile Archosauria	Alligatoridae	Chinese alligator *Alligator sinensis*	Unknown	100	100	C	n/a	n/a	272	NT	NT	XM_006022399.1spindlin-Z-like (LOC102374732), transcript variant X1, mRNA
Reptile Testudines	Trionychidae	Chinese soft-shelled turtle *Pelodiscus sinensis*	Unknown	100	100	C	n/a	n/a	275	NT	NT	XM_006131895.1spindlin-Z-like (LOC102450436), transcript variant X2, mRNA
Reptile Testudines	Emydidae	Western painted turtle *Chrysemys picta bellii*	Unknown	90	100	C	n/a	n/a	283	NT	NT	XM_005307632.2spindlin 1 (SPIN1), transcript variant X3, mRNA
Reptile Testudines	Cheloniidae	Green sea turtle *Chelonia mydas*	Unknown	90	100	C	n/a	n/a	285	NT	NT	XM_007059221.1spindlin-Z-like (LOC102939202), mRNA
Reptile Lepidosauria	Iguanidae	Anole lizard *Anolis carolinensis*	Chr. 2	85	94	C	n/a	n/a	263	NT	NT	Anole lizard AnoCar2.0 ENSEMBL genome assembly

T_m, melting temperature obtained from PRIMER3 v0.4.0 [Rozen and Skaletsky, 2000]; T_a, annealing temperature used for PCR; ₮, initially some species were amplified at 56°C (see Table 3) but we recommend 50°C (see text); bp, base pair; Ψ, Degen., the degenerate reverse primer base (y = C/T) leads to variation in the reverse primer melting temperature: T = 51.64°C and C = 54.36°C; Unk.*, expected allele size was *unknown* because no zebra finch W chromosome sequence is available; ¥, location of locus (as of 25th September 2014) in the chicken genome: Z chr., 42,647,956 bp and W random chr., 153,046 bp; in the zebra finch: Z chr., 7,529,968 bp; €, sexes of individuals were determined based on morphology of adult birds, behavior or other genetic markers (see Table 2); ₮, the lack of difference between male and female chickens may be because the chicken W allele is identical or very similar in size to the Z allele (predicted size difference ± 1 bp). Alternatively, it is possible that the W allele may be failing to amplify in chicken, although this is unlikely based on 100% primer-target homology; Exp., expected; Obs., observed; n/a, not available; NT, not tested.

[a]The degenerate base in the reverse primer (y = C/T) appeared to be chromosome specific in birds, existing as a "A" on the W chromosome and "T" on the Z chromosome (based on the known W homologs of three species and known Z homologs of seven species) and may assist in identifying the chromosome origin of each sequence).

[b]Male (ZZ) individual sequenced, so sequence must be that of the Z chromosome.

TABLE 3. Assessment of the *Z43B* marker for sex-typing birds

A. *Z43B* assessed in 61 non-ratite species belonging to 30 families in 15 bird orders

Order (sub-order)[a]	NCBI taxonomic classification	Species	Binomial name	*n*	Kn. F	Kn. M	Z43B W allele size (bp)	Z43B Z allele size (bp)	Sexed with Z43B	Notes	Ta (°C)	Samples supplied by
Aves; Neognathae	Non-ratites											
Anseriformes	Anatidae	Mallard	*Anas platyrhynchos*	5	2	3	263	272	Y		50	Emma Cunningham
”	”	Muscovy duck	*Cairina moschata*	6	3	3	261	272	Y		56	Moshen Vaez
Bucerotiformes	Bucerotidae	Monteiro’s hornbill	*Tockus monteiri*	8	2	4		272	N	No variation	56	David Richardson
Charadriiformes	Charadriidae	Kentish plover	*Charadrius alexandrinus*	4	2	2	266	272	Y		50	Clemens Küpper
”	”	Snowy plover	*Charadrius nivosus*	3	1	2	266	272	Y		50	Clemens Küpper
”	”	Ringed plover	*Charadrius hiaticula*	4	2	2	266	272	Y		50	Pavel Tomkovich
”	Chionidae (Chionididae)	Greater sheathbill	*Chionis alba*	4	1	2		272	N	No variation	50	Richard Phillips
”	Alcidae (Laridae)	Whiskered auklet	*Aethia pygmaea*	17	2	7	266	270, 274, 280, 282	Y	Z polymorphism	50	Fiona Hunter
”	Scolopacidae	Ruff	*Philomachus pugnax*	4	2	2	263	271	Y	All females were 263 homozygotes	50	David Lank
”	”	Curlew sandpiper	*Calidris ferruginea*	4	2	2		263	N	No variation	50	Jim de Fouw
”	”	Dunlin	*Calidris alpina*	2	1	1		263	N	No variation	50	Jim de Fouw
”	”	Little stint	*Calidris minuta*	3	1	2	266	272	Y		50	Jim de Fouw
”	”	Redshank	*Tringa totanus*	4	2	2	264	272	Y		50	Jim de Fouw
”	”	Terek sandpiper	*Xenus cinereus*	4	3	1	261	266, 272	Y	Z polymorphism	50	Jim de Fouw
”	”	Turnstone	*Arenaria interpres*	4	2	2		261, 264, 266	N	No W amp./no variation (all homozygotes)	50	Jim de Fouw
Columbiformes	Columbidae	Seychelles turtle dove	*Streptopelia picturata rostrata*	8	4	4		272	N	No variation	56	David Richardson
Coraciiformes	Coraciidae	European roller	*Coracias garrulus*	4	1	3	264	272	Y		56	Mercedes Molina Morales, Jesus M. Aviles, David Martín-Gálvez, Juan Gabriel Martinez
	Meropidae	European bee-eater	*Merops apiaster*	6	4	2	270	272	Y	All females were 270 homozygotes	50 and 56	Kate Lessels
Falconiformes	Accipitridae	Golden eagle	*Aquila chrysaetos*	7	4	3	268	271	Y		50 and 56	Brian Bourke
”	”	Spanish Imperial eagle	*Aquila adalberti*	7	2	5	268	271	Y		50	Begona Martinez-Cruz
”	”	White-tailed sea eagle	*Haliaeetus albicilla*	10	6	4	271	272	Y	1bp difference	50	Frank Hailer

continued

TABLE 3. (Continued)

A. *Z43B* assessed in 61 non-ratite species belonging to 30 families in 15 bird orders

Order (sub-order)[a]	NCBI taxonomic classification	Species	Binomial name	*n*	Kn. F	Kn. M	Z43B W allele size (bp)	Z43B Z allele size (bp)	Sexed with Z43B	Notes	Ta (°C)	Samples supplied by
”	”	Bonelli’s eagle	*Hieraaetus fasciatus*	10	7	3	268	271	Y		50	Sara Mira
”	”	Common buzzard	*Buteo buteo*	10	7	3	271	272	Y	1bp difference	50	Paul Johnson
”	”	Osprey	*Pandion haliaetus haliaetus*	50	20	30	268	272	Y		50	Colin Hewitt
”	Falconidae	Mauritius kestrel	*Falco punctatus*	3	1	2	266	269	Y		50	Jim Groombridge
”	”	Peregrine	*Falco peregrinus*	14	5	9	266	273	Y		50 and 56	Andy Dixon, Louise Gentle, Lucy Webster, Esther Kettel, Elizabeth Woodward, Derbyshire Wildlife Trust, Helen Hipperson, Sheffield Bird Study Group, Sorby Breck Ringing Group, David Wood
”	”	Saker	*Falco cherrug*	9	5	4	266	273	Y		50 and 56	Andy Dixon
”	”	Eleonora’s falcon	*Falco eleonorae*	18	13	5	266	271	Y		50 and 56	Claudie Doums
Galliformes	Megapodiidae	Australian brush-turkey	*Alectura lathami*	8	6	2	-	272	N	No variation	56	Darryl Jones
”	Phasianidae	Chicken (Crittenden breed)	*Gallus gallus*	8	6	2	-	266	N	No variation	56	Hans Cheng
”	”	Red grouse	*Lagopus lagopus scotica*	6	4	2		272	N	No variation	50	Paul Johnson
”	”	Common pheasant	*Phasianus colchicus*	5	3	2		272	N	No variation	50	Olivier Hanotte
Gruiformes	Gruidae	Blue crane	*Grus paradisea*	7	2	2	266	271	Y		56	Kate Carstens, Tiawanna Taylor
Passeriformes	(Passeri) Aegithalidae	Long tailed tit	*Aegithalos caudatus*	4	2	2		271	N	No variation	50	Douglas Ross, Ben Hatchwell
”	(Corvoidea), Corvidae	Black-billed magpie	*Pica pica*	23	12	11	260	272	Y		56	David Martín-Gálvez, Juan Gabriel Martinez
”	Paridae	Blue tit	*Parus caeruleus*	4	2	2		272	N	No variation	50	Iain Barr
”	(Passeroidea), Estrildidae/Passeridae	Zebra finch	*Taeniopygia guttata*	7	3	4	261	272	Y		56	Tim Birkhead
”	Fringillidae	Canary	*Serinus canaria*	8	3	5		271	N	No variation	50	Rupert Marshall
”	Parulidae	Seychelles warbler	*Acrocephalus sechellensis*	4	2	2		273	N	No variation	50	David Richardson

continued

TABLE 3. (Continued)

A. *Z43B* assessed in 61 non-ratite species belonging to 30 families in 15 bird orders

Order (sub-order)[a]	NCBI taxonomic classification	Species	Binomial name	*n*	Kn. F	Kn. M	Z43B W allele size (bp)	Z43B Z allele size (bp)	Sexed with Z43B	Notes	Ta (°C)	Samples supplied by
”	(Acanthisitti) Acanthisittidae	Rifleman	*Acanthisitta chloris*	10	4	6	261	272	Y		50	Steph Hodges, Ben Hatchwell
”	(Tyranni) Formicariida (Thamnophilidae)	Dusky antbird	*Cercomacra tyrannina*	8	1	9		273	N	No variation	50 and 56	Terry Burke
Pelecaniformes	Fregatidae	Magnificent frigatebird	*Fregata magnificens*	4	2	2	264	272	Y		50	Axa Rocha-Olivares
”	Phalacrocoracidae	South Georgia shag	*Phalacrocorax georgianus*	4	2	2	265	271	Y		50	Richard Phillips
Piciformes	Picidae	Acorn woodpecker	*Melanerpes formicivorus*	4	1	3		272	N	No variation	50 and 56	Joey Haydock
Procellariiformes	Diomedeidae (Procellariidae)	Wandering albatross	*Diomedea (exulans) exulans*	4	2	2	266	272	Y		50	Richard Phillips
”	Procellariidae	Southern giant petrel	*Macronectes (giganteus) giganteus*	4	2	2	267	270	Y		50	Fiona Hunter
”	”	Northern fulmar	*Fulmarus glacialis*	6	3	3	265	269	Y		50	Ewan Wakefield
Psittaciformes	Psittacidae	Thick-billed parrot	*Rhynchopsitta pachyrhyncha*	4	2	2	268	269	Y	1 bp difference	50	David Jeggo
”	”	Kea	*Nestor notabilis*	4	2	2		271	N	No variation	50	Terry Burke
”	”	Cape parrot	*Poicephalus robustus*	8	1	2	268	271	Y		50	Kerry Pillay, Tiawanna Taylor
”	”	Kakapo	*Strigops habroptilus*	5	2	3	266	272	Y		56	Bruce Robertson
”	”	Rock parrot	*Neophema petrophila*	2	1	1	268	271	Y		50	Tiawanna Taylor
”	”	Crimson rosella	*Platycercus elegans*	7	2	5	267	271	Y		50	Matt Berg
”	”	Rupelli’s parrot	*Poicephalus rueppellii*	2	1	1	268	271	Y		50	Tiawanna Taylor
”	”	Black headed caique	*Pionites melanocephalus*	4	2	2	268	271	Y		50	Tiawanna Taylor
”	”	Greater vasa parrot	*Coracopsis vasa*	9	5	4		269	N	No variation	50	Jon Ekstrom
Sphenisciformes	Spheniscidae	Adelie penguin	*Pygoscelis adeliae*	4	1	1	266	272	Y		50	Fiona Hunter
”	”	Gentoo penguin	*Pygoscelis papua*	14	9	5	266	272	Y		50	Richard Phillips
”	”	Macaroni penguin	*Eudyptes sclateri*	20	4	9	265	271	Y		50 and 56	Richard Phillips
”	”	Chinstrap penguin	*Pygoscelis antarctica*	3	1	2	266	272	Y		50	Tom Hart
Strigiformes	Tytonidae	Barn owl (Hungarian)	*Tyto alba guttata*	32	16	16		272	N	No variation	56	Akos Klein

TABLE 3. (Continued)

B. Z43B assessed in ratites and the saltwater crocodile (*Crocodylus porosus*)

Order (sub-order)[a]	NCBI taxonomic classification	Species	Binomial name	n	Kn. F	Kn. M	Z43B W allele size (bp)	Z43B Z allele size (bp)	Sexed with Z43B	Notes	Ta (°C)	Samples supplied by
Aves; Palaeognathae	Ratites											
Apterygiformes	Apterygidae	Brown kiwi	*Apteryx australis*	7	3	4	267	273	Y		56	Māori leaders council, New Zealand Department of Conservation
Casuariiformes	Casuariidae	Southern cassowary	*Casuarius casuarius*	3	0	0		273	–	No known sexes	56	Leon Huyen
”	Dromaiidae	Emu	*Dromaius novae*	5	0	0		273	–	No known sexes	56	Dominque Blache
Rheiformes	Rheidae	Lesser rhea (Darwin’s rhea)	*Rhea pennata (Pterocnemia pennata)*	3	0	0		273	–	No known sexes	56	Josephine Pemberton
Struthioniformes	Struthionidae	Ostrich	*Struthio camelus*	5	0	0		273	–	No known sexes	56	Jeff Graves
Reptiles	–	Saltwater crocodile	*Crocodylus porosus*	7	0	0		273	–	No known sexes)	56	Winston Kay

C. Summary of the success of the Z43B marker for sex-typing birds

	Non-ratites	Ratites	Total
Number of species tested with known sexes (known females and males for each species)	61	1	62
Number of species with no size variation observed between W and Z alleles (could be failure of W allele to amplify or identically sized W and Z alleles; not sexed)	18	0	18
Number of species with Z polymorphism preventing sexing	1	0	1
Number of species with Z polymorphism but still able to assign sex	2	0	2
Number of species where females were homozygous and W allele was a different size to Z allele (so still able to assign sex)	2	0	2
Number of species with a 1 bp difference between W and Z allele (still able to assign sex)	3	0	3
Total number of species which were successfully sex-typed	42 (69%)	1	43 (69%)
Number of orders tested with known sexes (known females and males)	15	1	16
Number of orders in which some species were sexed	10 (67%)	1	11 (69%)
Number of families tested with known sexes (known females and males)	30	1	31
Number of families in which some species were sexed	21 (70%)	1	22 (71%)

n, number of individuals genotyped; Kn. F; Kn. M, number of known females or males, respectively, genotyped (sex based on morphology, behavior or PCR-sexed with P2–P8 [Griffiths et al., 1998] and/or 2550F-2718R [Fridolfsson and Ellegren, 1999] and/or Z-002 [Dawson, 2007]; Y, yes; N, no; bp, base pair.

[a]Classification based on Sibley and Monroe [1990].

there are base mismatches between the target and primer sequence (DAD unpublished data). PCR amplification was performed using a DNA Engine Tetrad thermal cycler (Bio-Rad, Hemel Hempstead, UK). PCR amplification conditions were 94°C for 15 min; then 45 cycles of 94°C for 30 sec, 50°C (or 56°C) for 30 sec, 72°C for 30 sec; followed by one cycle of 72°C for 10 min. During the initial testing, an annealing temperature of 56°C was used successfully for some species but when tested in a larger number of species, an annealing temperature of 50°C produced stronger and more specific amplification. PCR products were diluted to 1:1500/1:1600 prior to separation on a 48-capillary ABI 3730 DNA Analyzer possessing Prism set D. The PCR product was first diluted to 1:150 or 1:160 (product: water) then 1 μl of this was added to 9 μl of HiDi Formamide that contained the ROX size standard (Applied Biosystems, Warrington, UK; 4.5/5 μl of ROX size standard was added to 1 ml of Formamide). Allele sizes were compared against ROX size standards and assigned using GeneMapper software (Applied Biosystems).

RESULTS

Sequence Alignments

After including a single degenerate base in the reverse primer, both primer sequences are an exact (100%) match to the 15 homologs in 14 avian species (including eight on the Z chromosome, six unmapped, the chicken W chromosome; and the saltwater crocodile) (Table 2A and B).

Success of the Z43B Marker for Sex-Typing Birds

Primer sequences, primer melting temperatures, and the expected and observed allele sizes in zebra finch and chicken are provided (Table 2A and B). The observed allele sizes in zebra finch were $W = 261$ bp and $Z = 272$ bp (Tables 2B and 3). The observed size of the Z allele exactly matched the expected size based on the zebra finch sequence (272 bp, Table 2B). The observed size of the zebra finch W allele amplified was 261 bp (Table 2B, no W sequence available for calculating the expected allele size). In those species for which known Z and W homologs were available, the observed allele sizes amplified matched those expected (for chicken, mallard, and turkey; $Z \pm 2$ bp, $W \pm 2$ bp; Tables 2A and 3). These matches between the expected and observed allele sizes confirm that the correct locus was amplified.

All of the 66 bird species tested amplified, as did the saltwater crocodile, demonstrating the conserved nature of the primer set (Table 3). A 12-well gradient PCR (41–65°C) revealed that zebra finch Z and W alleles were both amplified with annealing temperatures between 41 and 58°C but when the annealing temperature was above 58°C the W allele dropped out. For most species an annealing temperature of 50°C produced the strongest and most specific products and amplified both the W and Z alleles but some species could be successfully sexed when amplified with an annealing temperature of 56°C (Table 3). Forty-two of the 61 non-ratite species tested could be successfully sexed with the *Z43B* marker (69%), as they possessed amplifiable W and Z homologs of different allele sizes (Table 3). The species successfully sexed belonged to 10 of the 15 non-ratite orders tested and included ducks, shorebirds, cranes, eagles, falcons, passerines, penguins, and parrots (Table 3). Additionally, sex-typing was successful for a ratite species, the brown kiwi (Table 3). Several of the successfully sexed species were impossible/difficult to sex-type with the *P2–P8* marker set, including the black-billed magpie, albatross, petrel, eagle, falcon, crane, and penguin species (Table 3).

The size of the Z and W amplicons ranged between 260 and 282 bp (Table 3). The difference between the *Z43B* W and Z alleles, within a species, was relatively small in most species (1–16 bp); therefore, allele discrimination required resolution and analysis on an ABI DNA Analyzer (Applied Biosystems). In three species (an eagle, buzzard, and parrot), the size difference between the W and Z allele size was only one base-pair and required careful binning of the alleles (avoiding the automatic decimal place round-up performed by some software). We checked the accuracy of the sexing results in these three species by typing more individuals and found that the size of the W and Z alleles was chromosome-specific in all of the 10 white-tailed sea eagles (*Haliaeetus albicilla*), 10 common buzzards (*Buteo buteo*), and 4 thick-billed parrots (*Rhynchopsitta pachyrhyncha*) tested, confirming accuracy (Table 3).

For most of the species successfully sexed (38/42), females were always heterozygous and males always apparently homozygous (i.e., hemizygous). Two species displayed Z allele polymorphism but the W and Z allele sizes did not overlap so they could still be sexed (whiskered auklet *Aethia pygmaea*, Terek sandpiper *Xenus cinereus*) and for two species females (and males) were homozygous and the female (W) allele was a different size to that of the male (Z) allele so could also be sexed (ruff *Philomachus pugnax*, European bee-eater *Merops apiaster*; Table 3). In these last two cases, we presume the (smaller) W allele was amplified in females in preference to the Z allele [Toouli et al., 2000]. The diagnostic W allele was smaller than the Z allele in all species sexed, except for the black-billed magpie (Table 3).

For confidence in the accuracy of sex typing data, we recommend sex-typing using multiple markers (ideally designed from different loci), multiple individuals and populations and whenever possible, to include multiple known sex individuals (of both sexes).

Species That Could Not Be Sexed

Of the 19 homologs (16 species) originally compared in the alignment, only four homologs: the turkey W, turkey Z, mallard W, and Emperor penguin *Aptenodytes forsteri* homolog possessed mismatches to the primers. The turkey and mallard displayed only one to two primer base mismatches to one or both primers, The Emperor penguin (unknown chr.)

displayed poor homology to the CK309496 sequence, possibly due to sequence quality and was not used in the alignment. For the turkey, a comparison of the online Z and W sequences suggested two alleles differing by 10 bp were expected to be amplified, a Z allele (271 bp) and a second allele (261 bp) presumed to be the W allele (but currently assigned to a second location on the Z chromosome, Table 2A). Despite two base mismatches in the forward primer, the expected Z allele was still amplified in turkey (272 bp; Table 2A). Two base mismatches also exist between the *Z43B* reverse primer and the turkey presumed W allele (Table 2A), and these may or may not lead to the W allele failing to amplify in this species (no female turkey samples were available to test). A small number of base mismatches between the primer and target do not always cause amplification failure. For example, two base mismatches between the reverse primer bind site and the mallard W chromosome (different mismatches to those in turkey) did not prevent the mallard W allele from amplifying ($W = 263$ bp, $Z = 272$ bp; Table 3).

Nineteen of the 61 non-ratites species tested could not be sexed (Table 3). In some species, it may be that the W allele required a lower PCR annealing temperature to amplify. None of the four Galliform species tested could be sexed because there was no difference between the amplified allele sizes in males and females (chicken, red grouse *Lagopus lagopus scotica*, common pheasant *Phasianus colchicus*, and Australian brush-turkey *Alectura lathami*; Table 3). A small (1 bp) difference between the chicken Z and W homologes was calculated from the Z and W sequences available online (Table 3); however, since no size difference was observed between the amplified chicken Z and W alleles (and because the primers were an identical [100%] match to the chicken W and Z sequence) it was assumed this sequence length difference is a sequencing base-call error or a result of different chicken breed/strains sequenced and is not a sex associated size difference. In three orders (Charadriiformes, Passeriformes, Psittaciformes), some species could be sex-typed and others not. The *Z43B* marker amplified but failed to sex four species that were the single representatives of their order: a dove, an owl, a hornbill, and a woodpecker (Table 3).

DISCUSSION

Failure to Distinguish Between Sexes

Most of the species that could not be sexed displayed only a single allele of the same size in males and females (Table 3). Only one species could not be sexed due to Z polymorphism, the turnstone (*Arenaria interpres*), which displayed W and Z alleles that overlapped in size (Table 3). All of the species tested amplified, and for the majority of species all of the individuals tested amplified, including males (ZZ) and females (ZW), suggesting the Z allele is amplifying well. Failure of a species to be PCR sex-typed with the *Z43B* marker was mostly due to a lack of size difference between the Z and W alleles or failure of the W allele to amplify (Table 3). The comparison of allele sizes obtained when genotyping does not allow us to distinguish which of these two reasons was the cause of failure in the different species. However, this could be investigated if these regions were MiSeq sequenced in each species (ideally in a female individual and with sequencing extending across the primer-bind regions of the Z and W homolog).

Z-polymorphism

As for all (published) sex-typing markers, *Z43B* may display Z-polymorphism in any other untested species/populations or when assessed in a larger number of individuals, illustrating the need for alternative markers to check sex-typing data. We note that the whiskered auklet was observed to display Z-polymorphism with both the *Z43B* (this study) and *P2–P8* primer sets [Dawson et al., 2001], a point that may be of interest to those studying the evolutionary history of this and related species.

Utility of the Z43B Marker for Distinguishing Between Species

There was some variation in the sizes of the Z homologs in different species (±11 bp), when compared to the zebra finch ($Z = 272$ bp) and also variation in the W homologs (±10 bp), as compared with zebra finch ($W = 261$ bp), suggesting this marker possesses potential utility for distinguishing species or identifying hybrids (e.g., Lifjeld et al. [2010]).

CONCLUSION

The *Z43B* marker is of high utility for sex-typing most bird species. It is informative in species that are difficult to sex-type with other markers and provides a second marker to confirm the accuracy of sex-typing data.

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DATA ACCESSIBILITY

All sequence data used is available online in the EMBL, GenBank, and DDJB sequence databases and the EMBL sequence accession numbers are provided in the main text and Table 2B.

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