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TECHNICAL ADVANCES

New methods to identify conserved microsatellite loci and develop primer sets of high cross-species utility – as demonstrated for birds

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Abstract

We have developed a new approach to create microsatellite primer sets that have high utility across a wide range of species. The success of this method was demonstrated using birds. We selected 35 avian EST microsatellite loci that had a high degree of sequence homology between the zebra finch *Taeniopygia guttata* and the chicken *Gallus gallus* and designed primer sets in which the primer bind sites were identical in both species. For 33 conserved primer sets, on average, 100% of loci amplified in each of 17 passerine species and 99% of loci in five non-passerine species. The genotyping of four individuals per species revealed that 24–76% (mean 48%) of loci were polymorphic in the passerines and 18–26% (mean 21%) in the non-passerines. When at least 17 individuals were genotyped per species for four Fringillidae finch species, 71–85% of loci were polymorphic, observed heterozygosity was above 0.50 for most loci and no locus deviated significantly from Hardy–Weinberg proportions.

This new set of microsatellite markers is of higher cross-species utility than any set previously designed. The loci described are suitable for a range of applications that require polymorphic avian markers, including paternity and population studies. They will facilitate comparisons of bird genome organization, including genome mapping and studies of recombination, and allow comparisons of genetic variability between species whilst avoiding ascertainment bias. The costs and time to develop new loci can now be avoided for many applications in numerous species. Furthermore, our method can be readily used to develop microsatellite markers of high utility across other taxa.

Keywords: AVES, conserved, cross-species utility, expressed sequence tag (EST), microsatellite, Passerine

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Introduction

Microsatellite loci are much less abundant in birds than in some other taxa, such as mammals and fish (Primmer *et al.* 1997; Neff & Gross 2001). Therefore, studies in birds routinely use enrichment protocols to isolate sufficient microsatellite loci for analyses of parentage, population genetics or linkage mapping. Unfortunately, the isolation and development of microsatellites is a skilled and time-consuming task that can take weeks or months to complete and is therefore costly to perform. Microsatellite isolation is therefore often performed at specialist research facilities or by commercial laboratories.

Since the early demonstrations of avian microsatellite cross-utility (e.g. Primmer *et al.* 1996), one collective goal has been to identify a useful number of primer sets of high utility in a wide range of species. While a small number of such primer sets has been identified (e.g. Galbusera *et al.* 2000, see also the BIRDMARKER webpage http://www.sheffield.ac.uk/molecol/deborah-dawson), the bigger goal has proven elusive. If such a set of loci was identified, it would additionally be desirable to amplify the loci in a single-tube reaction using multiplex PCR.

We describe a simple method to develop microsatellite primer sets of high utility and demonstrate the success of the method using birds. The initial steps involved the identification of conserved zebra finch (*Taeniopygia guttata*) Expressed Sequence Tag (EST) microsatellite sequences and alignment to their chicken (*Gallus gallus*) homologues. It has long been recognized that microsatellite sequences can be isolated from EST sequences and this has been achieved in various different plant and animal species, including those species with a generally low abundance of microsatellites (Cordeiro *et al.* 2001; Kantety *et al.* 2002; Perez *et al.* 2005; Kong *et al.* 2007; Kim *et al.* 2008; Tang *et al.* 2008). In birds, EST sequence resources have been utilized to obtain galliform and passerine microsatellites (galliform: Ruyter-Spira *et al.* 1998; Dranchak *et al.* 2003; Mannen *et al.* 2005; passerine: Slate *et al.* 2007; Karaiskou *et al.* 2008). Recently, there has been renewed interest in the utility of EST microsatellite sequence data as a resource for genetic population analyses in various taxa (Ellis & Burke 2007; reviewed by Bouck & Vision 2007), partly fuelled by the recent submission of high volumes of EST sequence data to public data banks. Many EST sequences have now been identified in birds, including a passerine species, the zebra finch (e.g. Wada *et al.* 2006; Replogle *et al.* 2008). This EST sequence data can be mined for microsatellites. When primer sets have been designed simply from EST microsatellite sequence, without any pre-selection or additional primer set development, they have been shown to have only marginally higher cross-species amplification and polymorphism rates than anonymous microsatellite loci (Karaiskou *et al.* 2008). Other studies have found limited cross-utility of EST microsatellite loci, even when the protocol has included some additional components of primer development. Pashley *et al.* (2006) attempted to develop *Helianthus* sunflower EST microsatellite loci of high cross-species utility, but with limited success. Mismatches between the primer and target sequence have been shown to limit amplification success. Housley *et al.* (2006) designed dog–human primers for sequence-tagged site (STS) loci (i.e. non-microsatellite sequence) in exonic sequence and found primer mismatches to be the largest cause of PCR failure, with a 6–8% decrease in amplification per mismatch in primer pair.

To develop successfully primer sets that have the highest cross-utility, we suggest that the available sequence resources require more focused exploitation. The resources for birds include the assembled zebra finch and chicken genomes, along with the EST sequence data isolated from these and other avian species and avian microsatellite sequences isolated from genomic libraries. Here, we report the development of a method that enables the identification of conserved microsatellite loci that are informatively polymorphic across an unusually wide range of species, and that can be amplified using a single standard set of primers that allow these loci to be amplified under standard conditions. First, we identified those microsatellite loci of the highest potential. Sequences displaying high homology between source species and chicken have been found to display increased amplification levels across other species related to the source (Küpper *et al.* 2008). We therefore used zebra finch–chicken sequence homology to identify the most highly conserved microsatellite passerine loci and assigned these as being of the highest potential. Second, we developed primer sets for the selected loci that are identical in base-pair composition in both species and avoided the use of degenerate bases to maximize their potential for cross-species amplification. We illustrate the success of the method by developing a set of primers for 33 polymorphic microsatellite loci that are of the highest cross-species utility currently available for passerine birds. Additionally, we have designed the primer sets for these loci to have very similar melting temperatures and demonstrate that they can be amplified simultaneously at the same annealing temperature and PCR conditions.

Methods

Identification of highly conserved microsatellite loci

In order to attempt to identify the most conserved microsatellite loci in the avian genome, we compared homologous sequences in two species, the zebra finch and chicken. The two most genetically distant bird groups are

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the ratites and non-ratites. However, the zebra finch and chicken are also genetically very distantly related, having the highest recorded genetic distance for any two bird species based on DNA:DNA melting temperature (ΔT_m) hybridization distances (28.0, Sibley & Ahlquist 1990). We decided to use zebra finch EST microsatellite sequences for two reasons: (1) EST sequences (i.e. coding sequences) will be more conserved and have a higher homology to chicken than non-EST sequences and (2) a large number of zebra finch EST microsatellite sequences was available (n = 687, Slate *et al*. 2007).

We attempted to create a zebra finch–chicken consensus primer set for all autosomal zebra finch EST microsatellite sequences found to have an NCBI BLAST and WU-BLAST E-value of E-80 or better when compared with the chicken genome sequence (International Chicken Genome Sequencing Consortium, 2004). NCBI BLAST E-value scores were obtained from Slate *et al.* (2007) and compared with those obtained using an alternative WU-BLAST (using the distant homologies settings implemented on the ENSEMBL webpage at http://www.pre.ensembl.org/Gallus_gallus/index.html; methods as in Dawson *et al*. 2007). This check was performed because some (chromosome assignment) errors had previously been detected, (see Results) but additionally because the WU-BLAST software uses different criteria during sequence comparison, and has occasionally been found to be more sensitive than an NCBI BLAST (DAD unpublished data). The selected zebra finch EST microsatellite sequences were checked for duplication using BLASTN v.2.2.4 (Altschul *et al.* 1997) and all were found to be unique.

Creation of a consensus hybrid sequence and primer design

Homologous chicken sequences were identified by performing a WU-BLAST of zebra finch EST microsatellite sequence against the chicken genome sequence (using the distant homologies settings implemented on the ENSEMBL webpage http://www.pre.ensembl.org/Gallus_gallus/index.html; methods as in Dawson *et al.* 2007). Consensus zebra finch–chicken sequences were created by aligning homologous sequences using MEGA3 software (Kumar *et al.* 2004) and replacing mismatched bases and gaps with the code 'n' to represent an unknown base.

We used the zebra finch–chicken hybrid sequences to design consensus primer sets using PRIMER3 software (Rozen & Skaletsky 2000). All primer sets were 100% identical in zebra finch and chicken, with one exception (one base of the forward primer of locus *TG11-011* did not match with that of the chicken, Table 1). To enable efficient multiplex PCR, the primer sequences were designed to have a melting temperature as close as possible to 58 °C (range 54–61 °C). The melting temperatures of the forward and reverse primers of each pair were designed to be within 0.5 °C of each other. Degenerate bases were not used in the primer design, with one exception (one degenerate base was used in the forward primer of locus *TG01-000*, Table 1). The forward primer of each primer set was labelled with either a HEX or 6-FAM fluorescent dye (Table 1).

Nomenclature

The loci were named so as to refer to their source species and their position in the genome. The code 'TG' in the locus name refers to the first initials of the binomial names of the two species used: *Taeniopygia guttata* (zebra finch) and *Gallus gallus* (chicken). The numbers in the locus name represent its position on the chicken genome (v1.0); the first two digits represent the chromosome on which the locus is located and the last three digits refer to the position on that chromosome (in megabases).

Genome locations

All of the loci were assigned a chromosome location on the zebra finch genome by performing a BLAST search against the zebra finch genome assembly (using WU-BLAST 2.0 software and the *Taeniopygia guttata*-3.2.4 version of the map, released 14th July 2008 http://genome.wustl.edu/tools/blast/index.cgi; and proposed by the Zebra Finch Genome Consortium 2005). A figure displaying the locations of the loci on the zebra finch genome was created using MAPCHART software (Fig. 1; Voorrips 2002).

Genotyping

The primer sets developed were used to genotype individuals from 52 species selected from 15 different bird orders (classification following Sibley & Monroe 1990; Table 2). The species tested included 22 passerine and 30 non-passerine species and covered a wide range of genetic distances from the zebra finch (Table 2). For 21 species, only one individual was genotyped to assess cross-species amplification. A minimum of four individuals were genotyped at all 35 loci in 22 species, including zebra finch and chicken. The species tested included 17 passerine species (eight families) and five non-passerine species: Kentish plover *Charadrius alexandrinus*, rufous hummingbird *Selasphorus rufus*, barn owl *Tyto alba*, peach-faced lovebird *Agapornis roseicollis* and chicken. Four species that were tested with only a single individual were retested with four individuals (zebra finch, house sparrow *Passer domesticus*, great tit *Parus major* and chicken) to compare amplifi-

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Table 1 Details of 35 conserved autosomal microsatellite loci whose primer sets are 100% homologous in zebra finch *Taeniopygia guttata* and chicken *Gallus gallus*

Locus	EMBL accession number*	ZF chr †,‡	ZF position‡	ZF EST-ZF genome BLAST E-value	Repeat motif in ZF EST sequence	MR	Repeat motif in CH genome sequence	SR?	Primer sequences and fluoro-label (5′ - 3′)	M	D	T_m (°C)	Exp. ZF size (bp)	Obs. allele sizes in ZF (bp) (n=4)	Exp. CH size (bp)	Obs. allele sizes in CH (bp) (n=1)	Obs. allele sizes in wild CH (bp) (n=4)	1bp allele size incre ments in ZF (bp)
TG01-000	CK314156	1A (1A) (1A)	206,830 (206,830) (201,308)	8.3e-110	$(AT)_{8,8,3,2,3,8}$	8	$(AT)_9$	Y	F: [6-FAM]-TTGCTACCARAATGGAATGT R: TCCTAACCATGAGAAGCAGA	0	1 (F)	F: 55.67 R: 55.99	253	250, 251, 252, 253, 254, 256	190	189	189	Y
TG01-040	DV576233	1A	42,620,542	1.5e-109	$(AT)_2$ G $(AT)_7$ AC $(AT)_6$ TT $(AT)_2$	7	$(AT)_3$ & $(AT)_5$	Y	F: [6-FAM]-TGGCAATGGTGAGAAGTTTG R: AGAATTTGTACAGAGGTAATGCACTG	0	0	F: 59.69 R: 60.01	286	287, 288, 289, 290, 294	274	273	273, 274	Y
TG01-077	CK305147	1	95,581,733	3.3-e129	$(A)_{11}$ & $(CA)_3$	3	$(A)_{12}$ & (CA)	Y	F: [HEX]-GGTATGTCAGTTATCAAAAACAAGC R: AAATGGCAGGTAAGGATACTCTC	0	0	F: 58.28 R: 57.89	153	149, 150, 151	154	152	151, 152	Y
TG01-086	DV948966	1	102,491,753	3.8e-97	$(CT)_6$ TT $(CT)_6$	6	$(T)_4$ G $(T)_7$ G $(T)_4$ G $(T)_5$ G $(T)_3$ G $(T)_5$ G $(T)_{14}$	N	F: [6-FAM]-CCCAGCTTTAAATCCTTCCTG R: TACTGCCTCCAAGGCACAG	0	0	F: 60.08 R: 59.99	257	No amp.	235	No amp.	No amp.	–
TG01-092	DV958291	1	108,930,821	3.4e-146	$(AT)_3$ T $(AT)_6$ TT $(AT)_3$	6	$(AT)_3$ T $(AT)_4$ TT $(AT)_3$	Y	F: [6-FAM]-ATGTTGGTGAAAGTATTACAGCTCTC R: TCACCTTTTAAAAACCAATTTCAAC	0	0	F: 59.22 R: 59.72	183	182	179	178	179	–
TG01-114	CK301583	1	15,302,987	4.8e-115	$(AT)_3$ AA $(AT)_6$	6	$(AT)_3$ AA $(AT)_6$	Y (I)	F: [HEX]-TTGAAACATTGTGAAGCAG R: CAGATAGTGTCATAACAATACTTTTC	0	0	F: 53.07 R: 53.56	182	181, 182	180	181	179	Y
TG01-124	CK306631	1	34,320,381	2.1e-148	$(AT)_{11}$	11	$(AT)_5$	Y	F: [6-FAM]-AGTACTACTTGCCTGCAGAGTTTAT R: TGTGTATGGCAGCATTTACAA	0	0	F: 57.15 R: 57.74	403	400	391	389	389 (n=2)	–
TG01-147	CK315344	1 (1)	115,270,966 (65,886,305)	5.9e-158	$(AT)_5$ TT $(AT)_5$ TT $(AT)_3$ $(A)_6$ $(AT)_5$	5	$(AT)_4$ TT $(AT)_2$ GT $(A)_4$ $(AT)_8$	Y	F: [HEX]-TGAGCCACTACAGAGTGGAAA R: GCCACTACAATGAAGAAAATATTACAG	0	0	F: 58.51 R: 58.51	283	277, 285 (n=1)	278	276, 277	274, 278 (n=3)	N
TG01-148	CK301512	1	65,236,877	4.4e-144	$(AT)_8$ AA AT T $(AT)_5$	8	$(AT)_2$ GA TT $(AT)_2$ $(AC)_3$ $(AT)_5$ GA TT $(AT)_6$	Y	F: [HEX]-TTGCAACACATTCTAATATTGC R: TTTAAAGTACATCAAACAACAAAATC	0	0	F: 55.60 R: 55.99	195	196, 198	189	189	190	N
TG02-078	CK305233	2	82,845,909	1.9e-123	$(AT)_4$ AG $(AT)_7$ $(AC)_3$ $(AT)_6$	7	$(AT)_3$ ACT $(AT)_5$	Y	F: [HEX]-TGTTAAAGCCTGTTCCATAGG R: TTCCCCATAAAGTATGTACGC	0	0	F: 56.95 R: 56.73	308	315, 321	288	287	287	N
TG02-088	DV579347	2	93,538,047	1.9e-135	$(GT)_{15}$	15	GTGA $(GT)_7$ CTGT	Y	F: [6-FAM]-TGTGTGTTGACAGTATTCTCTTGC R: TTTAAACCTAATAAACGTCACACAGTC	0	0	F: 59.36 R: 59.09	266	263, 265, 268, 269	250	248	248	Y
TG02-120	DV945440	2	127,242,053	3.7e-84	$(AT)_4$ AA $(AT)_7$	7	$(AT)_4$ AA $(AT)_{10}$	Y	F: [6-FAM]-TTGGGCAAAGATGATATGAATG R: AGCCAGGTCCAGTTTCTAAGC	0	0	F: 59.79 R: 59.9	230	230	239	241	237, 239, 241	–

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Table 1 Continued

Locus	EMBL accession number*	ZF chr †,‡	ZF position‡	ZF EST-ZF genome BLAST E-value	Repeat motif in ZF EST sequence	MR	Repeat motif in CH genome sequence	SR?	Primer sequences and fluoro-label (5′ - 3′)	M	D	T_m (°C)	Exp. ZF size (bp)	Obs. allele sizes in ZF (bp) (n=4)	Exp. CH size (bp)	Obs. allele sizes in CH (bp) (n=1)	Obs. allele sizes in wild CH (bp) (n=4)	1bp allele size incre ments in ZF (bp)
TG03-002	DV575298	3	28,478,877	9.6e-139	$(AT)_{11}$	11	Multiple repeats $(TG)_6$ $(AT)_5$ AC $(AT)_5$ GC etc	Y	F: [6-FAM]-TCTTGCCTTTTTGGTATGAGTATAG R: TACAAAGCACTGTGGAGCAG	0	0	F: 58.09 R: 57.63	127	124, 126	282	282	283, 285	N
TG03-031	CK312587	3	32,407,799	1.7e-144	$(AT)_{12}$ TT $(AT)_4$	12	$(AT)_7$ TT $(AT)_4$	Y	F: [6-FAM]-ATTGCACATGAACCTGGAAG R: TCATTACTTGAAGCAGGTCTCTG	0	0	F: 58.57 R: 58.66	208	202, 204, 206	197	196	196	N
TG03-034	CK311260	3	44,506,669	5.3e-126	$(AT)_4$ AA $(AT)_{11}$	11	$(AT)_3$ CT AT AA $(AT)_6$ AA $(AT)_6$	Y	F: [6-FAM]-GAGATCGCCACCATCCTG R: AAGTCTACATTTCCCTTGTCTTGG	0	0	F: 60.16 R: 59.93	178	175, 177	168	167	167	N
TG03-035	DV578303	4	7,353,304	1.8e-151	$(AT)_4$ AA $(AT)_6$	6	$(GT)_3$ CT $(GT)_2$ GC $(GT)_2$	N	F: [HEX]-TGATGGCCAAATGCATACTC R: TATTTACAATATCTGCAGAAACAATCC	0	0	F: 59.5 R: 59.01	213	213	211	208	208	–
TG03-098	DV573670	3	102,966,495	1.3e-116	$(AG)_7$ AA AG GCG $(AG)_6$ AA $(AG)_6$	7	Multiple $(GT)_n$ & $(AT)_n$	N	F: [HEX]-TTTGCCTTAATTCTTACCTCATTTG R: TTGCAACCTCTGTGGAAGC	0	0	F: 59.92 R: 59.98	235	235, 236, 237	222	222	222	Y
TG04-004	DV946288	4A Un	6,999,782 8,894,282	3.0e-113 1.0e-99	$(AT)_{10}$ GT $(AT)_7$	10	$(AT)_7$	Y	F: [HEX]-CTGGAGCAGTATTTATATTGATCTTCC R: GAAGATGTGTTTCACAGCATAACTG	0	0	F: 59.83 R: 60.11	166	166, 168	148	147	145, 147, 149	N
TG04-012	CK306810	4A Un	17,044,351 132,142,465	6.1e-124 6.8e-122	$(GT)_4$ CT $(GT)_5$	5	$(AG)_5$ & $(AG)_4$ $(G)_6$ A $(AG)_5$	N	F: [HEX]-TGAATTTAGATCCTCTGTTCTAGTGTC R: TTACATGTTTACGGTATTTCTCTGG	0	0	F: 58.55 R: 58.63	150	150, 152, 154	137	135	135, 136	N
TG04-012A	CK309067	4A	16,934,636	2.9e-133	$(CT)_4$ TT $(CT)_5$ TTTT $(CT)_3$	5	$(AT)_6$	N	F: [6-FAM]-CGTTTTTGCAGTGATTGTGG R: AGCGAGGCCATGTTGAAG	0	0	F: 60.15 R: 59.94	236	235	227	225	237, 239, 241	–
TG04-041	CK316380	4	37,987,055	1.7e-98	$(AG)_7$ TG $(AG)_4$	7	$(CT)_4$ TT $(CT)_4$ TTTT $(CT)_2$= $(AG)_2$ AAAA $(AG)_4$ AA $(AG)_4$	Y	F: [HEX]-CTGAATTGTTGACCTTTGCTTAC R: GTCCTTTTAGAAAGCAGCACAG	0	0	F: 58 R: 58.34	173	172, 174	169	170	170, 178	N

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Table 1 Continued

Locus	EMBL accession number*	ZF chr †,‡	ZF position‡	ZF EST-ZF genome BLAST E-value	Repeat motif in ZF EST sequence	MR	Repeat motif in CH genome sequence	SR?	Primer sequences and fluoro-label (5′ - 3′)	M	D	T_m (°C)	Exp. ZF size (bp)	Obs. allele sizes in ZF (bp) (n=4)	Exp. CH size (bp)	Obs. allele sizes in CH (bp) (n=1)	Obs. allele sizes in wild CH (bp) (n=4)	1bp allele size incre ments in ZF (bp)
TG04-061	CK235034	4	20,910,894	4.6e-85	$(A)_7$ & $(GA)_{6,3,2}$	6	$(AG)_8$ AA $(AG)_4$ GT $(AG)_6$		F: [HEX]-GACAATGGCTATGAAATAAATTAGGC R: AGAAGGGCATTGAAGCACAC	0	0	F: 60.42 R: 60.26	186	183 , 184	210	210	208	Y
TG05-030	CK308028	5	32,518,629	4.7e-140	$(AT)_7$ CT $(AT)_3$	7	$(AT)_7$	Y	F: [HEX]-CTTCCCATCACATCTGTAAC R: GTAAACATTAATATGcAcTTTCTTAG	0§	0	F: 52.84 R: 53.22	186	183	178	177	179	–
TG05-046	DV957774	5	50,735,925	1.2e-128	$(AT)_8$ $(A)_4$ $(AT)_6$ $(A)_9$ $(AT)_2$	8	$(AT)_7$ AA $(AT)_6$	Y	F: [6-FAM]-AAAACATGGCTTACAAACTGG R: GCTCAGATAAGGGAGAAAACAG	0	0	F: 56.86 R: 57.26	337	335, 337	343	344	344, 345, 346	N
TG05-053	CK314425	5	61,275,962	3.1e-132	$(T)_4$ GA $(T)_6$ AA $(T)_{16}$ AA $(T)_4$ G $(T)_6$ & $T(AT)_8$ T $(AT)_4$ AA $(AT)_4$ TATACATA	8	$(A)_{10}$ GAG $(GA)_4$	N	F: [6-FAM]-GCATCATCTGGTTGAACTCTC R: ACCCTGTTTACAGTGAGGTGTT	0	0	F: 57.3 R: 57.63	196	194, 196	199	196, 197	196	N
TG06-009	CK315728	6 Un	3,612,453 76,856,260	6.0e-128 2.9e-127	$(AC)_3$ AT $(AC)_3$ AT $(AC)_3$ & $(GT)_4$ & $(AT)_2$ GT $(AT)_{10}$ GT $(AT)_3$	10	T $(AT)_7$ T $(AT)_4$ AA $(AT)_4$	Y	F: [6-FAM]-AAGCCTTGCTTACATTTTATGGTG R: GGGGTGGTAACTGAAATAAAGTATAGG	0	0	F: 60.72 R: 60.56	127	123, 125	126	122	120, 122	N
TG07-022	DV948210	7 (7) (7)	11,970,577 (11,939,763) (11,970,577)	1.4e-90	$(AT)_6$ AA $(AT)_4$	6	$(AC)_3$ AG ACT $(AC)_4$ & $(GT)_3$ & $(AT)_2$ $(GT)_3$ $(AT)_3$ $(GT)_4$ & $(AT)_{10}$	Y	F: [HEX]-CAGAAGACTGTGTTCCTTTTGTTC R: TTCTAATGTAGTCAGCTTTGGACAC	0	0	F: 59.36 R: 58.94	416	414, 416, 418, 420	437	437, 441	435, 437 (n=2)	N
TG08-024 (set 1)	CK314428	8	21,095,625	5.3e-127	$(AT)_4$ AG $(AT)_2$ AA $(AT)_3$ AA $(AT)_5$	5	$(AT)_6$ AA $(AT)_4$	Y	F: [HEX]-CCCACAAATCCTGAATTTCATATC R: ACTGGCTTATAAAGTCCATGGTTG	0	0	F: 60.75 R: 60.62	128	123	124	120	120	–
TG08-024 (set 2)	CK314428	8	21,095,625	5.3e-127	$(AT)_4$ AG $(AT)_2$ AA $(AT)_3$ AA $(AT)_5$	5	$(AT)_6$ AA $(AT)_4$	Y	F: [HEX]-CACAAATCCTGAATTTCATATCC R: AACAACGACAGCTATGAAAGAAC	0	0	F: 57.51 R: 57.64	243	240, 241	239	237	237, 238	Y

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Table 1 Continued

Locus	EMBL accession number*	ZF chr †,‡	ZF position‡	ZF EST-ZF genome BLAST E-value	Repeat motif in ZF EST sequence	MR	Repeat motif in CH genome sequence	SR?	Primer sequences and fluoro-label (5′ - 3′)	M	D	T_m (°C)	Exp. ZF size (bp)	Obs. allele sizes in ZF (bp) (n=4)	Exp. CH size (bp)	Obs. allele sizes in CH (bp) (n=1)	Obs. allele sizes in wild CH (bp) (n=4)	1bp allele size incre ments in ZF (bp)
TG09-014	DV948892	9	16,778,581	2.0e-101	$(AT)_4$ AG $(AT)_2$ AA $(AT)_3$ AA $(AT)_5$ AAAATAA $(AT)_4$ & $(A)_5$	6	$(AT)_4$ AG $(AT)_2$ AA $(AT)_3$ AA $(AT)_6$ AAAATA A $(AT)_4$ & $(A)_{13}$	Y	F: [6-FAM]-CCAAAGGTGAAGGAATCTATGG R: TCTGCCTGCAGAGTCCAAC	0	0	F: 59.83 R: 60.13	152	150	161	159	159	–
TG11-011	CK308096	11	19,380,799	9.7e-97	$(AT)_9$ AA $(AT)_6$ TA $(AT)_3$	9	$(AT)_5$	Y	F: [6-FAM]-ACAAACTAAGTACATCTATATCTgAAG R: TAAATACAGGCAACATTGG	1 (C)	0	F: 52.02 R: 52.07	223	221, 222	210	209	209	Y
TG12-015	DV953675	12	16,293,506	2.9e-145	$(AT)_{11}$ AA $(AT)_6$	11	$(AT)_4$ & $(AT)_6$ & $(AT)_3$	Y	F: [HEX]-ACAACAGTGGCTTTACTGTGTGA R: TACAGCAGCTGCAGCAAAGT	0	0	F: 59.76 R: 59.96	283	284, 288, 296	276	274	274	N
TG13-009	DV948691	13	3,672,471	1.3e-108	$(AT)_4$ GT $(AT)_5$	5	$(AT)_{13}$ AA $(AT)_6$	Y	F: [HEX]-TGTGGTGGGATAGTGGACTG R: CTGTAAAATGTGCAAGTAACAGAGC	0	0	F: 59.39 R: 59.46	195	195 (n=2)	187	185	186	–
TG13-016	CK308822	13	1,151,543	6.7e-143	$(A)_6$ & $(C)_4$ AA $(CA)_3$ $(GA)_2$	3	$(AT)_4$ GT $(AT)_5$	N	F: [6-FAM]-GATTGCTGAGGCTTGATTGC R: GCCTACGGCTTTATTTTACTTGC	0	0	F: 60.48 R: 58.28	136	130	143	136	136, 138 (n=3)	–
TG13-017	CK313422	13	18,542	1.5e-149	$(AT)_{10}$	10	$(AT)_5$	Y	F: [6-FAM]-GCTTTGCATCTTGCCTTAAA R: GGTAACTACAACATTCCAACTCCT	0	0	F: 58.19 R: 57.74	300	293, 295, 297	250	246	246	N
TG22-001	CK317333	Un 22 (Un)	157,424,056 1,428,098 (157,424,056)	3.0e-123 4.1e-123	$(AT)_5$ T $(AT)_6$	6	$(AT)_6$ GA $(AT)_3$ T $(A)_7$ $(AT)_2$	Y	F: [HEX]-TTGGATTTCAGAACATGTAGC R: TCTGATGCAAGCAAACAA	0	0	F: 55.39 R: 54.63	269	266, 268, 270	250	249	249	N

*The sequences were isolated by Replogle *et al.* 2008; Wada *et al.* 2006 and Wade *et al.* 2004.

†Duplicate hits to the ChrUnk (the unknown chromosome) were disregarded when these were identical to the hits to named chromosomes. These were considered to be residue sequences, which had not been deleted when sequence was assigned to named chromosomes.

‡Genome locations in the zebra finch were assigned using the WU GSC BLAST software provided on the Washington University server. Conflicting location assignments obtained by performing a WU-BLAST against the ENSEMBL zebra finch genome are recorded in parentheses.

§Two bases of the zebra finch EST sequence used in the reverse primer were unknown ('n') bases so the base in the chicken sequence was used. When the zebra finch EST sequence was compared against the recently released zebra finch genome sequence it was found that the previously unknown primer bases matched the chicken sequence.

ZF, zebra finch *Taeniopygia guttata*; CH, chicken *Gallus gallus* (the single individual tested belonged to a domesticated population); MR, maximum repeat run in the zebra finch EST sequence, i. e. longest number of uninterrupted tandemly repeating units; SR, same repeat motif type in zebra finch and chicken; I, repeat region composition and length identical in zebra finch and chicken; M, number of primer base mismatches; D, number of degenerate bases in primer sequence; Exp. ZF size; expected PCR product size based on the zebra finch EST sequence; Exp. CH size; expected PCR product size based on orthologous chicken genome sequence; Y, yes; N, no; No amp., no amplification.

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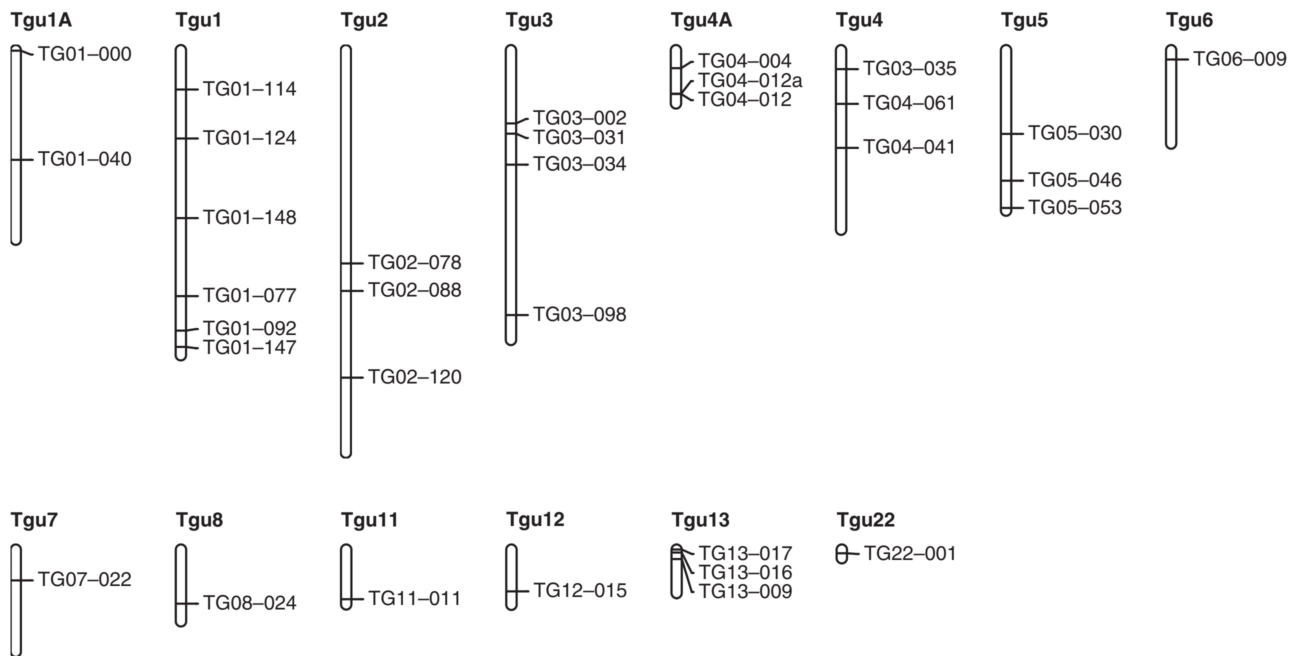

Fig. 1 Chromosomal locations in the zebra finch (*Taeniopygia guttata*) genome of 33 polymorphic conserved avian EST (expressed sequence tag) microsatellite loci for which primer sets were developed and found to be of high utility in passerine birds. Notes: The two loci of poor utility are not included (*TG01-086* and *TG09-014*). Locus *TG01-086* did not amplify in zebra finch, chicken or any of the other 27 species tested and locus *TG09-014* was monomorphic in all 21 species tested.

cation levels. Nine loci were tested in at least four individuals for 13 additional species of shorebird (Table 2).

All individuals genotyped were caught in the wild and belonged to a single population, with the exception of the zebra finch, Gouldian finch *Chloebia gouldiae*, ruff *Philomachus pugnax*, spotted thick-knee *Burhinus capensis* and the single cape parrot *Poicephalus robustus robustus* and single domesticated chicken tested (Table 2). These individuals were sampled in captive populations maintained at the University of Sheffield, the University of New South Wales (Sydney, Australia), Simon Fraser University (Burnaby, Canada), World of Birds (Cape Town, South Africa), belonging to a private breeder in South Africa and the United States Department of Agriculture (Agriculture Research Service, East Lansing, USA), respectively.

The blood samples collected from each individual were stored in absolute ethanol, Queen's Lysis buffer (Seutin *et al.* 1991) or Longmire's buffer (Longmire 1997). A feather was used for DNA extraction for the saker falcon *Falco cherrug*. Prior to DNA extraction, the feather was stored at room temperature. Genomic DNA was extracted using an ammonium acetate precipitation method (Nicholls *et al.* 2000), a salt extraction method (Bruford *et al.* 1998) or using Chelex-100 (Ceo *et al.* 1993; Harris 2007). Each DNA extraction was tested for amplification with the locus *LEI160* (Gibbs *et al.* 1997, Wardle *et al.* 1999), which has been found to amplify in all bird species tested (approximately 100 species; DAD unpublished data). PCR amplification was confirmed on 2% agarose gel stained with ethidium bromide or SYBR safe.

Each primer set was tested in isolation in all species, except for four finch species (see below). PCR reactions were performed in 10 μL volumes, with the exception of Berthelot's pipit *Anthus berthelotii*, which was amplified in a 2 μL PCR reaction (following Kenta *et al.* 2008). Each 10 μL PCR reaction contained approximately 20 ng of genomic DNA, 0.5 μM of each primer, 0.2 mM of each dNTP, 2.0 mM $MgCl_2$ and 0.25 units of *Taq* DNA polymerase (Bioline) in the manufacturer's buffer (final concentrations: 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25 °C), 0.01% Tween-20). We used the following PCR program: 94 °C for 3 min followed by 35 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s and finally 72 °C for 10 min. Amplification was performed using an MJ Research model PTC DNA Engine Tetrad thermal cycler.

Loci were fully characterized in a minimum of 17 individuals for four finch species: greenfinch *Carduelis chloris* (*n* = 21), common crossbill *Loxia curvirostra* (*n* = 17), Eurasian bullfinch *Pyrrhula pyrrhula* (*n* = 23) and chaffinch *Fringilla coelebs* (*n* = 20). The greenfinches were sampled at three locations: Kiev, Ukraine (*n* = 8), Oulu, Finland (*n* = 7) and Uppsala, Sweden (*n* = 6; Juha Merilä

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Table 2 Amplification of conserved microsatellite primer sets in 51 species and the genetic distance of each species from the zebra finch *Taeniopygia guttata* and chicken *Gallus gallus*

Species	Binominal name	Status	Tissue sampled and storage	Genetic distance to ZF (ΔT_mH)	Genetic distance to CH (ΔT_mH)	Order	Family (Sibley & Monroe 1990 / NCBI Taxonomy Database)	*n*	# loci tested	Loci amp. (%)*	DNA extractor and tissue supplier(s)
(a) Twenty-one species for which a single individual was tested											
Neognathae											
Passerines											
Zebra finch‡	*Taeniopygia guttata*	Captive	T/E	0	28	Passeriformes	Passeridae / Estrildidae	1	34	(100)*	Jon Chittock, Jayne Pellatt, Tim Birkhead
House sparrow‡	*Passer domesticus*	Wild	B/E	<5.4	28	Passeriformes	Passeridae	1	34	71	Nancy Ockenden
Reed bunting	*Emberiza schoeniclus*	Wild	B/E	10	28	Passeriformes	Fringillidae	1	34	50	Graeme Buchanan, Andrew Dixon
Long-tailed tit	*Aegithalos caudatus*	Wild	B/E	11.1	28	Passeriformes	Aegithalidae	1	34	82	Douglas Ross, Ben Hatchwell
Great tit‡	*Parus major*	Wild	B/E	11.1	28	Passeriformes	Paridae	1	34	56	Angharad Bickle
White-spectacled bulbul	*Pycnonotus xanthopygos*	Wild	B/E	11.1	28	Passeriformes	Pycnonotidae	1	34	76	John Wright
Capricorn silvereye	*Zosterops lateralis chlorocephala*	Wild	B/E	11.1	28	Passeriformes	Zosteropidae	1	34	71	Ian Owens
Starling	*Sturnus vulgaris*	Wild	B/E	11.7	28	Passeriformes	Sturnidae	1	34	85	Mike Double
Non-passerines											
Blue crane	*Grus paradisea*	Wild	B/L	21.6	28	Gruiformes	Gruidae	1	34	33	Kate Meares, Tiawana Taylor
Golden eagle	*Aquila chrysaetos*	Wild	B/E	21.6	28	Falconiformes	Accipitridae	1	34	79	Brian Bourke
Saker falcon	*Falco cherrug*	Wild	F/RT	21.6	28	Falconiformes	Accipitridae	1	34	47	Andrew Dixon
European turtle dove	*Streptopelia turtur*	Wild	B/E	21.6	28	Columbiformes	Columbidae	1	34	79	Pippa Thomson, Oliver Hanotte
Southern giant petrel (Antarctic giant petrel)	*Macronectes giganteus*	Wild	B/E	21.6	28	Procellariiformes	Procellariidae	1	34	82	Douglas Ross, Richard Phillips
Adelie penguin	*Pygoscelis adeliae*	Wild	B/E	21.6	28	Sphenisciformes	Spheniscidae	1	34	74	Fiona Hunter
Kea	*Nestor notabilis*	Wild	B/E	23.1	28	Psittaciformes	Psittacidae	1	34	68	Bruce Robertson
Cape parrot	*Poicephalus robustus robustus*	Captive	B/L	23.1	28	Psittaciformes	Psittacidae	1	34	(97)*	Kerusha Pillay, Tiawana Taylor
Greater spotted cuckoo	*Clamator glandarius*	Wild	B/E	23.6	28	Cuculiformes	Cuculidae	1	34	71	Juanga Martinez
Monterios's hornbill	*Tockus monteiri*	Wild	B/E	25	28	Bucerotiformes	Bucerotidae	1	34	71	David Richardson
Palaeognathae			B/E								
Chicken (domestic)‡	*Gallus gallus domesticus*	Captive	B/E	28	0	Galliformes	Phasianidae	1	34	(100)*	Nat Bumstead, Hans Cheng
Mallard	*Anas platyrhynchos*	Wild	B/E	28	22.9	Anseriformes	Anatidae	1	34	79	Emma Cunningham, Tim Birkhead
Ostrich (Ratite)	*Struthio camelus*	Wild	B/E	28	25.9	Struthioniformes	Struthionidae	1	34	76	Jeff Graves, Charles Kimwele, Dominique Blache, Leon Huynen, Irek Malecki

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Table 2 Continued

Species	Binominal name	Status	Tissue sampled and storage	Genetic distance to ZF (ΔT_mH)	Genetic distance to CH (ΔT_mH)	Order	Family (Sibley & Monroe 1990 / NCBI Taxonomy Database)	*n*	# loci tested	Loci amp. (%)*	DNA extractor and tissue supplier(s)
(b) Twenty-two species for which a minimum of four individuals were tested											
Neognathae											
Passerines											
Zebra finch‡	*Taeniopygia guttata*	Captive	T/E	0	28	Passeriformes	Passeridae / Estrildidae	4	34	100	Jon Chittock, Jayne Pellatt, Tim Birkhead
Gouldian finch	*Chloebia gouldiae*	Captive	B/E	<5.4	28	Passeriformes	Passeridae	8	34	100	Susannah Bird, Simon Griffith
Berthelot's pipit	*Anthus berthelotii*	Wild	B/E	8.3	28	Passeriformes	Passeridae	4	34	100	Lewis Spurgin, David Richardson, Juan Carlos Illera
House sparrow‡	*Passer domesticus*	Wild	B/E	8.5	28	Passeriformes	Passeridae	4	34	100	Nancy Ockenden
Greenfinch	*Carduelis chloris*	Wild	B/E	10	28	Passeriformes	Fringillidae	21	34	100	Kate Durrant, Juha Merilä
Common crossbill	*Loxia curvirostra*	Wild	B/E	10	28	Passeriformes	Fringillidae	17	33	97	Kate Durrant, Stuart Piertney
Chaffinch	*Fringilla coelebs*	Wild	B/E	10	28	Passeriformes	Fringillidae	20	34	100	Ben Sheldon
Eurasian bullfinch	*Pyrrhula pyrrhula*	Wild	B/E	10	28	Passeriformes	Fringillidae	23	34	100	Kate Durrant, Stuart Sharp, Simone Immler
Fairy martin	*Petrochelidon ariel* (*Hirundo ariel*)	Wild	B/E	11.1	28	Passeriformes	Hirundinidae	6	34	100	Ian Stewart, Greg Adcock, Simon Griffith
Great tit‡	*Parus major*	Wild	B/E	11.1	28	Passeriformes	Paridae	4	34	100	Louise Gentle, Angharad Bickle
Blue tit	*Cyanistes caeruleus* (*Parus caeruleus*)	Wild	B/E	11.1	28	Passeriformes	Paridae	4	34	100	Bengt Hansson
Great reed warbler	*Acrocephalus arundinaceus*	Wild	B/E	11.1	28	Passeriformes	Sylviidae	4	34	100	Bengt Hansson
Vinous-throated parrotbill	*Paradoxornis webbianus*	Wild	B/E	11.1	28	Passeriformes	Sylviidae / Muscicapidae (Timaliidae)	4	34	100	Jin-Won Lee, Ben Hatchwell
European blackbird	*Turdus merula*	Wild	B/E	11.7	28	Passeriformes	Muscicapidae / Turdidae	4	34	100	Michelle Simeoni, Ben Hatchwell
Chestnut-crowned babbler	*Pomatostomus ruficeps*	Wild	B/E	12.8	28	Passeriformes	Pomatostomidae / Timaliidae	6	34	100	Ian Stewart, Andrew Russell
Black-billed magpie	*Pica pica*	Wild	B/E	12.8	28	Passeriformes	Corvidae	4	34	100	David Martín-Gálvez
Apostlebird	*Struthidea cinerea*	Wild	B/E	12.8	28	Passeriformes	Corvidae / Corcoracidae	6	34	100	Ian Stewart, Simon Griffith
Non-passerines											
Kentish plover	*Charadrius alexandrinus*	Wild	B/Q	21.6	28	Charadriiformes	Charadiidae	4	34	100	Clemens Küpper, Tamás Székely
Rufous hummingbird	*Selasphorus rufus*	Wild	M/-70C & F/RT	22.5	28	Trochiliformes	Trochilidae	6–8	34	97	Ida Bacon, Josephine Pemberton
Barn owl	*Tyto alba*	Wild	B/E	22.5	28	Strigiformes	Tytonidae	8–20	34	100	Ákos Klein
Peach-faced lovebird	*Agapornis roseicollis*	Wild (& Captive)	B/E	23.1	28	Psittaciformes	Psittacidae	7 (& 2)	34	97	Andrew Krupa, Gemma Smith, Tim Birkhead
Palaeognathae											
Chicken (wild)‡	*Gallus gallus*	Wild	B/E	28	0	Galliformes	Phasianidae	4	34	100	Tommaso Pizzari

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Table 2 Continued

Species	Binominal name	Status	Tissue sampled and storage	Genetic distance to ZF (ΔT_mH)	Genetic distance to CH (ΔT_mH)	Order	Family (Sibley & Monroe 1990 / NCBI Taxonomy Database)	*n*	# loci tested	Loci amp. (%)*	DNA extractor and tissue supplier(s)
(c) Thirteen additional charadriiform species for which a minimum of four individuals were tested but with only 9 loci											
Neognathae											
Non-passerines											
Ruff	*Philomachus pugnax*	Captive	B/E	21.6	28	Charadriiformes	Scolopacidae	16	9	89	David Lank
Whiskered auklet	*Aethia pygmaea*	Wild	B/E	21.6	28	Charadriiformes	Laridae / Alcidae	16	9	89	Ian Hartley, Fiona Hunter
Collared pratincole	*Glareola pratincola*	Wild	B/E	21.6	28	Charadriiformes	Glareolidae	4	9	89	Auxi Villegas Sanchez
Brown (Antarctic) skua	*Catharacta lonnbergi*	Wild	B/E	21.6	28	Charadriiformes	Laridae / Stercorariidae	4	9	100	Douglas Ross, Richard Phillips
Gull-billed tern	*Gelochelidon nilotica* (*Sterna nilotica*)	Wild	B/E	21.6	28	Charadriiformes	Laridae	4	9	100	Douglas Ross, Richard Phillips, Auxi Villegas Sanchez
Red-necked phalarope	*Phalaropus lobatus*	Wild	B/E	21.6	28	Charadriiformes	Scolopacidae	4	9	100	Phil Whitfield
Great snipe	*Gallinago media*	Wild	B/E	21.6	28	Charadriiformes	Scolopacidae	4	9	100	Jon-Atle Kålas
Dunlin	*Calidris alpina*	Wild	B/E	21.6	28	Charadriiformes	Scolopacidae	4	9	100	Liv Wennerberg, Donald Blomqvist
Spotted thick-knee	*Burhinus capensis*	Captive	B/Q	21.6	28	Charadriiformes	Burhinidae	3	9	100	Tamás Székely, World of Birds, Cape Town, South Africa
Eurasian oystercatcher	*Haematopus ostralegus*	Wild	B/E	21.6	28	Charadriiformes	Charadriidae / Haematopodidae	4	9	100	Dik Heg
Avocet	*Recurvirostra avosetta*	Wild	B/E	21.6	28	Charadriiformes	Charadriidae / Recurvirostridae	4	9	100	Szabolcs Lengyel
Snowy plover†	*Charadrius alexandrinus nivosus*	Wild	B/Q	21.6	28	Charadriiformes	Charadriidae†	4	9	100	Clemens Küpper
Greater sheathbill	*Chionis alba*	Wild	B/E	21.6	28	Charadriiformes	Chionidae	4	9	100	Douglas Ross, Richard Phillips

*Of those species tested with one individual, amplification failures were re-amplified for the zebra finch, cape parrot and chicken only (Table 2a).
†Classified post Sibley & Ahlquist (1990), Küpper *et al.* (2009).
‡Four species that were tested with only a single individual were retested with four individuals (zebra finch, house sparrow, great tit and chicken).
T, tissue; B, blood; E, ethanol; L, Longmire's buffer (Longmire 1997); Q, Queen's Lysis buffer (Seutin *et al.* 1991); M, muscle; F, feather; RT, room temperature; n, number of individual tested; Amp., amplifying.
Genetic distance to ZF, genetic distance from species tested to zebra finch based on Sibley & Ahlquist (1990) and the classification of Sibley & Monroe (1990).
Genetic distance to CH, genetic distance from species tested to chicken (Sibley & Ahlquist 1990).
All of the PCR failures in the species tested in four individuals (Table 2b) were rechecked with the exception of the thirteen additional shorebird species (Table 2c).

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pers. comm.). The common crossbill individuals were sampled at three locations: Kielder, Northumberland, England ($n = 5$) and two locations near Rothiemurchus, Aviemore, Scotland ($n = 10$ & $n = 2$; Stuart Piertney pers. comm.). The Eurasian bullfinch individuals were sampled at three closely neighbouring locations in South Yorkshire, England: Sheffield ($n = 8$), Agden Reservoir ($n = 7$) and Denaby Ings Nature Reserve ($n = 8$; Simone Immler and Stuart Sharp pers. comm.). Finally, the chaffinch individuals were sampled in the breeding season at a single location near Whirlow Park, Sheffield, England (Ben Sheldon pers. comm.). The individuals genotyped for each species were presumed to belong to a single population, including the two species sampled from more widely-spaced locations (greenfinch and crossbill). For these four finch species, in most cases, two differently labelled primer sets were amplified simultaneously (multiplexed). Primer sets were checked for their potential to form hairpins and to identify any PCR incompatibilities due to primer sequence similarity using AUTODIMER software (Vallone & Butler 2004). No hairpins were detected in any primer sequences. Five pairs of primer sequences displayed some degree of homology and were avoided as multiplex combinations to prevent the risk of forming primer dimers (TG12-015F & TG02-088R, TG07-022F & TG02-088R, TG05-046F & TG02-120R, TG03-035R & TG01-114R, TG02-078R & TG01-124F). Each 4 μL multiplex PCR reaction contained approximately 20 ng of DNA, 0.5 μM of each primer and 2 μL of 2x QIAGEN Multiplex PCR Master Mix. The PCR program used for all loci when amplifying the finch species was 95 °C for 15 min followed by 35 cycles at 94 °C for 30 s, 56 °C for 90 s, 72 °C for 60 s, a final extension step of 60 °C for 6 min and an ambient holding temperature.

Products were diluted 1 in 500 prior to separation on an ABI DNA Analyzer and allele sizes were assigned using GeneMapper 3.7 software (Applied Biosystems). The same ABI 3730 DNA Analyzer at Sheffield was used for all species, with three exceptions. Two species, the blue tit *Cyanistes caeruleus* (*Parus caeruleus*) and the great reed warbler *Acrocephalus arundinaceus* were genotyped in a different laboratory at Lund University, Sweden using an ABI 9700 PCR machine and an ABI 3130 DNA Analyzer. The rufous hummingbird individuals were genotyped using a DYAD peltier thermal cycler and an ABI 3730 DNA Analyzer at the University of Edinburgh.

Different species were scored in different sessions by different individual researchers with three exceptions. The 21 species, for which only one individual was genotyped were scored by a single researcher (GH), the 15 charadiform species were all scored by one researcher (CK) and the greenfinch, crossbill, bullfinch and chaffinch genotypes were all scored by one researcher (GH). Alleles were scored separately for each species using species-specific allele bin sets.

Locus assessment, heterozygosity and linkage

Heterozygosity and estimated null allele frequencies were calculated using CERVUS v3.0 (Marshall *et al*. 1998; Kalinowski *et al*. 2007). Tests for departures from Hardy–Weinberg proportions and genotypic disequilibrium were conducted using the Markov-chain algorithm implemented in GENEPOP v3.4 (Raymond & Rousset 1995).

Results

Identification of highly conserved microsatellite loci and primer set design

Of the 687 zebra finch EST microsatellite sequences examined, 465 (68%) displayed homology with chicken and, among these, 135 (20%) had chicken sequence homologues with a BLAST E-value better than E-80 (data extracted from Slate *et al*. 2007). These 135 zebra finch sequences were aligned with their chicken homologues, and where possible, a consensus hybrid sequence created. Few hybrid sequences contained regions of 100% zebra finch–chicken consensus sequence of sufficient length from which to design primers. However, conserved primer sets could be created for 35 autosomal loci (5%) using the strict criteria outlined in the Methods section. The 35 sequences selected were isolated by Wade *et al*. (2004), Wada *et al*. (2006) and Replogle *et al*. (2008). The majority of homologous sequences displayed repeat regions in the chicken that were of the same motif type and were similar in composition to those observed in zebra finch. Details of the loci selected and primer sets developed are provided in Table 1.

Some of the selected EST loci possessed a relatively small number of uninterrupted dinucleotide repeat units (average length 7.4 repeat units, range 3–15, Table 1). In general, published polymorphic microsatellites possess at least nine repeats (based on the 550 avian microsatellite loci referenced by Dawson *et al*. 2006). However, we designed primer sets for all loci with at least three uninterrupted repeats because in most cases, several different repeat regions were present in the sequence (Table 1). Despite the small number of uninterrupted dinucleotide repeat units at some loci (Table 1), several loci were found to be polymorphic (Table 1, Table S1). For example, loci *TG01-000* and *TG22-001* possessed repeat runs of only eight and six repeats respectively, (Table 1) but displayed a high number of alleles (5–17) and heterozygosities greater than 0.70 in three of the four finch species tested (Table S2).

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Genome locations

All loci were assigned an autosomal location on the zebra finch genome based on sequence homology (Fig. 1). Two pairs of loci were assigned locations less than 5 Mb apart in the zebra finch genome and alleles at these loci may therefore tend to cosegregate and show linkage: *TG4-012* & *TG4-012A* and *TG13-016* & *TG13-017* (Table 1, Fig. 1).

Two loci were assigned to different locations in the chicken genome to those given by Slate *et al.* (2007). Locus *TG03-035* (DV578303) had been assigned to chicken chromosome 3 (Gga3), however, it was assigned to chromosome 4 in chicken, zebra finch and blue tit (Table 1, Fig. 1; Hansson *et al.* 2009). Locus *TG22-001* (CK317333) was assigned to a different base pair location, but to the same chromosome, Gga22.

Genome locations in the zebra finch, which were assigned using the Washington University server, were rechecked using the alternative WU-BLAST software provided by the ENSEMBL server. The locations assigned were identical with the exception of four loci. An additional hit to the same chromosome was assigned for *TG01-000* and *TG07-022*, an alternative location on the same chromosome was assigned for *TG01-147* and a location to the 'Unknown chromosome' only was assigned for *TG22-001* (Table 1).

Genotyping

All loci amplified in both zebra finch and chicken, except *TG01-086* (Table 1). Locus *TG01-086* failed to amplify any product in all 29 species tested (Table 1, Table S3 and unpublished data). The 34 amplifying primer sets included *TG01-000*, which contained a degenerate primer base, and *TG11-011*, which contained a single primer base that did not match chicken (Table 1).

In both zebra finch and chicken, the observed allele sizes were very similar to those expected based on the respective species sequences (Table 1). The maximum observed difference between the expected and observed allele sizes was seven base pairs (bp) for zebra finch and five base pairs for chicken (Table 1). The expected allele sizes in zebra finch when compared with those expected in chicken for each locus differed by a maximum of 24 bp, with the exception of loci *TG01-000*, *TG03-002* and *TG13-017*, which differed by 50–155 bp. For the vast majority of loci, the observed allele sizes in different species were very similar to those expected based on zebra finch and chicken and therefore of similar size in each species (normally ±1 to ±20 bp, 22 species checked at 34 loci; Table S3). This suggests that the correct target locus was being amplified in all species tested.

Cross-species amplification

A minimum of four individuals was genotyped in 17 passerine and five non-passerine species. On average, 100% of loci amplified per passerine species and 99% amplified per non-passerine species (zebra finch and chicken excluded; Fig. 1, Table S1). A maximum of four loci per species failed to amplify in the initial test and a repeat PCR was performed. For two loci (*TG01-147* and *TG12-015*), primer degradation was identified as the source of the initial amplification failure.

There was no decrease in amplification success with increasing genetic distance across species when the loci

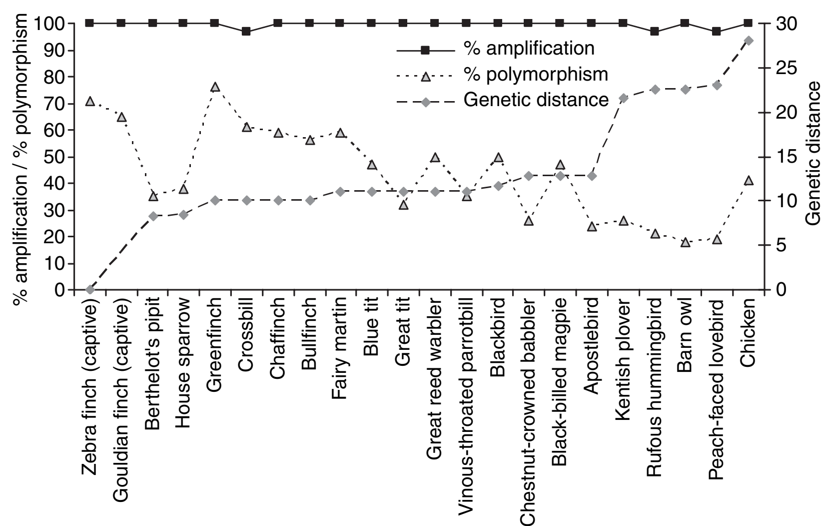

Fig. 2 Amplification and polymorphism of 34 conserved avian EST microsatellite loci in 22 species in relation to their genetic distance from zebra finch (*Taeniopygia guttata*)*. Genetic distance, DNA: DNA ΔT_m hybridisation distance (Sibley & Ahlquist 1990). *4 individuals were genotyped for each species at 34 loci.

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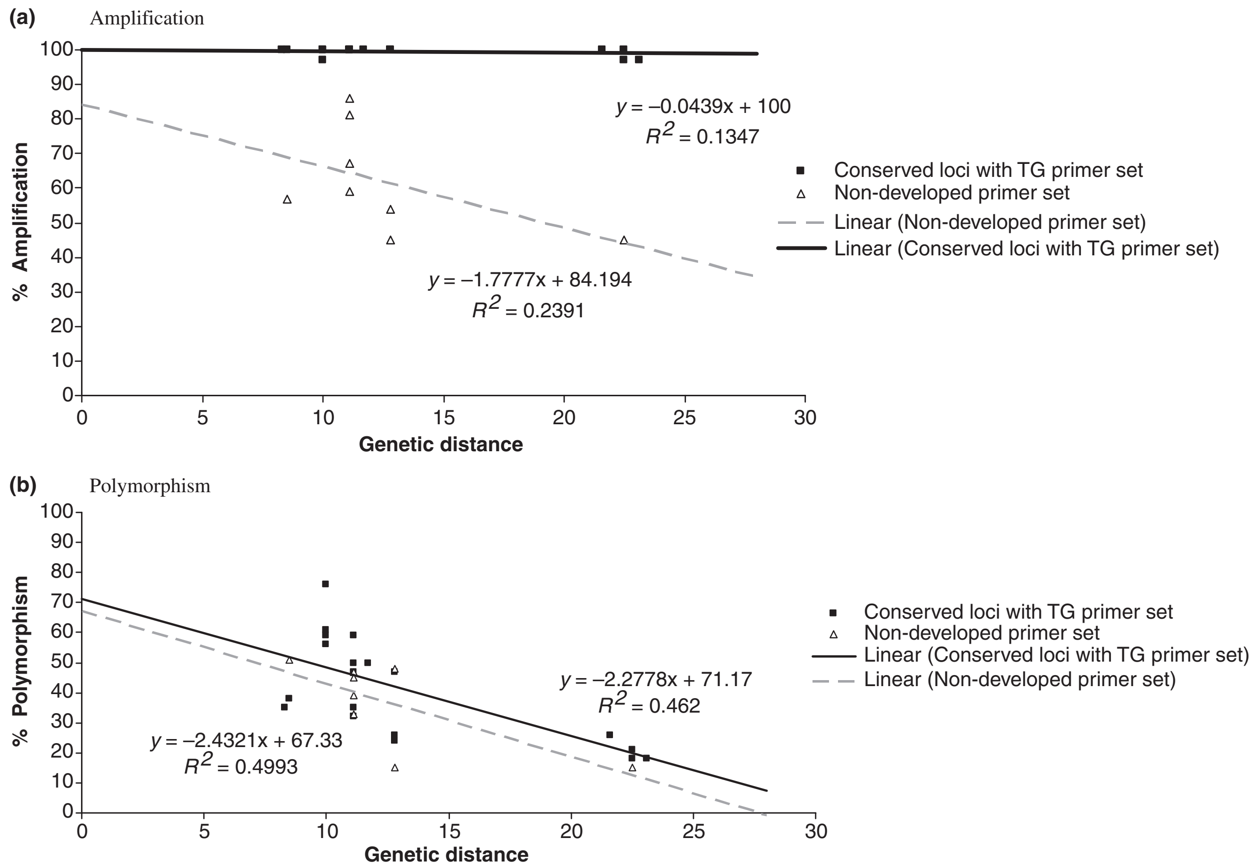

Fig. 3 Cross-species utility of conserved EST microsatellite loci when amplified with conserved TG primer sets vs. the utility of anonymous EST microsatellite loci amplified with non-developed primer sets*. Genetic distance between each species tested and the zebra finch (*Taeniopygia guttata*) based on DNA:DNA ΔT_m hybridization distance (Sibley & Ahlquist 1990) and the classification of Sibley & Monroe (1990). The DNA:DNA ΔT_m hybridization distance between Gouldian finch (*Chloebia gouldiae*) and zebra finch is less than 5.4, but the actual figure is unknown and therefore this data point was omitted from Fig. 3a, b. *34 conserved and developed TG EST primer sets were tested and four individuals were genotyped per species (19 species included, zebra finch, Gouldian finch and chicken results excluded) and 84 non-developed Tgu-EST primer sets were tested in four to eight individuals per species (eight species tested).

were tested in a minimum of four individuals per species (Figs 2 and 3). This was despite testing a wide range of species and including species that were distant from both the zebra finch and chicken (Figs 2 and 3). However, it should be noted that only four non-passerine species were included here (chicken data excluded).

A high proportion of loci amplified in each of the 21 species when just a single individual was tested at all 34 loci (eight passerines and 13 non-passerines, Table 2). None of the reactions failing to amplify were repeated, except zebra finch, cape parrot and chicken. On average, 70% of loci amplified in each passerine and 67% in each non-passerine when a single individual was tested (Table 2, zebra finch and chicken data excluded). However, we consider these estimates to be conservative due to detrimental effects on amplification levels of testing only one individual and poor DNA quality for some species (see Discussion).

Cross-species polymorphism

Only one locus (*TG09-014*) was monomorphic in all passerine and non-passerine species tested, displaying very similar allele sizes (148–159 bp) in the 38 species tested (Table S3 & unpublished data). The proportion of polymorphic loci per species, when four individuals were tested, ranged from 24 to 76% (mean 48%) in passerines and from 18 to 26% (mean 21%) in non-passerines (16 passerine and four non-passerine species tested, zebra finch, chicken and *TG09-014* data excluded, Table S1). Polymorphism decreased in passerines as the genetic distance from zebra finch increased (Figs 2 and 3). When assessed in four individuals per species, the highest levels of polymorphism were recorded for Passeridae and Fringillidae species (35–76% of loci polymorphic, mean 56%) dropping to 24% in species more distant from the zebra finch such as the apostle bird *Struthidea cinerea* (Fig. 2, Table S1).

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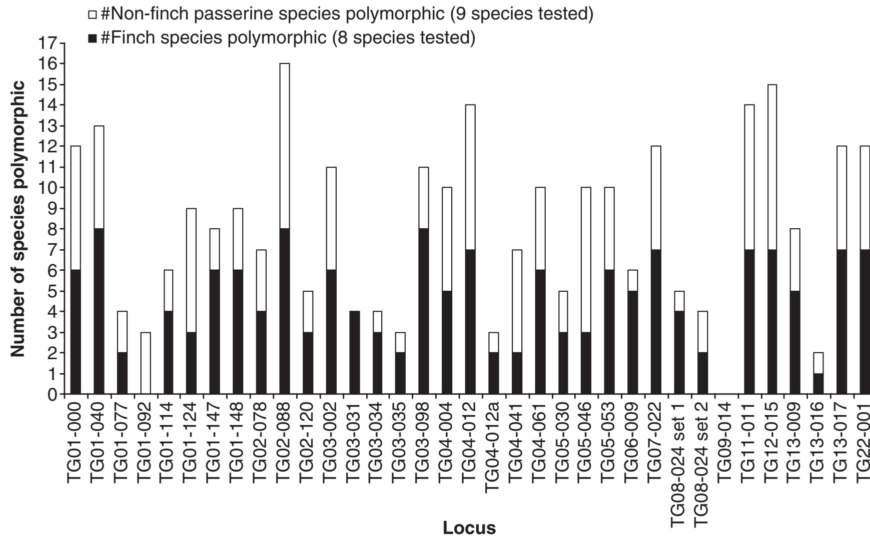

Fig. 4 Number of passerine species polymorphic at each conserved EST microsatellite locus when amplified with the conserved TG primer set*. 'Finch' indicates that species belong to the Passeridae and Fringillidae families and 'non-finch' indicates that species belong to other passerine families (classification following Sibley & Monroe 1990). *Each locus was tested in 17 species (including zebra finch *Taeniopygia guttata*). All loci amplified in all 17 species except locus *TG13-017* which failed to amplify in one species (common crossbill *Loxia curvirostra*). The data presented is based on the genotyping of four individuals per species.

A majority of loci were polymorphic in eight or more species (zebra finch included, Fig. 4). Seventeen loci were polymorphic in a minimum of 50% of the 17 passerine species tested (when all loci were assessed in four individuals/species; Fig. 4, Table S1). The highest performing loci included *TG01-040*, *TG02-088*, *TG04-012*, *TG11-011* and *TG12-015* which were polymorphic in 13–16 of 17 passerine species tested (76–94%; Fig. 4, Table S1).

Full locus assessment, heterozygosity and linkage

When 34 loci were characterized in at least 17 individuals from four Fringillidae finch species, high levels of polymorphism were observed and the majority of loci were considered to be easy to score (Table S2). Two loci failed to amplify consistently when part of a multiplex set: *TG11-011* in chaffinch and *TG13-016* in all four species (when co-amplified with *TG04-061* & *TG09-014*). However, these loci amplified well as singleplexes (Table S2). Locus *TG13-017* failed to amplify in crossbill when amplified as part of a multiplex set and in singleplex (as did *TG08-024* primer set 2). At several loci, the alleles observed differed by only a single base-pair. The majority of these alleles were confirmed not to be scoring artefacts by the presence of alleles differing by 1 bp in heterozygotes.

Thirty-three loci were polymorphic in at least one of the four Fringillidae finch species and the number of alleles ranged from 2 to 17 (Tables S2). On average, 27 of 34 markers tested (79%) were polymorphic in each finch species and 21 loci were polymorphic in all four species (Tables S1 and S2). For 21 loci, at least one finch species had an observed heterozygosity above 0.5 (Table S2). When the number of individuals genotyped was increased from four to 17–23 individuals, the mean proportion of polymorphic loci increased from 63% to 79% (3–8 additional polymorphic loci were found per species).

A small number of locus/species combinations were difficult to score due to complex chromatogram peaks or very high peak heights (Table S2). In some cases, scoring might have been improved by further diluting the amplified products prior to analysis. The conserved primer-species bind site homology appears to have reduced the presence of null alleles. Of the 132 locus/species combinations, only two had an estimated null allele frequency above 0.20, and 15 were above 0.10 (Table S2). The locus/species combinations exhibiting high null allele frequency estimates might be due to a Wahlund effect resulting from previously undetected population substructure, as they occurred in only greenfinch and crossbill and these samples originated from multiple localities (see also Merilä *et al.* 1996, 1997; Piertney *et al.* 2001). No loci deviated significantly from Hardy–Weinberg proportions after a sequential Bonferroni correction (Rice 1989).

Only two pairs of loci in one species displayed genotypic disequilibria after correction for multiple tests (*TG01-040* & *TG04-012* and *TG01-147* & *TG22-001*, both in the chaffinch, $P = 0.00049$ and $P = 0.00082$, respectively). However, when the test was repeated neither pair of loci displayed genotypic disequilibrium, presumably resulting from an artefact of the Markov-chain simulation.

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Genotypic disequilibria between pairs of loci may remain undetected as loci were genotyped in relatively few individuals for each species.

Sex linkage

The predicted map locations of these loci were all autosomal (Fig. 1) and no genotype-based evidence was found for sex-linkage. Thirteen female and nine male Eurasian bullfinch were genotyped (sex based on plumage coloration). Males (ZZ) always amplified, indicating that no loci were W-linked. Of the 24 loci polymorphic in bullfinch, 21 were heterozygous in some females, excluding Z-linkage. Z-linkage could not be excluded in this way for twelve loci that were either monomorphic in bullfinch (nine loci, Tables S1 and S2) or displayed low variability in the bullfinch (*TG03-034*, *TG04-004*, *TG13-009*; Table S2).

Utility of the loci in non-passerines

All 34 loci amplified in chicken and 41% (14 loci) were polymorphic when tested in four wild individuals (Table 1, Fig. 2, Table S1). These loci have been found to be of utility in other non-passerine species. The nearest avian order, in terms of genetic distance, to Passeriformes is the order Charadiiformes (shorebirds and their allies, Sibley & Ahlquist 1990). Our newly developed primer sets were found to be of utility in this order. All loci amplified and nine (26%) were found to be polymorphic in a shorebird species, the Kentish plover (four individuals tested; Fig. 2, Table S1). Nine loci were tested in 13 additional species of shorebird. The loci tested included seven loci that were polymorphic and two loci that were monomorphic in Kentish plover. Despite testing, only four individuals at nine loci, up to seven loci were found to be polymorphic per species (mean = 4, Table S4).

In species very distant to zebra finch, such as the rufous hummingbird and barn owl, 21% and 18% of markers were polymorphic; and when 6–20 individuals were tested per locus, this figure increased to 36% and 26% respectively (Table S1). Two loci have been found to display polymorphism across a wide range of owl species, *TG04-061* and *TG08-024* (*TG08-024* when amplified with primer set 2; Klein *et al.* 2009).

Discussion

Rapid locus assessment

The engineered ability of the primer sets to amplify all the loci at the same annealing temperature using the same PCR conditions facilitated rapid testing in a single PCR run. Four individuals of a species could easily be genotyped on an ABI Analyzer at all 34 loci within a week.

Factors affecting amplification

Amplification success when assessed (at all 34 loci) using a single individual was recorded as 7–44% lower than when four individuals were tested (Table 2). Amplification success rose dramatically in the two (non-source) passerine species that were initially assessed in a single individual and then in four individuals, from 71% to 100% in house sparrow and from 56% to 100% in great tit. However, for great tit, the DNA quality had also improved. Low DNA quality exacerbated the detrimental effect of testing a single individual. Amplification levels were only 30–55% when the DNA quality was low and only a single individual had been tested (e.g. reed bunting *Emberiza schoeniclus*, great tit, blue crane *Grus paradisea* and saker falcon). We would therefore recommend testing a minimum of four individuals to assess the utility of these loci.

Polymorphism levels

There was a wide range of polymorphism levels across the different species sampled (18–76%, Fig. 2, Table S1). Polymorphism dropped as the genetic distance between the species genotyped and zebra finch increased (Fig. 3). Other causes of the variation in the degree of polymorphism may include the different levels of genetic variation in different species or populations, perhaps due to a genetic bottleneck. In some cases, the expected level of polymorphism may be inflated due to the use of an incorrect phylogenetic classification. Some recent phylogenies of passerine birds have found conflicts for some species with the phylogeny proposed by Sibley & Ahlquist (1990; see Barker *et al.* 2004; Hackett *et al.* 2008).

The zebra finch and Gouldian finch samples were drawn from captive, potentially inbred populations that may have originated from a small number of founders. However, the numbers of loci polymorphic in these species were very similar to those observed in the wild populations of other finch species studied here (Fig. 2, Table S1).

Polymorphism was lower in the chicken (41%) than observed on average for passerine species (48%), but higher than the average observed for other non-passerines (21%). All loci displayed repeat regions in the chicken and the motif type was the same in the majority of cases (29/35, Table 1). The chicken often displayed fewer repeat units in the longest string of repeat units than found in its zebra finch orthologue

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(18 loci had fewer, six had more and nine the same number Table 1). The reduced number of repeats in the longest string may, in part, explain the low levels of polymorphism observed in chicken compared with zebra finch.

Non-repeat sources of variation

For locus *TG08-024* an alternative primer set (set 2) was designed using the same methods used for the other loci (Table 1). The product amplified by *TG08-024* set 2 was larger than that amplified by set 1 (243 bp vs. 128 bp). Unexpectedly, different species were found to be polymorphic using the different primer sets (Table 1, Table S1). The same individuals gave different genotypes. The inconsistencies observed suggest that in some species, there is variation in the region of sequence flanking the repeat region as opposed to the repeat region itself. Alternatively, as the alleles often differed by only 1 bp, this variation could be due to adenylation during PCR and it would be prudent to include a 'pigtail' on the reverse primer in each TG08-024 set (see Brownstein *et al.* 1996).

Superior utility to existing passerine microsatellite loci

As far as we are aware, the engineered high utility of our conserved microsatellite markers in distantly related species has never previously been achieved. This is a significant achievement, as we would normally expect a maximum of 20% of anonymous non-source passerine microsatellite loci to be usefully polymorphic in another passerine species (e.g. Dawson *et al.* 2005a; Melo & Hansson 2006; Griffith *et al.* 2007, Simeoni *et al.* 2007). In past studies, it was a common necessity to test over 70 anonymous non-source passerine microsatellite loci (often each with different annealing temperatures) to identify sufficient loci for a paternity or population study (e.g. Dawson *et al.* 2005a; Melo & Hansson 2006; Griffith *et al.* 2007, Simeoni *et al.* 2007, see also the BIRDMARKER webpage http://www.sheffield.ac.uk/molecol/deborah-dawson).

Amplification and polymorphism have been found to decrease as the genetic distance of the amplified species from the source species of the microsatellite increased (Primmer *et al.* 1996; Dawson *et al.* 2000, 2005a, 2005b). Cross-species amplification in this study was much more successful than has previously been observed for either anonymous or EST microsatellite loci (cf. Primmer *et al.* 1996; Dawson *et al.* 2000, 2005b; Karaiskou *et al.* 2008). Amplification did not decrease with increasing genetic distance from zebra finch, but remained high in passerine and non-passerine species (a minimum of 99% of loci amplified when assessed with four individuals).

Prior to this study, one of the most promising primer sets for passerine microsatellite cross-species utility was that designed from zebra finch EST microsatellite sequence by Karaiskou *et al.* (2008). These loci were tested in 2–8 individuals of six passerine species and one non-passerine (Tengmalm's owl *Aegolius funereus*). However, these markers were not developed to enhance their cross-species utility. Loci were not preselected based on those most conserved (by using for example sequence comparison BLAST E-value) and the primer sets designed were not consensus with any other species. This may explain why Karaiskou *et al.* found much lower amplification levels of 55–68% within passerines, which decreased to 46% for the single non-passerine tested, when compared with our averages of 100% for passerines and 99% for non-passerines (when a minimum of four individuals were genotyped). For three species – house sparrow, blue tit and great tit – amplification data are available for direct comparison from both studies. Karaiskou *et al.* tested four house sparrow, four blue tit and two great tit individuals, whereas we tested four individuals in each species. Amplification rates from our study compared with those of Karaiskou *et al.* were: house sparrow, 100% vs. 58%; blue tit, 100% vs. 69% and great tit, 100% vs. 60%. This clearly indicates that the method we have employed has improved the amplification success of EST microsatellite loci to the maximum 100% possible.

For the house sparrow, blue tit and great tit, we could again directly compare our polymorphism data with those of Karaiskou *et al.* For these three species, polymorphism levels were comparable between the two studies: 38% vs. 51% in house sparrow, 47% vs. 45% in blue tit and 32% vs. 33% in great tit (Fig. 2, Table S1 of our study vs. Karaiskou *et al.* Table 1, data extracted using zebra finch EST microsatellite loci only). The variation observed for house sparrow could be due to differences in the population source(s) of the individuals genotyped.

In general, we found higher polymorphism levels in passerines (mean 48%, range 24–76%) than Karaiskou *et al.* (mean 40%, range 19–51%; Fig. 3). This may be due to the more conserved nature of our primer sets and the engineered ability of our loci to amplify at the same temperature and therefore our primer sets are more likely to be amplifying the true target microsatellite locus. Several primer sets tested by Karaiskou *et al.* were reported as requiring a second PCR with a lower annealing temperature to enable the amplification or species-specific PCR optimization. In contrast to our primer sets, many of those designed by Karaiskou *et al.* amplified products different in size to that expected based on the zebra finch sequences (±40 bp in one or more test passerine species), and often these

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differently-sized products were monomorphic. These primer sets may be amplifying non-target (i.e. non-microsatellite) loci that are less likely to include detectable length variation than a microsatellite locus.

When our conserved EST microsatellite primer sets were genotyped in a wide range of species, polymorphism decreased with increased genetic distance at a similar rate to that previously shown for anonymous microsatellite loci and non-developed EST microsatellite loci (compare this study (Fig. 3) with Primmer *et al.* 2005; Karaiskou *et al.* 2008).

Identification of chromosome and gene order rearrangements

Cytogenetic studies and a comparison of passerine linkage maps with the sequenced chicken genome have revealed that the chromosome arrangement and (to a lesser extent) the gene order of the avian genome is well conserved between species (Shields 1982; Derjusheva *et al.* 2004; Dawson *et al.* 2006, 2007; Griffin *et al.* 2007; Backström *et al.* 2008, Stapley *et al.* 2008; Hansson *et al.* 2009). Therefore, the assigned locations of these loci are likely to be good estimations of their chromosome locations in many different species. These loci will allow the comparison of recombination levels and the identification of gene order rearrangements among many species.

Conclusion

We have illustrated the success of a new method to develop conserved microsatellite markers by developing primer sets for 33 polymorphic loci that are of high utility in passerine birds, with additional utility in shorebirds and other non-passerines. The microsatellite markers described here are particularly useful for genotyping in species belonging to the Passeridae and Fringillidae families, which encompass 1383 species (based on Sibley & Monroe 1990). We hope we have alleviated the requirement to use enrichment techniques to isolate microsatellite loci for paternity and population studies in these and many other species. These conserved loci are suitable for many uses, including, for example, studies of population structure, parentage, relatedness, for linkage mapping and, in the case of the less polymorphic loci, to distinguish between species and identify hybrids. The loci will also enable the comparison of different species at the same loci and so allow genetic variability and recombination to be compared directly between species, without ascertainment bias. The method employed is expected to be valuable for developing microsatellite markers of high utility across a wide range of taxa.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Polymorphism of 34 conserved avian microsatellite primer sets when amplified in 17 passerine and 5 non-passerine species

Table S2 Characterization of 34 conserved EST microsatellite loci in four finch species when amplified using the conserved TG primer sets

Table S3 Microsatellite allele sizes observed in 22 bird species when amplified using the 35 conserved TG primer sets

Table S4 Microsatellite allele sizes observed in 14 shorebird species when amplified using nine of the conserved TG primer sets

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