Cross-species utility of 22 microsatellite markers in the Melodious Warbler (*Hippolais polyglotta*)

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ABSTRACT

Microsatellites are a valuable tool in the analysis of population genetic structure. Utilising microsatellite markers that were originally isolated from other species (cross-species amplification) can prove an efficient way, in terms of time and cost, to obtain markers for genetic studies. Here, 55 avian microsatellite primer pairs were tested for the cross-amplification in the Melodious Warbler (*Hippolais polyglotta*). Thirty-five markers amplified, of which 22 were polymorphic, displaying two to nine alleles in the 15 individuals genotyped. The 35 markers which amplified in the Melodious Warbler were tested in its sister species the Icterine Warbler (*H. icterina*). Twenty-four markers were amplified, 14 of which were polymorphic in the five *H. icterina* individuals genotyped. Thirteen loci were polymorphic in both species. The polymorphic loci identified are suitable for analysing the genetic population structure and assigning parentage.

Keywords: cross-species amplification, *Hippolais*, microsatellite, simple tandem repeat

1. INTRODUCTION

The Melodious Warbler (*Hippolais polyglotta*) and the Icterine Warbler (*H. icterina*) are small, long distance migrating passerines breeding in open to semi-open scrublands or open woodland in the Western Palaearctic (Glutz von Blotzheim and Bauer, 1991). The distributions of both species are parapatric with a narrow contact zone spanning throughout West and Central Europe, where both species hybridise occasionally (Secondi *et al.,* 2006). The Melodious Warbler occurs west of the contact zone covering large parts of southwestern Europe and coastal Maghreb. The Icterine Warbler occurs east of the contact zone and covers most of Eastern Europe to southern Scandinavia and the Ural. For at least 70 years the Melodious Warbler has been expanding its range north-eastwards, whereas the boundary of the western range of the Icterine Warbler has been receding in the same direction (Secondi *et al.,* 2006).

Recent range shifts of extant species are interesting model systems to study the evolutionary and genetic consequences of global change, including, for example, founder effects, hybridisation and niche evolution. However, natural range expansions of indigenous species over large distances and within a short timescale are–with the exception of invasive species–rather rare events but of increased interest for empirical studies (*e.g.* Duckworth, 2009; Hochkirch and Damerau, 2009; Garroway *et al.,* 2011; Schulte *et al.,* 2013). Due to detailed documentation of the range expansion of the Melodious Warbler in the past (Faivre *et al.,* 2002; Secondi *et al.,* 2006), this species provides an ideal model organism to study both extrinsic (*e.g.* effects of climate change or interspecific interaction, Engler *et al.*, 2013) and intrinsic factors (*e.g.* genetic or behavioural effects) that might facilitate range expansion in this species.

In order to study the genetic consequences of range expansions, such as the spatiotemporal changes in population structure, inbreeding or outbreeding during the colonisation process, a set of highly variable genetic markers, such as microsatellites, is required. The isolation of novel species-specific microsatellite loci is time consuming and costly, however, it can be avoided by identifying markers by the cross-amplification of existing primer pairs isolated in related species or by using markers purposely designed to be of high cross-species utility. Numerous studies have shown that, particularly in birds, marker sets can be developed from cross-species

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amplification without requiring the redesign of the original primer sets (e.g. Bourke and Dawson, 2006; Lee *et al.,* 2009; Mukesh *et al.,* 2011). However, when the genetic distance between the source and target species is high, a large number of markers need to be tested to identify a sufficient number of loci for a population or parentage study (e.g. Klein *et al.,* 2009; Martín-Gálvez *et al.,* 2009; Salmona *et al.,* 2010; Simeoni *et al.,* 2007, 2009). Recently enhanced avian markers have been specifically designed from new genome resources to have high cross-species utility in birds (Dawson *et al.,* 2010). These markers have been successfully utilised in a wide range of species, especially in passerines, in many cases reducing the numbers of markers that need to be tested to identify a set suitable for studying parentage and population structure (Dawson *et al.,* 2010; Durrant *et al.,* 2010; Vangestel *et al.,* 2011). Markers isolated by cross-species testing have also been successfully used to identify hybrids in other bird species (Lifjeld *et al.,* 2010; Hansson *et al.,* 2012). For these reasons, the cross-species amplification of a set of 55 microsatellite markers was conducted for the Melodious and the Icterine Warbler. We focused on the Melodious Warbler (*i.e.* the expanding species). For the Icterine Warbler, we tested only those markers which amplified in the Melodious Warbler and used a smaller subset of individuals in order to check for diagnostic loci with potential for enabling species delimitation and hybrid assignment.

2. MATERIAL AND METHODS

Initially, all 55 primer sets were tested on a total of five individuals of *H. polyglotta* stemming from three different populations under conditions as stated below. These samples were collected in 2008 during a local study investigating range edge dynamics in populations of *H. polyglotta* in Germany (Elle *et al.,* 2009; herein called local samples, Table 1). Loci which successfully amplified were analysed in 10 additional individuals stemming from five additional populations of *H. polyglotta* distributed across France and Spain. Finally, markers which amplified in *H. polyglotta* were tested in five *H. icterina* individuals to check for species-specific alleles that might be useful for the identification of *H. polyglotta*–*H. icterina* hybrids. The five *H. icterina* individuals tested comprised of one individual from each of five different populations distributed from the contact zone eastwards to the Baltic Sea. The samples of both species used herein were collected between 2001 and 2003 as part of a long-term study investigating the *Hippolais* hybrid zone (Faivre *et al.,* 1999; Secondi *et al.,* 2006; herein called 'global samples', Table 1).

During sampling, 10–50 μL blood was taken from the brachial vein of each individual and mixed with either 50 μL for local samples or 500 μL of buffer (0.15 M NaCl, 0.05 M Tris-HCl, 0.001 M EDTA, pH = 8.0) for global samples. Samples were stored in the laboratory at –20°C until DNA extraction was performed. Genomic DNA was extracted using a Roche High Pure PCR (polymerase chain reaction) Template Preparation Kit for local samples and with a phenol–chloroform procedure (Sambrook *et al.,* 1987) for global samples.

Table 1 Origin of the *Hippolais* warbler blood samples used for this study

Species	ID	Population	Region	Sex
Local samples				
H. polyglotta	6957	Trier	SW Germany	Male
H. polyglotta	6964	Trier	SW Germany	Male
H. polyglotta	6968	Trier	SW Germany	Male
H. polyglotta	6990	Mayen	SW Germany	Male
H. polyglotta	6992	Bingen	SW Germany	Male
Global samples				
H. polyglotta	AA4	Madrid	Spain	Male
H. polyglotta	AC8	Madrid	Spain	Male
H. polyglotta	AA9	Chizé	W France	Female
H. polyglotta	AD9	Chizé	W France	Male
H. polyglotta	BF6	Caen	NW France	Female
H. polyglotta	BL0	Caen	NW France	Male
H. polyglotta	AO6	Auxonne	E France	Female
H. polyglotta	AU0	Auxonne	E France	Male
H. polyglotta	BK3	Le Quesnoy	NE France	Male
H. polyglotta	BT9	Le Quesnoy	NE France	Female
H. icterina	BK9	Le Quesnoy	NE France	Female
H. icterina	AB1	Magdeburg	E Germany	Male
H. icterina	BR5	Rostock	NE Germany	Male
H. icterina	AY5	Öland	S Sweden	Female
H. icterina	BD1	Rybachy	W Russia	Female

Nine of the 55 markers tested were originally isolated from the Seychelles Warbler (*Acrocephalus seychellensis*), some of which have been shown to be also polymorphic in other passerines (Richardson *et al.,* 2000). *Acrocephalus* and *Hippolais* warbler species are separated by a relatively small genetic distance and belong to the same family (Sylviidae; Fregin *et al.,* 2009); a high proportion of markers would therefore be expected to cross-amplify (Primmer *et al.,* 1996). Additionally, 46 primer sets were used that were shown to be of high cross-species utility in many other bird species, particularly passerines (Dawson *et al.,* 2010; Dawson *et al.,* unpublished). Thirty-four of these primer sets are exceptionally highly conserved in the genome. Identical sequences were observed in the Zebra Finch *Taeniopygia guttata* Expressed Sequence Tags (EST) and the chicken *Gallus gallus* genome and have been found to amplify in 99% of all bird species tested (Dawson *et al.,* 2010; the "*TG*" loci, Table 2). The forward primer of each pair was labelled with either a HEX or 6-FAM fluorescent dye. Singleplex

Table 2 Characterisation of 35 microsatellite loci in the Melodious Warbler (*Hippolais polyglotta*).; *n* is the number of individuals werefor which the locus successfully amplified (from a total of 15 individuals);. *k* refers to the number of different alleles. Primer sequences are given for forward (F) and reverse (R) primer respectively; HWE gives the p values for the Chi²-Test on deviations from Hardy-Weinberg-Equilibrium.

Marker (source species)	Reference for primer set and EMBL accession no.	Repeat motif[a]	Primer sequence (5′ –3′) [fluoro-label]	T_a (°C)	Observed allele size (bp)	*n*	*k*	H_O	H_E	HWE p-value*P*
Ase9 (*Acrocephalus seychellensis*)	Richardson *et al.*, 2000 AJ287392	$(GA)_{15}$	F: [6-FAM]-GAC TGA AGT CCT TTC TGG CTT C R: CAC CAG GAA TAC AAG TCC ATT G	60	119–155	12	9	0.42	0.85	0.06
Ase19 (*Acrocephalus seychellensis*)	Richardson *et al.*, 2000 AJ276376	$(CA)_4GA(CA)_5$	F: [6-FAM]-TAG GGT CCC AGG GAG GAA G R: TCT GCC CAT TAG GGA AAA GTC	60	177, 179	15	2	0.27	0.39	0.22
Ase34 (*Acrocephalus seychellensis*)	Richardson *et al.*, 2000 AJ276636	$(CT)_{11}$	F: [HEX]-GTT AAT TCT TTT GGC CCT CAG C R: GGA GAC ACC ACA CCA ATG C	54	204–218	14	6	0.79	0.67	0.99
Ase37 (*Acrocephalus seychellensis*)	Richardson *et al.*, 2000 AJ276639	$(AC)_9$	F: [6-FAM]-TAA TTC ATG GAG AAG CCC AG R: TCA AAA CAA CAG TTT TCA CAG C	58	236, 238	14	2	0.21	0.38	0.11
Ase46 (*Acrocephalus seychellensis*)	Richardson *et al.*, 2000 AJ276775	$(TG)_{13}$	F: [HEX]-CTG GCT GTA TCT TGG TGT GC R: CAG TGT TTT AGG TCT CCT GCT G	57	241–245	15	3	0	0.34	0.00
Ase56 (*Acrocephalus seychellensis*)	Richardson *et al.*, 2000 AJ276785	$(GT)_{18}$	F: [6-FAM]-TTC ACT GAG AAG TGA GAA TGT G R: GTC CTT GAT TGA TTA CAG GCT	54	294–310	14	7	0.79	0.81	0.83
TG01-000 (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al.*, 2010 CK314156	$(AT)_{8,8,3,2,3,8}$	F: [6-FAM]-TTG CTA CCA RAA TGG AAT GT R: TCC TAA CCA TGA GAA GCA GA	56	218–224	13	3	0.08	0.32	0.01
TG01-077 (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al.*, 2010 CK305147	$(A)_{11}\&(CA)_3$	F: [HEX]-GGT ATG TCA GTT ATC AAA AAC AAG C R: AAA TGG CAG GTA AGG ATA CTC TC	56	153	12	1	0	0	-
TG01-092 (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al.*, 2010 DV958291	$(AT)_3T(AT)_6TT(AT)_3$	F: [6-FAM]-ATG TTG GTG AAA GTA TTA CAG CTC TC R: TCA CCT TTT AAA AAC CAA TTT CAA C	56	185	15	1	0	0	-
TG01-114 (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al.*, 2010 CK301583	$(AT)_3AA(AT)_6$	F: [HEX]-TTG AAA CAT TGT GAA GCA G R: CAG ATA GTG TCA TAA CAA TAC TTT TC	56	177, 181	14	2	0.07	0.32	0.89
TG01-124 (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al.*, 2010 CK306631	$(AT)_{11}$	F: [6-FAM]-AGT ACT ACT TGC CTG CAG AGT TTA T R: TGT GTA TGG CAG CAT TTA CAA	56	407, 409	10	2	0.10	0.10	0.87

Table 2. (Continued)

Marker (source species)	Reference for primer set and EMBL accession no.	Repeat motif[a]	Primer sequence (5′ –3′) [fluoro-label]	T_a (°C)	Observed allele size (bp)	*n*	*k*	H_O	H_E	HWE p-value*P*
TG01-148 (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al*., 2010 CK301512	$(AT)_8AAATT(AT)_5$	F: [HEX]-TTG CAA CAC ATT CTA ATA TTG C R: TTT AAA GTA CAT CAA ACA ACA AAA TC	56	189	5	1	0	0	-
TG02-078 (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al*., 2010 CK305233	$(AT)_4AG(AT)_7(AC)_3(AT)_6$	F: [HEX]-TGT TAA AGC CTG TTC CAT AGG R: TTC CCC ATA AAG TAT GTA CGC	56	291	8	1	0	0	-
TG03-002 (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al*., 2010 DV575298	$(AT)_{11}$	F: [6-FAM]-TCT TGC CTT TTT GGT ATG AGT ATA G R: TAC AAA GCA CTG TGG AGC AG	56	121, 123	15	2	0.07	0.06	0.89
TG03-031 (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al*., 2010 CK312587	$(AT)_{12}TT(AT)_4$	F: [6-FAM]-ATT GCA CAT GAA CCT GGA AG R: TCA TTA CTT GAA GCA GGT CTC TG	56	199	8	1	0	0	-
TG03-034 (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al*., 2010 CK311260	$(AT)_4AA(AT)_{11}$	F: [6-FAM]-GAG ATC GCC ACC ATC CTG R: AAG TCT ACA TTT CCC TTG TCT TGG	56	177	10	1	0	0	-
TG03-035 (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al*., 2010 DV578303	$(AT)_4AA(AT)_6$	F: [HEX]-TGA TGG CCA AAT GCA TACT C R: TAT TTA CAA TAT CTG CAG AAA CAA TCC	56	215	10	1	0	0	-
TG04-004 (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al*., 2010 DV946288	$(AT)_{10}GT(AT)_7$	F: [HEX]-CTG GAG CAG TAT TTA TAT TGA TCT TCC R: GAA GAT GTG TTT CAC AGC ATA ACT G	56	168–176	14	5	0.43	0.46	0.77
TG04-012A (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al*., 2010 CK309067	$(CT)_4TT(CT)_5TTTT(CT)_3$	F: [6-FAM]-CGT TTT TGC AGT GAT TGT GG R: AGC GAG GCC ATG TTG AAG	56	236	10	1	0	0	-
TG04-041 (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al*., 2010 CK316380	$(AG)_7TG(AG)_4$	F: [HEX]-CTG AAT TGT TGA CCT TTG CTT AC R: GTC CTT TTA GAA AGC AGC ACA G	56	178, 182	5	2	0.20	0.18	0.80
TG04-061 (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al*., 2010 CK235034	$(A)_7$&$(GA)_{6,3,2}$	F: [HEX]-GAC AAT GGC TAT GAA ATA AAT TAG GC R: AGA AGG GCA TTG AAG CAC AC	56	192–198	14	7	0.36	0.60	0.00
TG05-030 (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al*., 2010 CK308028	$(AT)_7CT(AT)_3$	F: [HEX]-CTT CCC ATC ACA TCT GTA AC R: GTA AAC ATT AAT ATG CAC TTT CTT AG	56	177	5	1	0	0	-
TG05-046 (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al*., 2010 DV957774	$(AT)_8(A)_4(AT)_6(A)_9(AT)_2$	F: [6-FAM]-AAA ACA TGG CTT ACA AAC TGG R: GCT CAG ATA AGG GAG AAA ACA G	56	336	13	1	0	0	-
TG05-053 (*Taeniopygia guttata* & *G. gallus*)	Dawson *et al*., 2010 CK314425	$(T)_4GA(T)_6AA(T)_{16}AA(T)_4G(T)_6$ &$T(AT)_8T(AT)_4AA(AT)_4TATACATA$	F: [6-FAM]-GCA TCA TCT GGT TGA ACT CTC R: ACC CTG TTT ACA GTG AGG TGT T	56	210–214	14	3	0.50	0.48	0.68

Table 2. (Continued)

Marker (source species)	Reference for primer set and EMBL accession no.	Repeat motif[a]	Primer sequence (5′ –3′) [fluoro-label]	T_a (°C)	Observed allele size (bp)	*n*	*k*	H_O	H_E	HWE p-value*P*
TG06-009	Dawson *et al.*, 2010	$(AC)_3AT(AC)_3AT(AC)_3\&(GT)_4$	F: [6-FAM]-AAG CCT TGC TTA CAT TTT ATG GTG	56	120, 122	14	2	0.24	0.19	0.65
(*Taeniopygia guttata & G. gallus*)	CK315728	$\&(AT)_2GT(AT)_{10}GT(AT)_3$	R: GGG GTG GTA ACT GAA ATA AAG TAT AGG							
TG08-024 (Set 1)	Dawson *et al.*, 2010	$(AT)_4AG(AT)_2AA(AT)_3AA(AT)_5$	F: [HEX]-CAC AAT CCT GAA TTT CAT ATC C	56	125, 127	14	2	0	0.34	0.00
(*Taeniopygia guttata & G. gallus*)	CK314428		R: AAC AAC GAC AGC TAT GAA AGA AC							
TG09-014	Dawson *et al.*, 2010	$(AT)_4AG(AT)_2$	F: [6-FAM]-CCA AAG GTG AAG GAA TCT ATG G	56	151	14	1	0	0	-
(*Taeniopygia guttata & G. gallus*)	DV948892	$AA(AT)_3AA(AT)_5AAAATAA(AT)_4\&(A)_5$	R: TCT GCC TGC AGA GTC CAA C							
TG11-011	Dawson *et al.*, 2010	$(AT)_9AA(AT)_6$	F: [6-FAM]-ACA AAC TAA GTA CAT CTA TAT CTG AAG	56	209–213	13	3	0.23	0.21	0.97
(*Taeniopygia guttata & G. gallus*)	CK308096	$TA(AT)_3$	R: TAA ATA CAG GCA ACA TTG G							
TG13-017	Dawson *et al.*, 2010	$(AT)_{10}$	F: [6-FAM]-GCT TTG CAT CTT GCC TTA AA	56	215, 217	14	2	0.07	0.07	0.89
(*Taeniopygia guttata & G. gallus*)	CK313422		R: GGT AAC TAC AAC ATT CCA ACT CCT							
Z-037	Dawson *et al.*, unpubl.	$(AT)_6TT(AT)_7TT(AT)_7AC(AT)_6A(AT)_2$	F: [6-FAM]-AAA ACA CCT TGT AAT TTA AAA CTG G	56	162	6	1	0	0	-
(*Taeniopygia guttata & G. gallus*)	DV945670	$\&(AT)_6(AC)_4$	R: CAT AGA TAC ATA TCA ATA CAG CAC ATT C							
Ase55-CEST	Dawson *et al.*, unpubl.	$(TG)_9$	F: [HEX]-AGCTGGATTGGCATCGTG	55	278–282	11	3	0.55	0.60	0.49
(*Acrocephalus seychellensis*)	AJ276784		R: TCATTACAGCAATTACCATTGAGC							
ApCo46-ZEST	Dawson *et al.*, unpubl.	$(AG)_3AA(AG)_{11}$	F: [6-FAM]-GCT GCC AGC ACT CTG AAT GTC	56	213, 215	15	2	0.33	0.36	0.79
(*Aphelocoma coerulescens*)	AF520885		R: GAT TCA GCA AAA TAG GGG TCA GAA G							
Calex-08-CEST	Dawson *et al.*, unpubl.	$(CA)_2TACATA(CA)_2CGTA(CA)_3$	F: [HEX]-AAGAGGGCCAATGTGCTTCTC	60	207	14	1	0	0	-
(*Charadrius alexandrinus*)	AM072456	$TA(CA)_3\ TG(CA)_3TA(CA)_8$	R: AAGCGGAATATTAAGTAGAGGCTTCC							
Tc.11B4E-CEST (Set 2)	Dawson *et al.*, unpubl.	$(GT)_{10}$	F: [6-FAM]-CCT GGT GAT AGC AGT GAA TGT	65	396–402	11	4	0.64	0.62	0.97
(*Telespiza cantans*)	AF036266		R: TAG CGA GAT GCC TGT GTA TG							
DkiB119-CEST	Dawson *et al.*, unpubl.	$(CAT)_9$	F: [6-FAM]-CAT ACA ACT TCA TGA CTA CCA TAG CAC	60	232–237	13	4	0.23	0.51	0.03
(*Dendroica kirtlandii*)	AY769677		R: TCC ATA GTG ACA TAG AAC GAG CTG							

[a] Repeat motif as observed in source species; comma-separated repeat lengths indicate non-repeat sequence in between the repeat motifs.
k refers to the number of different alleles. Primer sequences are given for forward (F) and reverse (R) primer respectively; HWE gives the *P* values for the chi-squared-test on deviations from Hardy–Weinberg equilibrium.

PCRs were performed. Each 10 μL PCR contained, 4.4 μL Hot Master Mix 2.5x (5PRIME), 5.8 μL ultrapure H_2O, 0.2 μL of each forward and reverse primer at 5 μM, and 0.5 μL of DNA (concentration 10–40 ng μL^{-1}). The PCR was run in a Mastercycler thermocycler (Eppendorf). The conditions for PCR followed the manufacturers' protocol with annealing temperatures between 54 and 65°C as recommended for each locus (Richardson *et al.,* 2000; Dawson *et al.,* 2010; Dawson, unpublished data; Table 2). Initially five *H. polyglotta* individuals were used to check for successful amplification using agarose gel. A 3 μL volume of each PCR product was loaded on a 1.4% agarose gel stained with SYBR Green (Applied Biosystems). Successfully amplified primer sets were subsequently used for genotyping the remaining individuals of both species. Fragment lengths were determined on a MegaBACE 1000 automated DNA sequencer (GE Healthcare). Primer lengths were scored using Fragment Profiler v.1.2 (Amersham Biosciences). Expected (H_e) and observed (H_o) heterozygosities as well as Chi²-tests on deviations from Hardy Weinberg Equilibrium (HWE) were calculated in GenAlEx v.6.4 (Peakall and Smouse, 2006). Null allele frequencies were estimated for loci using Microchecker v.2.2.3 (van Oosterhout *et al.,* 2004).

3. RESULTS AND DISCUSSION

Of the 55 markers tested, amplification failed in 20 of the primer pairs when tested in five *H. polyglotta*, *Ase13*, *Ase29*, *Ase48* (Richardson *et al.,* 2000); *TG01-040*, *TG01-147*, *TG02-088*, *TG02-120*, *TG03-098*, *TG04-012*, *TG07-022*, *TG08-024(set 2)*, *TG12-015*, *TG13-009*, *TG13-016*, *TG22-001* (Dawson *et al.,* 2010); *Z-040*, *Asu15-EST*, *Tc.11B4E-CEST (set 1)*, *SAP47-EST*, *Pte24-EST* (Dawson *et al.,* unpublished). It was expected that all the *TG* loci and *Z* loci should have amplified since these amplify in virtually all birds (Dawson *et al.,* 2010; Dawson *et al.,* unpublished) and the reasons for the PCR failure remain unknown. The 35 primer pairs which amplified successfully in *H.*

Table 3 Observed allele sizes of 24 microsatellite loci that amplified successfully in both, the Icterine Warbler (*Hippolais icterina*; *n* = 5 individuals) and the Melodious Warbler (*Hippolais polyglotta*; *n* = 15 individuals) *n* is the number of individuals were the locus successfully amplified, *k* refers to the number of different alleles, the two markers *TG01-092* and *Calex-08-CEST* were monomorphic in both species but displayed different allele sizes in each species and are therefore expected to be of high utility for species identification

	Hippolais icterina			*Hippolais polyglotta*		
Locus	Obs. allele sizes (bp)	*n*	*k*	Obs. allele sizes (bp)	*n*	*k*
Ase9	**129**, **131**, **133**, 135, 141	5	5	**119**, **127**, 135, **137**, 141, **143**, **151**, **153**, **155**	12	9
Ase19	**169**, 177	5	2	177, **179**	15	2
Ase34	212, 214, 218, **222**, **226**, **228**, **230**, **240**	4	8	**204**, **208**, **210**, 212, 214, 218	14	6
Ase37	**234**	2	1	**236**, **238**	14	2
Ase46	**247**	4	1	**240**, **242**, **244**	15	3
Ase56	**286**, **290**, 294, **296**, 300, 302, **312**, **320**	5	8	294, **298**, 300, 302, **304**, **306**, **310**	14	7
TG01-000	216, 218, **220**	5	3	216, 218, **222**	13	3
TG01-092	183	5	1	185	15	1
TG01-114	181	5	1	**177**, 181	14	2
TG01-124	407, 409	5	2	407, 409	10	2
TG03-002	123, **125**	2	2	**121**, 123	15	2
TG04-004	168, 170, 172	5	3	168, 170, 172, **174**, **176**	14	5
TG04-061	192, 195	5	2	192, **193**, **194**, 195, **196**, **197**, **198**	14	7
TG05-046	336, **338**	4	2	336	13	1
TG05-053	210	3	1	210, **212**, **214**	14	3
TG06-009	122, **124**	5	2	**120**, 122	14	2
TG08-024 (Set 1)	125, 127	5	2	125, 127	14	2
TG11-011	211	3	1	**209**, 211, **213**	13	3
TG13-017	215	5	1	215, **217**	14	2
Ase55-CEST	278, 280	5	2	278, 280, **282**	11	3
ApCo46-ZEST	213	4	1	213, **215**	15	2
Calex-08-CEST	199	2	1	207	14	1
Tc.11B4E-CEST (Set 2)	398	5	1	**396**, 398, **400**, **402**	11	4
DkiB119-CEST	236, 237	5	2	**232**, **235**, 236, 237	13	4

n is the number of individuals where the locus is successfully amplified, *k* refers to the number of different alleles.
Allele sizes unique to each species are highlighted in bold and underlined).
The two markers *TG01-092* and *Calex-08-CEST* were monomorphic in both species but displayed different allele sizes in each species and are therefore expected to be very useful for species identification.

polyglotta (Table 2) were tested in *Hippolais icterina* and 24 amplified (Table 3). When assessed in the 15 *H. polyglotta* individuals (from eight populations), 22 loci were polymorphic and the number of alleles per locus ranged from two to nine. Expected heterozygosities ranged from 0.06 to 0.85 and observed heterozygosities ranged from 0.07 to 0.79 (Table 2). Since the genetic structure among the Melodious Warbler's range is panmictic (Engler *et al*., unpublished), we were formally able to calculated deviations from HWE based on the complete sampling data set (15 samples). Deviations from HWE were detected in five of the markers (Table 2). The sex of all the individuals genotyped was known from sex-specific characteristics (*i.e.* song in males, breeding patch in females). All polymorphic loci were autosomal in both *Hippolais* species based on the presence of heterozygotes in both sexes. The occurrence of null alleles was suggested for *H. polyglotta* in six of the 35 amplified primer pairs (Oosterhout probability, *Ase9*, 0.26, *Ase46*, 0.34, *TG01-000*, 0.27, *TG04-061*, 0.20, *TG08-024*, 0.35, *DkiB119-CEST*, 0.27).

Of the 22 loci that were polymorphic in *H. polyglotta*, nine displayed new, previously unseen, alleles in *H. icterina* and 14 were polymorphic although only a few individuals were tested (Table 3). Genotyped birds originated from several populations but from a small number of individuals (a maximum of 15 *H. polyglotta* and five *H. icterina*). Therefore, the genotyping of additional individuals of each species is required to confirm these diagnostic loci. Two loci, *TG01-092* and *Calex-08-CEST* were monomorphic in both species and displayed different allele sizes in each species and are therefore expected to be particularly useful to identify hybrids (Table 3). Locus *TG01-092* is monomorphic in all non-finch passerines tested so far ($n = 12$ species, Dawson *et al.,* 2010; Lifjeld *et al.,* 2010) and therefore is not expected to display any variability and should remain diagnostic when more individuals are tested. The number of alleles displayed in the five *H. icterina* individuals ranged between two and eight but is expected to increase when more individuals are tested.

The 22 polymorphic loci derived from the cross-species amplification are expected to be useful for population genetic analyses of *H. polyglotta*. Furthermore, the identification of primer pairs with diagnostic alleles for each species will enable the identification of possible hybrids between the two species.

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