Sexual selection and reproductive strategies in songbirds

Berg, Mathew Leslie

Chapter 2

Cross-species amplification of microsatellites in winter wrens andAustralian reed warblers

Mathew L. Berg, Marco van der Velde & Jan Komdeur

ABSTRACT

Microsatellites are the current markers of choice for studies of parentage and relatedness between individuals. Their main practical disadvantage is the need to develop species-specific loci. One solution to this problem is to test microsatellite markers developed in closely related species. However, there have been relatively few published attempts to examine cross-species amplification on a broad scale, using primers developed from a wide range of source taxa. We tested 114 microsatellite primer pairs from a variety of avian source taxa for use in two new passerine bird species. A high proportion of markers were successful in cross-amplification. Of 108 markers, 69 (64%) gave a product in winter wrens (*Troglodytes troglodytes*) of which 23 (33%) were polymorphic. In the Australian reed warbler (*Acrocephalus australis*) 84 of 87 markers (97%) gave a product of which 31 (37%) were polymorphic. Fifty-five of the 81 primers tested in both target species amplified a product (68%), of which 13 (24%) were polymorphic in both species. From these, we identified five markers which should have good utility in wrens for studies of population genetics and parentage analysis. Our results suggest that cross-amplification can be a valuable source of microsatellite markers for new studies, and that success may be enhanced by choosing source species closely related to the target species.

INTRODUCTION

The use of molecular markers for analysing genetic variation is an important tool for studies of evolutionary relationships, population genetics and genome organisation. Microsatellite markers have become the preferred tool for many of these applications, in particular of studies of relatedness and parentage (Bruford & Wayne 1993; Queller *et al.* 1993; Jarne & Lagoda 1996). These markers have many advantages for these applications, including hypervariability, abundance throughout the genome and efficient polymerase chain reaction (PCR) based typing procedures.

Microsatellites are 2–5 base pair repeat sequences that are highly polymorphic and predominately selectively neutral. They are abundant throughout the genomes of all higher organisms (Tautz & Renz 1984; Tautz 1989). PCR techniques and gel electrophoresis allow small amounts of even poor-quality DNA to be accurately typed to single nucleotide resolution. This permits easy comparison of results (allele sizes) between studies and laboratories. Fluorescently labelled microsatellite primers and automated genotyping systems that can efficiently type numerous loci are now widely available (Reed *et al.* 1994). In addition, software packages such as CERVUS (Marshall et al 1998; Slate *et al.* 2000) and KINSHIP (Goodnight & Queller 1999) facilitate the accurate assignment of relatedness and parentage based on a likelihood approach (Blouin 2003; Butler *et al.* 2004). For these reasons, microsatellites the current markers of choice for studies on parentage and relatedness between individuals.

The main practical disadvantage of microsatellite markers for ecological studies is the usual need to develop species-specific loci. This is because PCR primers require a high degree of homology to the target sequence in order to function. However, mutations in microsatellite flanking sequences will eventually prevent amplification in one species with primers developed from another species (Jarne & Lagoda 1996). Unless a genetic map is already available for the study species concerned the development of species specific markers can be time-consuming and expensive, and requires laboratory equipment and expertise beyond that needed for basic PCR-based genotyping (Hammond *et al.* 1998; Slate *et al.* 1998).

One solution to this problem is to attempt to use microsatellite markers developed in closely related species. Some microsatellite flanking regions are relatively highly conserved, and will amplify homologous products in some closely related species. Several studies have utilized this capability, at least in part, for genetic studies (e.g. Moore *et al.* 1991; Ellegren 1992; Pêpin *et al.* 1995; Engel *et al.* 1996; Küln *et al.* 1996; Slate *et al.* 1998; Fernández *et al.* 2000; Galan *et al.* 2003; Loyau *et al.* 2005), including several for parentage analysis. However, most studies reporting on cross-species amplification (i.e. notes reporting the development of new microsatellite markers) have focused on applying a small set of primers developed in one species to a narrow set of other closely related species. There have been relatively few published attempts to examine cross-species amplification on a broader scale, using primers developed from a wider range of source taxa (Primmer *et al.* 1996a; Slate *et al.* 1998; Dallimer 1999; Galbusera *et al.* 2000; Primmer *et al.* 2005).

The purpose of this study is to report the results of an intensive screen of two species of passerine birds with a panel of 114 polymorphic avian microsatellite markers developed in other passerine taxa, and to evaluate their suitability for use in ecological studies. The order Passeriformes is the largest and most diverse commonly recognised clade of birds (Sibley & Ahlquist 1990). The two target species that we used are a Wren, the winter wren (*Troglodytes troglodytes*; order: Passeriformes; family: Troglodytidae) and an Old World Warbler, the Australian reed warbler (*Acrocephalus australis*; order: Passeriformes; family: Sylviidae). Many studies documenting the isolation and characterization of novel microsatellites from a particular species (such as those cited above) report cross-amplification success in a small number of other, usually closely related species. This study takes the opposite approach by testing a large number of existing microsatellite primers in wrens and reed warblers. Broad cross-species amplification studies are particularly useful to identify loci which might be usable over a broad range of species, and to potentially use microsatellites for comparative maps (O'Brien *et al.* 1993; Primmer *et al.* 1996a; Slate *et al.* 1998). Such markers are highly desirable as they greatly reduce the requirement to develop microsatellites for each new study species and may allow useful comparisons between species (Gemmell 1997; Slate *et al.* 1998).

Our primary interest is in the assessment of relatedness and parentage in free-living populations of wrens. The application of a panel of microsatellite markers from this study to parentage analysis in winter wrens as reported in chapter 7. To our knowledge, only one study has so far examined extra-pair mating in the winter wren, using multilocus (minisatellite) DNA fingerprinting to analyze parentage in 19 complete broods. We replicated this study with larger sample of broods collected over four years, and using the single locus microsatellite markers described here.

METHODS

Field work

We collected data during the main breeding seasons from families of free-living winter wrens (1999-2002) and Australian reed warblers (1997-2000). Data was collected from two nearby study sites for wrens, Friescheveen and Vosbergen forest reserves, Paterswolde, The Netherlands (*ca.* 50 ha, 53°08' N, 6°35' E), and two study sites (*ca.* 50km apart) for reed warblers, Cherry Lake, Altona North (6 ha, 37°53' S, 144°48' E) and Edithvale Wetlands (10 ha, 38°02' S, 145°07' E), Melbourne, Australia. We caught adults in mist-nets and nestlings on the nest. We collected 50-100 μl of blood from both adults and nestlings, which was stored in 1–1.5 ml of 95% ethanol at room temperature. Blood was collected by puncture of the brachial vein of adults or medial metatarsal vein of nestlings, using a sterile needle. Care was taken to avoid contamination with maternal or other tissue. Tissue samples from unhatched embryos and other dead individuals were collected and stored at –20 °C for several weeks before transfer into 1–1.5 ml of 95% ethanol for storage at room temperature. Blood and tissue sampling techniques and storage were identical for both species.

We found nests by searching systematically through occupied territories at least weekly. The nests of both species are highly distinctive. For wrens, all potential nest sites (i.e. vegetation or structures such as tree stumps above ground) were examined carefully for nests. In the case of reed warblers, nest searching was done on 5 m transects through the reed beds (the minimum distance that can be seen through the reeds), parting the vegetation carefully to minimise disturbance. We marked all nests with a small piece of green or yellow weather-proof plastic tape. For wrens, this was attached to a twig *ca.* 5 m either side of the nest, and for reed warblers this was attached to a reed stem supporting the nest. These methods allowed exact relocation of each nest even if it disappeared due to depredation or adverse weather. Nest location coordinates were also determined with a handheld satellite global positioning system (Garmin GPS 12®) to a precision of < 5 m.

DNA extraction and purification

DNA was extracted from either whole blood samples or tissue samples (embryos or dead nestlings) stored in ethanol. We used a salt extraction method followed by precipitation in ethanol, as described in Richardson *et al.* (2001). DNA extraction was identical for both species to minimise the chance of species-specific variation in DNA quality, which could bias the outcomes of PCR amplifications.

Markers tested

We tested markers for a total of 114 avian microsatellite loci, which had been derived (cloned) from 12 source species representing 11 genera and 11 families of passerine birds in previous studies (Table 2.1). These loci were presumed to be selectively neutral. Due to time constraints or PCR failure, not all markers were tested in both species (108 markers were tested on winter wrens and 87 were tested on Australian reed warblers). These markers were chosen from existing avian markers, based on their availability and their success in cross-amplification among other passerine taxa. Our panel of markers included six of the nine loci recently identified by Primmer *et al.* (2005) as being unusually successful at cross-species amplification in passerines. Primers were screened on a panel comprising high-quality DNA from 3-6 (see Table 2.2) presumably unrelated individual adult wrens (5.9 ± 0.5 SD individuals) and reed warblers (5.9 ± 0.4 SD individuals). All markers were screened initially for cross-amplification and polymorphism in the target species at the Sheffield Molecular Genetics Facility by MB using identical protocols in both species.

PCR protocol

For cross-amplification tests, PCR amplification reactions were performed in a final 10 μl volume containing approximately 20-50 ng of DNA, 0.2-1.0 μM of each primer, 0.2mM of each dNTP, 0.05 units of *Taq* DNA polymerase (Advanced Biotechnologies) and 2 mM $MgCl_2$ in supplied reaction buffer. PCR amplification was performed using a Hybaid thermal cycler using the following program: one cycle of 94 °C for 2 min, then 35 cycles of 94 °C for 20 s, 55 °C for 30 s and 72 °C for 60 s, followed by 72 °C for 5 min. The protocols used were identical for both species and whenever possible both species were tested concurrently in the same PCR run for each marker to be tested.

Table 2.1. Summary of the 114 primer pairs tested for cross-amplification in this study. Single allele sizes are from the cloned individual in source species (observed size range in parentheses, if known). H_O is the observed heterozygosity in the source species. A dash indicates the information was not available from published literature. Classifications and common names follow Sibley & Ahlquist (1990). Primer pairs are generally named with an abbreviation of the original organism's scientific name followed by the number of the clone (e.g. Ase = *Acrocephalus sechellensis*). All primer sequences are previously published except for *Pca6* (EMBL accession no. AJ279808), PK11 and PK12; see sources for primer sequences and GenBank/EMBL accession numbers.

Family	Scientific name	Common name	Locus	Size in source species, bp (range)	HO in source species	Source
Corvidae	Pica pica	Magpie	*Ppi*2	–	0,85	Martinez *et al.* 1999
Emberizidae	*Melospiza melodia*	Song sparrow	*Mme*12	(188-240)	0,53	Jeffery *et al.* 2001
Fringillidae	*Emberiza schoeniclus*	Reed bunting	*Escμ*1	148	0.89[a]	Hanotte *et al.* 1994
Fringillidae	*Emberiza schoeniclus*	Reed bunting	*Escμ*2	210	0.86[a]	Hanotte *et al.* 1994
Fringillidae	*Emberiza schoeniclus*	Reed bunting	*Escμ*3	158	0.86[a]	Hanotte *et al.* 1994
Fringillidae	*Emberiza schoeniclus*	Reed bunting	*Escμ*4	152	0.92[a]	Hanotte *et al.* 1994
Fringillidae	*Emberiza schoeniclus*	Reed bunting	*Escμ*5	204	0.00[a]	Hanotte *et al.* 1994
Fringillidae	*Emberiza schoeniclus*	Reed bunting	*Escμ*6	196	0.90[a]	Hanotte *et al.* 1994
Hirundinidae	*Hirundo rustica*	Barn swallow	*Hr*U2 (STG4)	(131-139)	0,47	Ellegren 1992; Primmer *et al.* 1995
Hirundinidae	*Hirundo rustica*	Barn swallow	*Hr*U3	(214-300)	0,89	Primmer *et al.* 1995
Hirundinidae	*Hirundo rustica*	Barn swallow	*Hr*U4	(88-102)	0,76	Primmer *et al.* 1995
Hirundinidae	*Hirundo rustica*	Barn swallow	*Hr*U5	(118-137)	0,93	Primmer *et al.* 1995
Hirundinidae	*Hirundo rustica*	Barn swallow	*Hr*U6	(153-465)	0,97	Primmer *et al.* 1995
Hirundinidae	*Hirundo rustica*	Barn swallow	*Hr*U7	(142-168)	0,39	Primmer *et al.* 1995
Hirundinidae	*Hirundo rustica*	Barn swallow	*Hr*U8	(176-194)	0,93	Primmer *et al.* 1995
Hirundinidae	*Hirundo rustica*	Barn swallow	*Hr*U9	(295-»800)	0,95	Primmer *et al.* 1996b
Hirundinidae	*Hirundo rustica*	Barn swallow	*Hr*U10	(187-380)	0,95	Primmer *et al.* 1996b
Maluridae	*Malurus cyaneus*	Superb fairy-wren	*Mcyμ*1	270	0,65	Double *et al.* 1997
Maluridae	*Malurus cyaneus*	Superb fairy-wren	*Mcyμ*2	199 (165-206)	0,65	Double *et al.* 1997; Double *et al.* 2005
Maluridae	*Malurus cyaneus*	Superb fairy-wren	*Mcyμ*3	250 (228-268)	0,71	Double *et al.* 1997; Double *et al.* 2005
Maluridae	*Malurus cyaneus*	Superb fairy-wren	*Mcyμ*4	170 (140-188)	0,94	Double *et al.* 1997; Beck *et al.* 2003
Maluridae	*Malurus cyaneus*	Superb fairy-wren	*Mcyμ*5	99	0,88	Double *et al.* 1997
Maluridae	*Malurus cyaneus*	Superb fairy-wren	*Mcyμ*6	113	0,82	Double *et al.* 1997
Maluridae	*Malurus cyaneus*	Superb fairy-wren	*Mcyμ*7	349 (169-401)	0,94	Double *et al.* 1997; Beck *et al.* 2003
Muscicapidae	*Ficedula hypoleuca*	Pied flycatcher	*Fh*U1 (PTC2)	–	0.49[a]	Ellegren 1992; Primmer *et al.* 1996b
Muscicapidae	*Ficedula hypoleuca*	Pied flycatcher	*Fh*U2 (PTC3)	–	0.76[a]	Ellegren 1992; Primmer *et al.* 1996b
Muscicapidae	*Ficedula hypoleuca*	Pied flycatcher	*Fh*U3	(168-177)	0.50[a]	Primmer *et al.* 1996b
Muscicapidae	*Ficedula hypoleuca*	Pied flycatcher	*Fh*U4	(179-237)	0.90[a]	Primmer *et al.* 1996b
Muscicapidae	*Ficedula hypoleuca*	Pied flycatcher	*Fh*U5C+D	(174-223)	1.00[a]	Primmer *et al.* 1996b
Muscicapidae	*Ficedula hypoleuca*	Pied flycatcher	*Fh*U6	(92-138)	0.68[a]	Primmer *et al.* 1996b
Muscicapidae	*Phylloscopus occipitalis*	Large-crowned leaf warbler	*Pocc*1	229	–	Bensch *et al.* 1997
Muscicapidae	*Phylloscopus occipitalis*	Large-crowned leaf warbler	*Pocc*2	195	–	Bensch *et al.* 1997
Muscicapidae	*Phylloscopus occipitalis*	Large-crowned leaf warbler	*Pocc*3	174	–	Bensch *et al.* 1997
Muscicapidae	*Phylloscopus occipitalis*	Large-crowned leaf warbler	*Pocc*4	214	–	Bensch *et al.* 1997
Muscicapidae	Phylloscopus trochilus	Willow warbler	*Phtr*2	(97-125)	0,72	Fridolfsson *et al.* 1997
Paridae	*Parus caeruleus*	Blue tit	*Pca*1	125	0,00	Dawson *et al.* 2000
Paridae	*Parus caeruleus*	Blue tit	*Pca*2	291	0,63	Dawson *et al.* 2000
Paridae	*Parus caeruleus*	Blue tit	*Pca*3	191	0,75	Dawson *et al.* 2000
Paridae	*Parus caeruleus*	Blue tit	*Pca*4	191	1,00	Dawson *et al.* 2000
Paridae	*Parus caeruleus*	Blue tit	*Pca*5	132	0,75	Dawson *et al.* 2000
Paridae	*Parus caeruleus*	Blue tit	*Pca*6	-	-	D. A. Dawson pers. comm.
Paridae	*Parus caeruleus*	Blue tit	*Pca*7	127	0,63	Dawson *et al.* 2000
Paridae	*Parus caeruleus*	Blue tit	*Pca*8	295	0,88	Dawson *et al.* 2000
Paridae	*Parus caeruleus*	Blue tit	*Pca*9	131	1,00	Dawson *et al.* 2000
Paridae	*Parus caeruleus*	Blue tit	PK11	-	-	SM Tanner, H Richner, D Schuemperli (unpubl.); EMBL accession no. AF041465
Paridae	*Parus caeruleus*	Blue tit	PK12	-	-	SM Tanner, H Richner, D Schuemperli (unpubl.); EMBL accession no. AF041466
Parulidae	*Dendroica petechia*	Yellow warbler	*Dpu*16	162	0.63[b]	Dawson *et al.* 1997
Passeridae	*Passer domesticus*	House sparrow	Pdoμ5	230	0,92	Griffith *et al.* 1999

Family	Scientific name	Common name	Locus	Size in source species, bp (range)	HO in source species	Source
Sylviidae	*Acrocephalus arundinaceus*	Great reed warbler	Aar1	176 (166-176)	0,49	Hansson *et al.* 2000
Sylviidae	*Acrocephalus arundinaceus*	Great reed warbler	*Aar*2	132 (130-142)	0,12	Hansson *et al.* 2000
Sylviidae	*Acrocephalus arundinaceus*	Great reed warbler	*Aar*3	194 (180-284)	0,77	Hansson *et al.* 2000
Sylviidae	*Acrocephalus arundinaceus*	Great reed warbler	*Aar*4	118 (112-124)	0,52	Hansson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*1	64	–	Richardson *et al.* 2000[c]
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*2	97	0,71	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*3	101	0,86	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*4	103	0,40	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*5	110	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*6	119	0,76	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*7	123	0,83	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*8	125	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*9	125	0,40	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*10	127	0,64	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*11	128	0,40	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*12	128	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*13	132	0,52	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*14	140	–	Richardson *et al.* 2000[c]
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*15	150	–	Richardson *et al.* 2000[c]
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*16	155	1,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*17	175	–	Richardson *et al.* 2000[c]
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*18	176	0,56	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*19	177	0,88	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*20	178	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*21	180	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*22	181	0,50	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*23	186	–	Richardson *et al.* 2000[c]
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*24	186	-	Richardson *et al.* 2000[c]
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*25	187	0,76	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*26	203	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*27	204	0,64	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*28	204	–	Richardson *et al.* 2000c
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*29	207	0,14	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*31	216	–	Richardson *et al.* 2000c
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*32	218	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*33	220	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*34	220	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*35	224	0,44	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*36	225	0,20	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*37	226	0,32	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*38	226	0,50	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*39	227	–	Richardson *et al.* 2000[c]
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*40	230	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*41	231	–	Richardson *et al.* 2000[c]
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*42	243	0,32	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*43	250	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*44	250	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*45	265	–	Richardson *et al.* 2000[c]
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*46	265	0,24	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*47	267	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*48	270	0,56	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*49	272	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*50	272	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*51	277	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*52	278	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*53	285	0,43	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*54	291	–	Richardson *et al.* 2000[c]
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*55	292	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*56	298	0,44	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*57	299	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*58	311	0,76	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*59	318	–	Richardson *et al.* 2000[c]
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*61	369	0,40	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*62	372	0,00	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*63	400	0,29	Richardson *et al.* 2000
Sylviidae	*Acrocephalus sechellensis*	Seychelles warbler	*Ase*64	412	0,50	Richardson *et al.* 2000

[a] HE is presented because H_O was not available from published literature
[b] We present the mean of the H_O values presented for two populations in Dawson *et al.* 1997
[c] See also http://www.shef.ac.uk/misc/groups/molecol/SeychellesWarbler*ASE*PRIMERTABLE1.xls

Table 2.2. Cross-amplification of the 114 microsatellite loci examined in winter wrens and Australian reed warblers. For each loci and test species, the number of individuals tested (presumably unrelated), the number of alleles observed and the number of unique banding patterns generated (for polymorphic loci, where recorded) are shown. – indicates not tested (number of individuals and alleles) or not known (number of banding patterns).

	Winter wren			Australian reed warbler		
Locus	No. individuals tested	No. alleles	No. banding patterns	No. individuals tested	No. alleles	No. banding patterns
*Aar*1	6	1	1	6	1	1
*Aar*2	6	1	1	-	-	-
*Aar*3	6	0	0	6	7	-
*Aar*4	4	3	-	6	3	-
*Ase*1	6	1	1	6	1	1
*Ase*2	6	1	1	6	1	1
*Ase*3	6	1	1	6	1	1
*Ase*4	6	0	0	6	1	1
*Ase*5	6	1	1	6	1	1
*Ase*6	6	1	1	6	1	1
*Ase*7	-	-	-	6	2	2
*Ase*8	6	7	6	6	3	3
*Ase*9	6	2	2	6	3	4
*Ase*10	6	>2	>1	5	9	5
*Ase*11	6	1	1	6	7	6
*Ase*12	6	1	1	6	4	3
*Ase*13	6	2	2	5	5	4
*Ase*14	6	1	1	6	1	1
*Ase*15	6	2	2	6	1	1
*Ase*16	-	-	-	6	5	5
*Ase*17	6	0	0	6	1	1
*Ase*18	6	5	4	6	1	1
*Ase*19	6	3	3	6	3	2
*Ase*20	6	1	1	6	1	1
*Ase*21	6	0	0	6	9	5
*Ase*22	6	1	1	6	1	1
*Ase*23	6	1	1	6	1	1
*Ase*24	6	1	1	6	1	1
*Ase*25	6	0	0	6	1	1
*Ase*26	6	1	1	6	1	1
*Ase*27	6	1	1	6	1	1
*Ase*28	6	0	0	6	1	1
*Ase*29	6	5	3	6	1	1
*Ase*31	6	1	1	6	1	1
*Ase*32	6	1	1	6	1	1
*Ase*33	6	1	1	6	1	1
*Ase*34	6	2	2	5	3	4
*Ase*35	6	0	0	6	1	1
*Ase*36	6	1	1	6	1	1
*Ase*37	6	3	3	6	2	2
*Ase*38	6	0	0	-	-	-
*Ase*39	6	0	0	6	1	1
*Ase*40	5	2	2	6	3	-
*Ase*41	6	0	0	6	1	1
*Ase*42	6	1	1	6	1	1
*Ase*43	6	3	2	6	2	2
*Ase*44	6	1	1	6	1	1
*Ase*45	6	0	0	-	-	-
*Ase*46	6	3	4	6	1	1
*Ase*47	6	1	1	6	4	2
*Ase*48	6	0	0	5	7	5
*Ase*49	6	0	0	6	1	1
*Ase*50	6	0	0	6	1	1
*Ase*51	6	1	1	6	1	1
*Ase*52	6	1	1	6	1	1
*Ase*53	6	0	0	6	1	1
*Ase*54	6	1	1	6	1	1
*Ase*55	6	5	4	6	1	1

Table 2.2. Continued

Locus	Winter wren			Australian reed warbler		
	No. individuals tested	No. alleles	No. banding patterns	No. individuals tested	No. alleles	No. banding patterns
*Ase*56	6	6	5	6	5	6
*Ase*57	6	1	1	6	6	5
*Ase*58	6	0	0	6	1	1
*Ase*59	-	-	-	6	1	1
*Ase*61	6	0	0	6	0	0
*Ase*62	6	1	1	6	1	1
*Ase*63	-	-	-	6	2	2
*Ase*64	-	-	-	6	7	5
*Dpu*16	6	1	1	6	1	1
*Escμ*1	6	1	1	-	-	-
*Escμ*2	6	0	0	6	1	1
*Escμ*3	6	1	1	-	-	-
*Escμ*4	6	0	0	-	-	-
*Escμ*5	6	0	0	-	-	-
*Escμ*6	6	0	0	6	3	-
*Fh*U1 (PTC2)	6	0	0	-	-	-
*Fh*U2 (PTC3)	6	4	-	6	1	1
*Fh*U3	6	0	0	-	-	-
*Fh*U4	6	0	0	-	-	-
*Fh*U5	6	0	0	-	-	-
*Fh*U6	6	0	0	-	-	-
*Hr*U2 (STG1)	6	3	-	6	1	1
*Hr*U3	6	1	1	6	6	-
*Hr*U4	6	1	1	6	1	1
*Hr*U5	6	0	0	6	1	1
*Hr*U6	6	4	4	6	5	5
*Hr*U7	6	1	1	5	2	2
*Hr*U8	6	0	0	6	0	0
*Hr*U9	6	0	0	6	1	1
*Hr*U10	6	0	0	6	0	0
*Mcyμ*1	6	1	1	-	-	-
*Mcyμ*2	6	3	3	-	-	-
*Mcyμ*3	6	0	0	-	-	-
*Mcyμ*4	6	4	4	6	4	4
*Mcyμ*5	6	0	0	-	-	-
*Mcyμ*6	6	1	1	-	-	-
*Mcyμ*8	6	1	1	-	-	-
*Mme*12	6	1	1	6	3	2
*Pca*1	6	0	0	-	-	-
*Pca*2	6	1	1	6	1	1
*Pca*3	6	0	0	6	1	1
*Pca*4	6	5	4	-	-	-
*Pca*5	3	0	0	3	1	1
*Pca*6	6	1	1	-	-	-
*Pca*7	6	0	0	6	1	1
*Pca*8	6	0	0	-	-	-
*Pca*9	6	0	0	6	2	2
*Pdoμ*5	6	1	1	6	1	1
*Phtr*2	6	1	1	-	-	-
PK11	6	1	1	-	-	-
PK12	6	0	0	6	7	5
*Pocc*1	-	-	-	6	1	1
*Pocc*2	6	1	1	-	-	-
*Pocc*6	6	1	1	-	-	-
*Pocc*8	6	6	5	-	-	-
*Ppi*2	3	1	1	6	5	5

For detailed investigation of allele frequencies (wrens only), PCR amplification reactions were the same as for cross-amplification, except for optimisation of annealing temperatures and $MgCl_2$ concentrations in some cases (Table 2.4). Amplification was performed using a Thermolyne Amplitron II or Corbett Research thermal cycler. The following program was used: one cycle of 94 °C for 2 min, then 35 cycles of 94 °C for 30 s, 45 s at the annealing temperature (Table 2.4) and 72 °C for 45 s, followed by 72 °C for 3 min. For touchdown program, the annealing temperature started at 60 °C and decreased by 1 °C every cycle until 49 °C, and finished with 19 cycles at 48 °C. Samples were amplified using the forward primer 5' end-labelled with fluorescent phosphoramidite (6-FAM, HEX or NED).

Visualization of PCR products

For cross-amplification testing, products were run on 6% polyacrylamide gels and visualized with silver staining (Promega). If products were not near the bottom of the gel and thus maximally separated they were rerun for a longer period based on the distance down the gel in the first run. We recorded by eye the number of different alleles for each locus and, in some cases, the number of unique banding patterns displayed across the panel of test individuals. If no product was seen after two PCR amplications, then the locus concerned was considered not conserved in the test species. If a single-band or polymorphic PCR product was found, it was considered to be the target microsatellite providing the band showed a 'stutter' band typical of a microsatellite and was close to the size range in the original species.

For detailed investigation of allele frequencies in wrens we used a subset comprising the five most successful loci, based on the variability observed in the prior tests and the expected size range (to permit multiplexing). One μl of each sample was denatured (heated at 100 °C for 3 min and placed directly on ice) and then electrophoresed on a 10% denaturing polyacrylamide gel, with an internal lane standard (ROX500, Applied Biosystems). The DNA fragments were detected using an Applied Biotechnologies (ABI) 377 XL DNA sequencer. We included negative controls on all gels along with our samples to ensure there was no spurious amplification. The fluorescently labelled DNA fragments were analysed using GENESCAN software (Applied Biosystems). In some cases, samples were organized into multiplex loading groups containing 3-5 loci of non-overlapping allele lengths with a single fluorescent dye, and in total three fluorescent dyes were used. For the subset of five loci chosen for further analysis in wrens (see also chapter 7), electrophoresis of PCR products was conducted by GreenomicsTM (Plant Research International B.V., The Netherlands). Due to PCR failure, not all 215 individual wrens were typed at all loci (0 loci: 4 individuals, 2 loci: 2 individuals, 3 loci: 6 individuals, 4 loci: 26 individuals, 5 loci: 177 individuals).

Data analyses

Microsatellite banding patterns were analysed with GENOTYPER software (Applied Biosystems). Basic genetic statistics (observed (H_O) and expected heterozygosity (H_E), polymorphic information content (PIC), parental exclusion probabilities, Hardy-Weinberg

probability and the estimation of null allele frequency) were calculated using CERVUS 2.0 software (Marshall *et al.* 1998). The observed heterozygosity (H_O) represents proportion of heterozygous individuals at each locus, while the expected heterozygosity (H_E) is the unbiased expected heterozygosity calculated from the observed allele frequencies, assuming Hardy-Weinberg equilibrium. Polymorphic information content (PIC) is an additional measure of the informativeness of a locus, which is related to expected heterozygosity and also assumes Hardy-Weinberg equilibrium (Hearne 1992). Parental exclusion probabilities are calculated from the observed allele frequencies, and represent the power of the locus to exclude a randomly-selected unrelated candidate parent from parentage of an arbitrary offspring, given the genotype of either only the offspring (P_{E1}), or of the offspring and the parent of the opposite sex (P_{E2}). Hardy-Weinberg (H-W) p-values indicate whether the frequency of genotypes in the population deviates significantly from Hardy-Weinberg equilibrium. Statistical analyses were performed in SPSS 12.0.1 for Windows (SPSS Inc., USA) and followed Quinn & Keough (2002). All *P*-values are two-tailed and the null hypothesis was rejected when $P < 0.05$.

RESULTS

Microsatellite marker cross-amplification

Table 2.2 shows the cross-species amplification success of all of the tested primers in each target species. We found that 69 of 108 (64%) of the primers tested in wrens amplified a product, and of these 23 (33%) were polymorphic. In reed warblers, 84 of 87 (97%) of the primers tested amplified a product, of which 31 (37%) were polymorphic. Fifty-five of the 81 primers tested in both target species amplified a product (68%), of which 13 (24%) were polymorphic in both species.

Table 2.3 summarises the cross-species amplification success of the microsatellite markers used in this study, categorised by the Family of the source species (the species in which the microsatellite was originally cloned). It shows the number and proportion of loci amplifying in each of our target species and the number and proportion of loci displaying polymorphism in each of our two target species. The rate of successful amplification and polymorphism from the total panel of markers tested was considerably higher in reed warblers (97% and 36%, respectively) than wrens (64% and 21, respectively). This was the case across all source families, with the exception that neither of the two Muscicapidae loci tested in our panel of six reed warblers were polymorphic. There was no correlation between the proportion of loci from each family amplifying (Spearman rank correlation: $r_s = 0.107$, $n = 10$, $P = 0.768$) or polymorphic ($r_s = 0.073$, $n = 10$, $P = 0.841$) in wrens and reed warblers.

The majority of loci tested in the Australian reed warbler (72%) were sourced from the same family (Sylviidae). In contrast, no loci tested in wrens were sourced from the same family (Troglodytidae). As expected from this, amplification success was considerably higher in reed warblers than wrens in our study, including with Sylviidae primers (98% in reed warblers and 72% in wrens; Table 2.3). However, amplification success was

Table 2.3. Summary of cross-amplification success of (a) 108 microsatellite loci tested in winter wrens, and (b) 87 microsatellite loci tested in Australian reed warblers. For each family, the number of loci tested, the number and proportion of loci amplifying a product, and the number and proportion of polymorphic loci are shown.

a Winter wren

Family	No. loci tested	No. loci amplifying	% amplifying	No. loci polymorphic	% polymorphic
Corvidae	1	1	100%	0	0%
Emberizidae	1	1	100%	0	0%
Fringillidae	6	2	33%	0	0%
Hirundinidae	9	5	56%	2	22%
Maluridae	7	5	71%	2	29%
Muscicapidae	10	5	50%	2	20%
Paridae	11	4	36%	1	9%
Parulidae	1	1	100%	0	0%
Passeridae	1	1	100%	0	0%
Sylviidae	61	44	72%	16	26%
Total	108	69	64%	23	21%

b Australian reed warbler

Family	No. loci tested	No. loci amplifying	% amplifying	No. loci polymorphic	% polymorphic
Corvidae	1	1	100%	1	100%
Emberizidae	1	1	100%	1	100%
Fringillidae	2	2	100%	1	50%
Hirundinidae	9	7	78%	3	33%
Maluridae	1	1	100%	1	100%
Muscicapidae	2	2	100%	0	0%
Paridae	6	6	100%	2	33%
Parulidae	1	1	100%	0	0%
Passeridae	1	1	100%	0	0%
Sylviidae	63	62	98%	22	35%
Total	87	84	97%	31	36%

just as high or higher in loci sourced from other families as those from Sylviidae, and the proportion of polymorphic loci was relatively similar between wrens and reed warblers (Table 2.3).

Relatedness and parentage assessment

Table 2.4 describes the five loci chosen for further analysis in wrens, along with the PCR conditions that were used (annealing temperature and $MgCl_2$ concentration) and allele frequency statistics calculated by CERVUS. These statistics are derived from a larger sample of wrens (n = 180-211 individuals depending on the locus). All loci had relatively high

H_O and H_E values (≥ 0.635). No significant deviations from H-W equilibrium were reported for any of the loci selected for further analysis in wrens, and the estimates of null allele frequency were low in all cases (≤ 0.049). However, in one case (*Ase*8), the deviation from H-W equilibrium was not calculated by CERVUS. The allele frequencies observed in these five loci are shown in figure 1.

Using the panel of five markers described in Table 2.4 to analyse parentage in 29 wren families, we found that 11 nests (37.9%) contained extra-pair young, accounting for 25 of 134 typed offspring (18.7%). The analysis of parentage and individual genetic diversity in wrens is reported in detail in chapter 7.

DISCUSSION

Cross-amplification and the genetic distance between taxa

In this study we examined cross-species amplification and polymorphism of 114 microsatellite markers developed from a wide range of passerine birds in two other passerine species, the winter wren and the Australian reed warbler. Our results show that a large proportion of microsatellite primers will amplify a microsatellite across families within the order Passeriformes, and that many of these will be polymorphic to some extent. When grouped by source family, there was no correlation between the proportion of primers amplifying or polymorphic in wrens and reed warblers.

The degree of genetic distance between the source and tested species is generally considered the most important factor in determining the success of cross-species amplification and polymorphism (Primmer *et al.* 2005). However, there is some debate as to the rate at which cross-species amplification will decrease with increasing genetic distance (Dallimer 1999; Primmer *et al.* 2005). The results of several previous studies of diverse taxa have indicated that for species that diverged *ca.* 10-20 million years ago 40-50% of primer sets will amplify, of which *ca.* half will be polymorphic (Moore *et al.* 1991; Pêpin *et al.* 1995; Primmer *et al.* 1996a; Gemmell et al 1997). Recently, Primmer *et al.* (2005) have suggested that divergence times for 50% amplification and polymorphism success in passerines would be approximately 8.5-24 and 5-14.3 million years ago, respectively. With the origin of passerine family level clades thought to be approximately 21-25 million years ago (Sibley & Ahlquist 1990), these values would seem to be broadly in agreement with the level of success in our study. However, it should also be noted that our study was based on a biased sample of loci, because we initially selected the panel of primers to test based on ease of use and high cross-species polymorphism success in passerines. The tendency of certain microsatellite loci to be much more highly conserved across species than others is well known (Primmer *et al.* 1996a; Primmer *et al.* 2005), and this could be expected to inflate our rates of cross-species amplification and polymorphism considerably.

Furthermore, most of the loci tested in the Australian reed warbler (72%) were sourced from the same family as reed warblers, while no loci tested in wrens were sourced from the wren family. As expected, amplification success was considerably higher in reed warblers than wrens in our study, including with Sylviidae primers (Table 2.3).

Table 2.4. Characteristics of the five microsatellite markers chosen for parentage, heterozygosity and relatedness analysis in winter wrens (see chapter 7), with the results of an allele frequency analysis. Table presents the fluorescent label used for each marker, PCR conditions (annealing temperature (T_a), and magnesium chloride ($MgCl_2$), concentration), allele frequency and size data, polymorphism information content (PIC) values, exclusion probabilities for the first (P_{E1}) and second (P_{E2}) parents, Hardy-Weinberg (H-W) *p*-values, and estimates of the frequency of null alleles (NA). These variables are described fully in the text (see Methods). Genetic statistics were calculated using CERVUS 2.0 software were derived from the complete datasets for each species (*i.e.* based on all individuals typed in the study for each locus). TD = touchdown program (see Methods).

Locus	Label	Ta, °C	$MgCl_2$ conc., mM	No. alleles (no. ind.)	Size range, bp	H_O	H_E	PIC	P_{E1}	P_{E2}	H-W *P*-values	NA freq. estimate
*Ase*8	HEX	TD	1.5	30 (208)	63 (124-187)	0.894	0.939	0.933	0.773	0.872	NA	0.022
*Ase*55	NED	62	1.5	10 (210)	28 (285-313)	0.652	0.668	0.611	0.257	0.419	NS	0.012
*Ase*56	6-FAM	60	1.5	16 (180)	45 (291-336)	0.761	0.844	0.824	0.523	0.689	NS	0.049
FhU2	6-FAM	50	1.5	7 (211)	18 (122-140)	0.635	0.588	0.521	0.186	0.330	NS	-0.047
Mcyμ4	NED	55	1.5	18 (211)	44 (133-177)	0.773	0.774	0.743	0.399	0.578	NS	-0.002
Overall				16.2 (204)	39.4	0.705	0.719	0.675	0.961	0.993		0.003

However, amplification success of loci sourced from families other than Sylviidae were often just as high or higher than those from Sylviidae in both reed warblers and wrens (Table 2.3), which suggests that this alone was not the cause of the unusually high success rate in reed warblers. Furthermore, the proportion of polymorphic loci was more similar between wrens and reed warblers than the proportion of loci amplifying. This suggests that the use of a large proportion of loci sourced from the same family did not enhance cross-species polymorphism success in reed warblers to the same degree as it enhanced amplification success. More detailed analyses based on genetic distances between the source and target species (e.g. derived from DNA-DNA hybrization studies) will be required to fully quantify the influence of genetic distance in cross-species amplification and polymorphism in our study.

The utility of cross-amplified markers

When considering the utility of new markers for further genetic studies, it is essential to consider a large sample of genotypes from presumably unrelated individuals of the target species. This is necessary to make accurate estimates of population allele frequencies (Blouin 2003) and, of particular importance to parentage and population studies, to examine deviations from Hardy-Weinberg equilibrium and the occurrence of non-amplifying (null) alleles (Pemberton *et al.* 1995). The heterozygosities of the five loci chosen for further analysis in wrens (Table 2.4) compared favourably to that in similar studies (e.g. Dallimer 1999; Galan *et al.* 2003; Loyau *et al.* 2005), and the high exclusion probabilities (0.96 for the first parent and 0.99 for the second parent) will ensure a high confidence level in parentage studies (Marshall *et al.* 1998). No significant deviations from Hardy-

Weinberg equilibrium were detected and the estimated null allele frequencies were low (see chapter 7 for further assessment of the occurrence of null alleles). Therefore, these loci should have good utility in studies of population genetics, genetic diversity, and relatedness or parentage in natural populations of this species (see chapter 7). In the future, a set of microsatellite markers selected from this study will also be applied to parentage analysis of Australian reed warblers.

It should be noted that we have assumed that the PCR products that we scored in the target species were homologous loci. This is likely to be the case. However, to fully demonstrate that we were amplifying homologous loci would require sequence comparisons of the products between the source and target species (Slate *et al.* 1998). Therefore, we can not claim to be amplifying homologous loci with complete certainty. All of the products that we scored in the target species were a similar size to that reported in the source species and displaying the stutter band patterns typical of microsatellites. In a similar study of cross-amplification in passerine birds, Primmer *et al.* (1996a) sequenced 10 amplification products and all were found to be true homologues of the original loci.

Despite this caveat, the large number of primer sets that amplified polymorphic loci in winter wrens and Australian reed warblers augers well for future studies on the molecular ecology of these species. Furthermore, some of the markers identified as successful in this study, in particular those that worked well in both wrens and reed warblers, may prove useful in other passerine species for which insufficient loci have been developed. More detailed analyses of the data presented here may reveal more intricate associations between cross-amplification success or heterozygosity and species relatedness or characteristics of the loci such as allele size, repeat type or repeat number (Primmer *et al.* 1996a). To further our knowledge of microsatellite conservation across taxa, still more studies are required that test a large number of loci on a large number of target species (Primmer *et al.* 2005).

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