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CROSS-AMPLIFIED POLYMORPHIC MICROSATELLITES FOR THE SPOTLESS STARLING *STURNUS UNICOLOR*

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Summary.—*Cross-amplified polymorphic microsatellites for the spotless starling* Sturnus unicolor.

Aims: To test the real suitability of a set of 18 cross-amplified microsatellite primers for individual genotyping of the spotless starling and, therefore, to expand the panel of available microsatellite really useful for genetic studies of this species.

Location: Villalba, Madrid, Spain.

Methods: 18 microsatellite primers isolated from 7 different bird species were used as starting point. Their Polymerase Chain Reaction (PCR) was optimized. The polymorphism and genetic variability of primers that produced bands of expected size and had a clear electropherogram and segregation pattern was checked. To test the real suitability of these primers in genetic studies, they were used to analyze the frequency of extra-pair paternity (EPP), intra-specific brood parasitism (IBP) and pseudo-parasitism in 30 breeding pairs and 206 offspring.

Results: Six of the 18 initial primers were selected by their polymorphism (average alleles per locus: 8), heterozygosity (expected heterozygosity: 0.656), segregation and unambiguous electropherogram pattern. EPP was 16.5 % of offspring and 51 % of clutches. Pseudo-parasitism was 0.97 % of offspring and 3.5 % of clutches. IBP was not detected.

Conclusions: This paper provides 6 cross-amplified polymorphic markers for spotless starling and tests their suitability for genetic studies in this species in a reasonable number of individuals, confirming their utility in possible future studies of genetic diversity, population structure, individual identification and reproductive strategies.

Key words: cross-amplified microsatellites, extra-pair paternity, pseudo-parasitism, *Sturnus unicolor*.

Resumen.—*Microsatélites polimórficos de amplificación cruzada para el estornino negro* Sturnus unicolor.

Objetivos: Contrastar la validez real de 18 cebadores para microsatélites de amplificación cruzada para el genotipado de individuos de estornino negro Sturnus unicolor y, por tanto, expandir el panel de microsatélites realmente útiles para realizar estudios genéticos en esta especie.

Localidad: Villalba, Madrid, Spain.

Métodos: Se usaron como punto de partida 18 cebadores para microsatélites aislados a partir de 7 especies distintas de aves. Su reacción en cadena de la polimerasa (PCR) fue optimizada. Se analizó el polimorfismo y la variabilidad genética de aquellos cebadores que producían bandas del tamaño esperado y tenían un electroferograma y patrón de segregación claro. Para testar la verdadera utilidad de estos ce-

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badores en estudios genéticos, se decidió usarlos para analizar la frecuencia de paternidad extra-pareja, parasitismo intra-específico y pseudo-parasitismo en 30 parejas y 206 pollos.

Resultados: De los 18 microsatélites originales, se seleccionaron 6 por su polimorfismo (promedio 8 alelos por locus), heterocigosis (heterocigosis esperada: 0,656) y claro patrón de segregación. Se detectaron casos de extra-paternidad (16,5 % de los pollos y el 51 % de las puestas) y pseudo-parasitismo (0,97 % de los pollos y 3,5 % de las puestas). No se detectó ningún caso de parasitismo intra-específico.

Conclusiones: Este trabajo proporciona 6 marcadores polimórficos de amplificación cruzada en el estornino negro y testa su validez para estudios genéticos en esta especie en un número razonable de individuos, confirmado así, su utilidad en posibles estudios futuros de diversidad genética, estructura de la población, identificación de individuos y estrategias reproductivas.

Palabras clave: microsatélites de amplificación cruzada, paternidad extra-pareja, pseudo-parasitismo, *Sturnus unicolor.*

Introduction

The spotless starling *Sturnus unicolor* is a facultatively polygynous passerine living in large permanent social groups which is being intensively studied (Moreno *et al.*, 1999; Veiga *et al.*, 2001, 2002, 2004; Polo *et al.*, 2004; Polo and Veiga, 2006). Their laying period extends from mid April to mid June and most females lay two clutches per breading season. Both males and females defend nest boxes against competitors and intra-sexual competition for nest sites is heavy in both males and females. Breeding is mostly synchronous and alternative breeding strategies such as promiscuity and intra-specific brood parasitism (IBP) had been detected using DNA multilocus fingerprinting (Cordero *et al.*, 2003). Also by using DNA multilocus fingerprinting Aparicio *et al.* (2001) provided a new index for individual homozygosity based on the frequency of bands shared between parents and offspring used for studies on heterozygosity fitness correlations (Aparicio *et al.*, 2001; Cordero *et al.*, 2004). However, the use of this type of DNA marker and others like RAPDs, and single-locus minisatellites can have significant methodological or logistic disadvantages, as they are more time consuming and provide less accurate information on alleles segregation limiting the utility of their application to a particular population study (Dawson *et al.*, 1997).

Microsatellites are polymorphic markers PCR-based requiring minimal amounts of DNA and therefore less invasive methods for sampling. They are relatively cheap, their analysis is faster than for traditional genetic markers and they present locus-specificity and co-dominance. The advantages of the use of microsatellites as genetic markers seem to be evident, and their characteristics match with those described by Quellar *et al.* (1993) for the ideal genetic marker. Furthermore, microsatellite DNA is relatively abundant in the genomes of many taxa and loci have been isolated from a variety of wild vertebrates (Amos *et al.*, 1992; Taylor *et al.*, 1994; Paetkau and Strobeck, 1994) including avian species (Ellegren, 1992, Hanotte *et al.*, 1994; McDonald and Potts, 1994; Primmer *et al.* 1995, 1996; Gibbs *et al.*, 1996). One of the drawbacks for the use of microsatellites is that they require a complete and time-consuming process for isolation from the focal study species. Recently, nine specific microsatellites have been developed and tested for efficiency in the spotless starling (Celis *et al.*, 2007). However, increasing the panel of amplifiable microsatellites is beneficial for the analyses of individual heterozygosity and pair genetic similarity. An alternative to specific microsatellites isolation is testing the cross-amplification of microsatellites already available from different taxa (Galbusera *et al.*, 2000). This is a cheaper and faster method to obtain

amplifiable microsatellites for a study species and it has been used for a number of avian species (Arruga *et al.*, 1996; Dallimer, 1999; Dawson *et al.*, 2000; Galbusera *et al.*, 2000; Loyau *et al.*, 2005; Primmer *et al.*, 2005). In this paper 18 cross-amplified microsatellites are tested for their suitability in individual genotyping at the spotless starling to try to expand the panel of available microsatellites useful for this species, in which there are still many evolutionary questions to be solved on genetic diversity and fitness, sexual selection and mating strategies.

Material and methods

DNA extraction

Blood samples were obtained from adults when they were captured and from the nestlings when they were about 5-6 days old, by puncturing the jugular vein. About 0.1 ml of blood was stored in the field in absolute ethanol and then kept at -40ºC. DNA was extracted and purified from blood samples using NucleoSpin® Tissue Kit (Macherey-Nagel).

Primers´s amplification

Seven primers isolated from *Acrocephalus sechellensis, Aphelocoma ultramarina, Catharus ustulatus, Fycedula hypoleuca, Parus caeruleus* and *Poecille atricapillus* and 11 primers initially developed for *Lamprotornis superbus* and tested for amplification, but not polymorphism, in the spotless starling (Rubenstein, 2005) were used to test for their cross amplification in spotless starling. (Table 1, Appendix 1). Polymerase chain reaction (PCR) was optimized and carried out on a Mastercycler Epgradients (Eppendorf) thermal cycler using a 40 - 60ºC annealing temperature gradient. Approximately 5 - 10 ng of template DNA were added to 15 µl reaction volumes containing 1X Biotools standard buffer (75 mM Tris HCl (pH 9.0), 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, Biotools), 0.2 mM of each dNTP, 0.2 µM of each primer and 0.5 U of Taq DNA polymerase (Biotools). The PCR programme used was 4 min denaturing at 94ºC followed by 30 cycles of 30 s at 94ºC, 45s at the annealing temperature and 45 s at 72ºC, ending with 5 min elongation stage at 72ºC. PCR products were visualized under UV light after electrophoresis on a 2.5 % agarose gel stained with ethidium bromide.

Primers's selection and individual genotyping

Primers producing bands of expected size were labelled with fluorescent dyes (FAM, HEX or NED) at the 5´end and checked for polymorphism in 70 unrelated individuals of the total sample studied. Approximately 10 ng of template DNA were amplified in 13-µl reaction volumes containing Ecostart® Reaction Buffer 1X((16 mM $(NH_4)_2SO_4$; "67 mM Tris-HCl (ph 8.3 a 25ºC); 0.01% Tween-20), 0.2 mM dNTPs, 0.25 µmol of each labelled primer and 0.02 U of Taq DNA polymerase (Ecostart®, Ecogen). Polymerase chain reaction (PCR) was run on a GeneAmp® PCR System 9700 (Applied Biosystem) thermal cycler following a denaturation step at 95ºC for 7 min followed by 40 cycles of 30s at 94ºC; 45s at the annealing temperature and 45s at 72ºC, ending with a 5 min elongation stage at 72ºC. Finally, 5 µl of the PCR product was run on a 2.5 % agarose gel to verify amplification. PCR products were sized using a ROX labelled size standard in a single-capillary automated DNA sequencer (ABI 310® Genetic Analyzer, Applied Biosystems) and genotypes were scored using GeneScan 3.7 (Applied Biosystems). In cases where product signal was too low for accurate allele sizing, PCR on the sample was repeated. The primers which have a clear electropherogram pattern were selected and their variability was analyzed using

CERVUS 2.0 (Marshall *et al.*, 1998), GENEPOP 3.4 (Raymond and Rousset, 1995) and CERNICALÍN (Aparicio, Ortego and Cordero, 2006).

Parentage analysis

The selected primers were used to analyze the frequency of EPP, IBP and pseudo-parasitism in a breeding colony of Villalba (Madrid, Spain; Moreno *et al.*, 1999) during four breeding seasons. The genetic relationships of 206 nestlings, 20 breeding females and 27 putative social males not submitted to any hormonal manipulation experiment (Veiga *et al.*, 2004) was determined by comparing nestling genotypes firstly to that of the attending female and then to the territorial male at the six loci. Within-pair young was considered if nestlings alleles matched (*i.e.* if they possessed alleles of the size of each parent and segregation was compatible with Mendelian inheritance) to the social parents alleles on all six loci or had one mismatched allele assuming that this mismatch could be originated through mutation. Nestlings that mismatched the social male allele at two or more loci were considered to be extra-pair offspring (Johnsen *et al.*, 1998). Those that mismatched the social female allele at two or more loci but matched the social male were considered as pseudo-parasite offspring. Nestlings that mismatched both social male and female alleles at two or more loci were considered to be intra-specific-parasite offspring. The offspring (if the other siblings matched) or the parents (if all siblings mismatched in two or more loci) were genotyped again for all parent-offspring relationships with two or more mismatches.

Results and Discussion

Identification and characterization of microsatellite loci useful for genetic analyses

Table 1

Characterization of 6 selected cross-amplified microsatellite loci for the spotless starling. Primer name, initial species, original primer source reference, GenBank Accession Numbers, primer sequences, primer-dye, annealing temperatures (T^a), number of individuals tested, number of alleles, size range, observed heterozygosity (H_O), expected heterozygosity (H_E), exact P-values and SE of the Hardy-Weinberg equilibrium test for six cross-amplified microsatellite loci selected in spotless starling.

Locus	Initial species	Ref	GenBank Accession no.	Primer sequences (5´-3´)
Pca-7	*Parus caeruleus*	*Dawson et al.,2000*	AJ279809	F-TGA GCA TCG TAG CCC AGC AG R-GGT TCA GGA CAC CTG CAC AAT G
Ase-18	*Acrocephalus sechellensis*	Richardson et al.,2000	AJ276375	F-ATC CAG TCT TCG CAA AAG CC R-TGC CCC AGA GGG AAG AAG
SS 2.32	*Lamprotornis superbus*	Rubenstein, 2005	AY906889	F-GGT ATC ACC ATA TCT GCT GCC AGT A R-CAG GCT TTT GCT GAC AAT TAT TTT G
SS 1.6	*Lamprotornis superbus*	Rubenstein, 2005	AY906875	F-TTT CAC TGG CTG GAT CTG GTA AAC C R-CTA GCA ACA TAT AGC CCA AGC TGT ATT GAT
SS 2.106	*Lamprotornis superbus*	Rubenstein, 2005	AY906877	F-TGT GTT ATC CCA TTG TAA GGG CTC TTT R-GAC TCT AGG TGG AAA CCC CCA TTT T
SS 2.130	*Lamprotornis superbus*	Rubenstein, 2005	AY906884	F-CTG AAG GCA CCC AGC AGG TTC T R-AGA CCC ACT GTG ATA ATT ACC ACT TCT CTG

Of the 18 initial primers 15 were polymorphic in spotless starling and of these 6 were selected for their unambiguous and clear electropherogram and their segregation pattern. (Table 1, Appendix 1). These loci produced 48 alleles and were polymorphic with an average of 8 alleles per locus (range: 4 - 12). Average proportion of heterozygotes was 0.669. The frequency of expected (0.656; range: 0.431 - 0.815) and observed (0.682; range: 0.457 - 0.841) heterozygosity did not differ (χ^2_{12} = 4.1; P = 0.9822) indicating no departure from Hardy-Weinberg equilibrium and an absence of a significant proportion of null alleles (*sensu* Pemberton *et al.*, 1995; Primmer *et al.*, 1995). All tests for pair-wise linkage disequilibrium between loci were non-significant. The probability of parental exclusion at each locus ranged between 0.269 and 0.651 and the combined probability of parental exclusion for all six loci together was 0.977.

Use of microsatellite markers for parentage analysis

The real suitability of the 6 selected microsatellites for genetic studies was assessed using them to analyze extra-pair paternity, pseudo-parasitism and intraspecific-brood parasitism in the study population. The 16.5 % (34 / 206) of chicks resulted from extra-pair fertilization and 51 % of clutches (29 / 57) contained at least one extra-pair young. Pseudo-parasitism occurred in 0.97 % (2 / 206) of chicks from 3.5 % of clutches (2 / 57) but we did not detect IBP, results congruent with previous work (Cordero *et al.*, 2003).

In sum, the primary result of this paper is that it provides a set 6 cross-amplified microsatellites for spotless starling that have been already tested in a full population (253 individuals) showing that they are efficient for studies of parentage and could be also used for studies of genetic diversity, dispersal, population

TABLE 1 CONT.

[Caracterización de los 6 loci microsatélite seleccionados para su amplificación cruzada en el estornino negro. Nombre del primer, especie en la que se obtuvo, su referencia bibliográfica, Número de Acceso a GenBank, secuencia de nucleótidos de los primers, fluoróforo, temperatura de anillamiento (T_a), número de individuos testados, numero de alelos, rango de tamaños, heterocigosidad observada (H_o), heterocigosidad esperada (H_e), valores de P y error Standard (SE) del test del equilibrio de Hardy-Weinberg para 6 loci microsatélite de amplificación cruzada seleccionados para el estornino negro.]

Primer-Dye	T_a (°C)	No. Of indiv.	No. Of alleles	Size range (bp)	Ho	He	HW p-value	HW SE
HEX	56	70	12	92-128	0.457	0,431	0.8518	0.0206
NED	50	70	7	176-198	0.771	0,8	0.7504	0.0048
FAM	58	69	12	228-254	0.841	0,815	0.7798	0.0098
HEX	60	70	8	188-204	0.814	0,757	0.4113	0.0091
FAM	56	70	4	272-284	0.514	0,525	0.8698	/
NED	58	70	5	242-252	0.700	0,608	0.7343	0.0048

substructure and genetic correlates of mating and breeding success in this species.

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Appendix 1

Characterization of 12 microsatellite loci tested for cross amplification in the spotless starling that were discarded for one or more of the following reasons: poor or no amplification in spotless starling, non-specific amplification products with multiband profiles, non scoreable patterns or detection of only one or two alleles.

Locus	Initial species	Ref.	GenBank Accession no.
Ase-19	*Acrocephalus sechellensis*	Richardson et al., 2000	AJ276376
Cuu-28	*Catharus ustulatus*	Lisle et al., 1999	AF122894
Patmp2.43	*Poecille atricapillus*	Otter et al., 1998	AM056064
SS1.11	*Lamprotornis superbus*	Rubenstein, 2005	AY906870
SS1.12	*Lamprotornis superbus*	Rubenstein, 2005	AY906871
SS2.68	*Lamprotornis superbus*	Rubenstein, 2005	AY906905
SS2.80	*Lamprotornis superbus*	Rubenstein, 2005	AY906910
SS2.83	*Lamprotornis superbus*	Rubenstein, 2005	AY906912
SS3.42C	*Lamprotornis superbus*	Rubenstein, 2005	AY906917
FhU-3	*Ficedula hypoleuca*	Primmer et al., 1996	X84362
Mjg-1	*Aphelocoma ultramarina*	Li et al., 1997	U82673
SS2.71B	*Lamprotornis superbus*	Rubenstein, 2005	AY906906

[APÉNDICE 1] CONT.

[Caracterización de 12 loci microsatélites testados para su amplificación cruzada en el estornino negro que descartamos por una o más de las siguientes razones: amplificación débil o inexistente en el estornino negro, amplificación de productos no específicos con patrones de múltiples bandas, patrones no medibles o deteccion de solo 1 o dos alelos en el locus.]

Primer sequences (5´-3´)	Ta(ºC)	No.of indiv.	Amplification	No.of alleles	Size range (bp)
F-TAG GGT CCC AGG GAG GAA G R-TCT GCC CAT TAG GGA AAA GTC	57	14	yes	3	148-172
F-GAG GCA CAG AAA TGT GAA TT R-ACT TAT TTC AGT CCT AAA TTC ACC	60	10	yes	2	127-131
F-ACA GGT AGT CAG AAA TGG AAA G R-GTA TCC AGA GTC TTT GCT GAT G	63	20	yes	4	120-134
F-AAA TTT GAA CCG ATC CAG CCT GTT TA R-CTC GCT CCC TCT CCC TCT TTC AC	61	17	yes	5	176-188
F-TAT TTT CCC TTT TCT TCC CTT AGC AG R-GAA GCC AAC AAT ATG TAC AGA ATG TGC	60	7	yes	4	205-213
F- AAC TTG CTG GTT GAA AAT TTT AAT G R-TGT TCT TTA ATT GTT ACT CAG AAG TGA A	60	16	yes	2	134-136
F-ACC CAC TTT TAC CTA CCT AGC ATG TTC TGT R-ATT AGA GTG CCC AAG GAC TTG TTC TCA	58	19	yes	5	304-314
F-TGT CCC AGT TTT ACA TTT TTG TTA A R-GAC TTT TAA TAT GGA GCC TGC TCT	58	8	yes	4	158-164
F-TAT ATC CCA GGG AGG GTT GTG GTG TG R-ATC AAA CTG CAG CAG GAC TCT GAC TGT G	60	14	yes	5	142-150
F-ATA TTC CCC ATA AGA TAA TGG R-ATA GTG TTG TCT TAA GGT CTC T	49	15	no	–	–
F-CCC GGG AAA GGC TTC GTC TTC R-GGA GAT TTT ATA TCG GTG GC	60	10	no	–	–
F-CAC ACC CAA CAT GTA ACA AAT CTT ACA R-CTT TGA GCC TCT GCT TTT AGA AAT TG	62	11	no	–	–