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Notas Breves

NOVEL HIGHLY POLYMORPHIC LOCI AND CROSS-AMPLIFIED MICROSATELLITES FOR THE LESSER KESTREL *FALCO NAUMANNI*

NUEVOS LOCI ALTAMENTE POLIMÓRFICOS Y AMPLIFICACIONES CRUZADAS DE MICROSATÉLITES PARA EL CERNÍCALO PRIMILLA *FALCO NAUMANNI*

Joaquín Ortego* [1], Elena G. González**, Inés Sánchez-Barbudo*, José M. Aparicio* and Pedro J. Cordero*

The lesser kestrel *Falco naumanni* is a colonial and migratory species breeding in part of the Mediterranean Basin, including Spain, Italy, Greece, North Africa, Middle East and part of Central Asia and North-East of China (Del Hoyo *et al*., 1994). The species suffered an important decline during the 1970s and it is actually catalogued as globally endangered (Biber, 1996). Their mating system is markedly monogamous with low levels of extra-pair fertilisations (Alcaide *et al*., 2005). Colonies form breeding entities in which phylopatry, immigration and dispersal are crucial to understand their dynamics and evolution. The applicability of a minimal panel of optimised microsatellites would allow evaluating their genetic characteristics in order to understand different aspects of their conservation in relation with dispersal and colonisation, genetic aspects of mating and breeding performance (Aparicio *et al*., 2007).

Blood samples (100µl) were obtained by venipuncture of the brachial vein of adults and chicks and preserved in ~1200 µl ethanol 96 % at -20 ºC. QIAamp DNA Blood Mini Kits (QIAGEN) were used to extract and purify genomic DNA from the blood. Microsatellite loci for the lesser kestrel were isolated by constructing a genomic library enriched for GT and GATA repeats based on protocols of Ostrander *et al*. (1992) and Hamilton *et al*. (1999), with minor modifications (for details of the procedure see González *et al*., 2005). To design primer pairs PRIMER3 software (Rozen and Skaletsky, 2000) was used. Assessment was also carried out of 19 microsatellites isolated from peregrine falcon *Falco peregrinus*, gyrfalcon *Falco rusticolus*, Northern goshawk *Accipiter gentilis* and barn swallows *Hirundo rustica* for polymorphism in lesser kestrels (Table 1). Some primers initially developed for peregrine falcon have

* Grupo de Investigación de la Biodiversidad Genética y Cultural, Instituto de Investigación en Recursos Cinegéticos - IREC (CSIC, UCLM, JCCM), Ronda de Toledo s/n, Ciudad Real, E-13005 Spain.

** Departamento de Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC), José Gutiérrez Abascal 2, Madrid, E-28006 Spain.

[1] Corresponding author: joaquin.ortego@uclm.es

TABLE 1

Locus name, initial species, original locus source reference, GenBank accession numbers, primer sequences, annealing temperatures (T_a), number of individuals (Villacañas subpopulation/Consuegra subpopulation), size range, number of alleles, observed heterozygosity (H_O) and expected heterozygosity (H_E) for three novel and nineteen cross-amplified microsatellites tested in lesser kestrels *Falco naumanni*. Repeat motifs for novel microsatellite loci are $(AG)_{15...}((TG)_XTC)_Y$ for Fn1-11, $((TCTA)_XA)_Y$ for Fn2-14 and $(GT)_{12}...(GT)_{11}$ for Fn2-5.

Locus	**Initial species**	**Ref**	**GenBank accession no.**
Fn2-14	*Falco naumanni*	Present study	EF152565
Fn1-11	*Falco naumanni*	Present study	EF152566
Fn2-5	*Falco naumanni*	Present study	EF152567
Fp5	*Falco peregrinus*	Nesje *et al*., 2000	AF118420
Fp13	*Falco peregrinus*	Nesje *et al*., 2000	AF118421
Fp31	*Falco peregrinus*	Nesje *et al*., 2000	AF118422
Fp46-1	*Falco peregrinus*	Nesje *et al*., 2000	AF118423
Fp79-4	*Falco peregrinus*	Nesje *et al*., 2000	AF118427
Fp82-2	*Falco peregrinus*	Nesje *et al*., 2000	AF118428
Fp86-2	*Falco peregrinus*	Nesje *et al*., 2000	AF118429
Fp89	*Falco peregrinus*	Nesje *et al*., 2000	AF118430
Fp92-1	*Falco peregrinus*	Nesje *et al*., 2000	AF118430
Fp107	*Falco peregrinus*	Nesje *et al*., 2000	AF118430
Age5†	*Accipiter gentilis*	Topinka and May, 2005	AY312455

† This primer did not amplify with the final reaction mix described in the text. It only amplified under the conditions used to test initially for PCR amplification. [*Este cebador no amplificó con el mix de reacción descrito en el texto. Sólo lo hizo en las condiciones usadas para probar inicialmente la amplificación por PCR.]*

¶ Data from Villacañas and Consuegra subpopulations combined. *[Datos combinados para las subpoblaciones de Villacañas y Consuegra.]*

TABLE 1

[Nombre del locus, especie en la que se obtuvo, referencia bibliográfica, número de acceso al GenBank, secuencia de oligos, temperatura de anillamiento (T_a), número de individuos (subpoblación de Villacañas/subpoblación de Consuegra), rango de tamaños, número de alelos, heterocigosidad observada (H_O) y heterocigosidad esperada (H_E) para tres microsatélites nuevos y nueve de amplificación cruzada testados en cernícalo primilla Falco naumanni. *Los motivos de repetición para los nuevos microsatélites son* $(AG)_{15...}((TG)_XTC)_Y$ *para Fn1-11,* $((TCTA)_XA)_Y$ *para Fn2-14 y* $(GT)_{12}...(GT)_{11}$ *para Fn2-5.]*

Primer Sequence (5´-3´)	**Ta (ºC)**	**No. of indiv.**	**Size range (bp)¶**	**No. of alleles¶**	**HO¶**	**HE¶**
F: TTGCCAGCTTTTGAACCCTAA R: AAATTCAGGCCACCCACATC	56	288/181	178-1300	172	0.902*‡	0.985
F: TTCTATTGTAGGAATCCTGGAAACTT R: GGCTGTTATTTATTGGAAGAGTGA	59	288/200	256-426	23	0.770	0.768
F: CAATAACCGGGCATAAAGAG R: ATACCCACACCCACTCACACT	53	14/10	259	1	-	-
F: CCGTTCTGGAGTCAAAAC R: CATGCAGCACTTTATTCAG	55	288/197	99-109	6	0.629	0.618
F: AGCTTGATTGAGGCTGTG R: CCAAATTCCCTGCTGAAG	55	287/201	87-107	4	0.557*	0.629
F: ATCACCTGCACATAGCTG R: TTTAGCTCCTCTCTCTCAC	55	288/201	126-144	8	0.648	0.659
F: TTAGCCTCGCAGCTTCAG R: GTAATGAAAAGTCTTTGGGG	55	288/198	117-141	11	0.593	0.598
F: TGGCTTCTCTTATCAGTAAC R: GGCTGGGTGGAATTAAAG	55	287/199	127-195	33	0.907	0.936
F: CTGCACGAGGAGATGATG R: CCAGATAGCTGTGAAATGG	53	235/170	130-138	5	0.267*‡	0.552
F: GTAAATAAGCCTCCAAAAGG R: CATGCTTCCTGATTACTTC	54	287/199	138-142	3	0.317*‡	0.494
F: CTCTGCCCTGAATACTTAC R: GAATCTTGTTTGCATTGGAG	54	288/199	117-123	4	0.503	0.519
F: TTACTAGAAGGCTGCTCAG R: CGTATTCCAAACTTTATGGC	55	123/80	98-128	14	0.508*	0.791
F: ACAGATTTGATTGCCAGG R: TGCCATGTCACATTCATAC	55	108/80	186-406	19	0.606*‡	0.887
F: ACGTTACAGACACCGATTACTTCC R: AGCCACGCGTCTGATACTTT	54	33/15	148-170	9	0.625	0.637

* Significant heterozygote deficit after Bonferroni correction in Villacañas subpopulation. *[Déficit significativo de heterocigotos después de la corrección por Bonferroni en la subpoblación de Villacañas.]*
‡ Significant heterozygote deficit after Bonferroni correction in Consuegra subpopulation. *[Déficit significativo de heterocigotos después de la corrección por Bonferroni en la subpoblación de Consuegra.]*

TABLE 1 CONT.

Locus name, initial species, original locus source reference, GenBank accession numbers, primer sequences, annealing temperatures (T_a), number of individuals (Villacañas subpopulation/Consuegra subpopulation), size range, number of alleles, observed heterozygosity (H_O) and expected heterozygosity (H_E) for three novel and nineteen cross-amplified microsatellites tested in lesser kestrels *Falco naumanni*. Repeat motifs for novel microsatellite loci are $(AG)_{15...}((TG)_XTC)_Y$ for Fn1-11, $((TCTA)_XA)_Y$ for Fn2-14 and $(GT)_{12}...(GT)_{11}$ for Fn2-5.

Locus	Initial species	Ref	GenBank accession no.
Age7	*Accipiter gentiles*	Topinka and May, 2005	AY312457
Fr34	*Falco rusticolus*	Nesje and Røed, 2000	AF200200
Fr142	*Falco rusticolus*	Nesje and Røed, 2000	AF200201
Fr144-2	*Falco rusticolus*	Nesje and Røed, 2000	AF200202
Fr164-1	*Falco rusticolus*	Nesje and Røed, 2000	AF200203
Fr203	*Falco rusticolus*	Nesje and Røed, 2000	AF200206
Fr206	*Falco rusticolus*	Nesje and Røed, 2000	AF200207
HrU2	*Hirundo rustica*	Primmer *et al*., 1995	X84087

been previously proved to be polymorphic in lesser kestrels by Groombridge *et al*. (2000) and Alcaide *et al*. (2005). Here, the suitability of these and other microsatellite loci to genotype lesser kestrels was re-evaluated, testing the specific and positive cross-amplified microsatellites for mendelian inheritance, pair-wise linkage disequilibrium between loci and Hardy-Weinberg assumption for heterozygosity with a higher number of typed individuals.

Both novel and cross-amplified primer pairs were used for lesser kestrel samples collected in two subpopulations ("Villacañas" subpopulation: 39º 30´N, 03º20´W, 16 colonies; "Consuegra" subpopulation: 39º 35´N, 03º 40´W; 6 colonies) separated by 30 km and located in La Mancha, Central Spain. Polymerase chain reactions (PCR) were optimized in five individuals and carried out on a Mastercycler EpgradientS (Eppendorf) thermal cycler using a 40-60 ºC annealing temperature gradient. Approximately 5 ng of template DNA were added to 10-µL reaction volumes containing 1X buffer (67 mM Tris-HCL, pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01 % Tween-20, 2.5 mM $MgCl_2$, BIORON), 0.5 mM of each dNTP, 0.5 µM of each primer and 0.25 U of *Taq* DNA polymerase (BIORON). The PCR programme used 2 min denaturing at 94 ºC followed by 30 cycles of 30 s at 94 ºC, 45 s at the annealing temperature and 45 s at 72 ºC, ending with a 5 min final elongation stage at 72 ºC. PCR products were

TABLE 1 CONT.

[Nombre del locus, especie en la que se obtuvo, referencia bibliográfica, número de acceso al GenBank, secuencia de oligos, temperatura de anillamiento (T_a), número de individuos (subpoblación de Villacañas/subpoblación de Consuegra), rango de tamaños, número de alelos, heterocigosidad observada (H_O) y heterocigosidad esperada (H_E) para tres microsatélites nuevos y nueve de amplificación cruzada testados en cernícalo primilla Falco naumanni. *Los motivos de repetición para los nuevos microsatélites son $(AG)_{15...}((TG)_XTC)_Y$ para Fn1-11, $((TCTA)_XA)_Y$ para Fn2-14 y $(GT)_{12}...(GT)_{11}$ para Fn2-5.]*

Primer Sequence (5´-3´)	**Ta (ºC)**	**No. of indiv.**	**Size range (bp)¶**	**No. of alleles¶**	**HO¶**	**HE¶**
F: GGGGCATTGTGCTATTAGAAGTGA R: GGAGGCCCCCAGACAAAAG	40-60	18/12	-	-	-	-
F: TATTTCAGCCTGGTTTCCTAT R: TTTAGTATCTCAAAGACCCTGTGT	54	40/22	151	1	-	-
F: CCACCCCTCTGCCACTCA R: CCCCTGTCAGCTAAACACATCAC	54	40/22	182	1	-	-
F: GGGCTTTAGGTCTTCTTATTTTC R: GCCTACTATTTCCGTTTACTGG	40-60	7/3	-	-	-	-
F: CTGTTCGGATGGTTCCTACAACTT R: CTCACAGGGAGGCAGGTTACTT	46	40/22	123	1	-	-
F: CAGACCTGGCTGCAATGAGGA R: GACGACCCACGGACTACAGCTTT	46	18/12	244	1	-	-
F: ATCTAATGGGCTTTCCTGGATTT R: GACATTTTCCTCATAGGCAACTGA	40-60	7/3	-	-	-	-
F: CATCAAGAGAGGGATGGAAAGAGG R: GAAAAGATTATTTTTCTTTCTCCC	54	18/12	126	1	-	-

controlled under UV light after electrophoresis on a 2.5 % agarose gel stained with ethidium bromide.

Primers producing visible and expected bands were labelled with fluorescent dyes (FAM, HEX or NED) at the 5' end to determine whether they were polymorphic by amplifying 24-489 unrelated individuals from both Consuegra and Villacañas subpopulations with the optimized PCR profile (Table 1). In this case, approximately 5 ng of template DNA was added to 10-µL reaction volumes containing 1X reaction buffer (67 mM Tris-HCL, pH 8.3, 16 mM $(NH_4)_2SO_4$, 0.01 % Tween-20, Ecostart Reaction Buffer, Ecogen), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 µM of each primer and 0.1 U of *Taq* DNA EcoStart Polymerase (Ecogen). The PCR programme used was 9 min denaturing at 95 ºC followed by 30 cycles of 30 s at 94 ºC, 45 s at the annealing temperature (see Table 1) and 45 s at 72 ºC, ending with a 5 min final elongation stage at 72 ºC. Amplification products were electrophoresed using an ABI 310 Genetic Analyser (Applied Biosystems) and genotypes were scored using GeneScan 3.7 (Applied Biosystems).

Two out of three novel primers sets with positive amplifications were highly polymorphic (Table 1). Locus Fn2-14 showed a complex structure partially composed by a tetra-nucleotide microsatellite in variable tandem repeats of the sequence similar to a micro- with

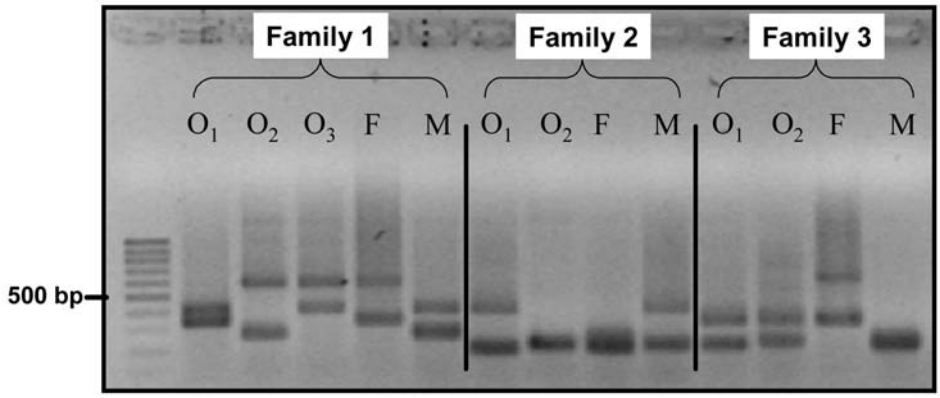

FIG. 1.—Mendelian segregation for PCR products of three different lesser kestrel families (A, B and C, Full-siblings = O, fathers = F and mothers = M) for locus Fn2-14 and run on agarose gel (2.5 %). Offspring band sizes of all three families match to either band of their respective parents. In the left lane is DNA size ladder (1 kb DNA ladder Biotools) with the position of the 500 nucleotide fragment indicated. *[Segregación mendeliana para los productos PCR de tres familias distintas de cernícalo primilla (A, B and C; respectivamente, hijos = O, padres = F and madres = M) para el locus Fn2-14 corridos en un gel de agarosa normal al 2.5 %. El tamaño de las bandas de los descendientes de las tres familias coinciden con al menos una de las bandas de sus respectivos padres. En la izquierda, se muestra el marcador escalado por tamaños (1kb DNA ladder Biotools) con la posición del fragmento de 500 nucleótidos indicada.]*

a mini-satellite arrangement that could explain its extraordinary range size and variability. This is, as far as is known, the most variable amplifiable locus described to date in birds. Its allele diversity is so high that it allows the estimation of accurate levels of heterozygosity (see Aparicio *et al*., 2006), and becomes very useful for population studies and paternity assessment, allowing the identification of several bands which can be scored on standard 2.5 % agarose gels (Fig. 1). This particular property of the Fn2-14 locus, to a lesser extent also found in Fn1-11, makes it very useful for preliminary and rapid paternity exclusions using scorable bands without or prior fragment analysis genotyping (Fig. 1). The combination of both primers may be useful for immediate scoring of individuals after PCR amplification. A preliminary screening may be performed for individual identification and exclusion of paternity according with the time and length PCR products are run on an agarose gel (Fig. 1). With a simple and standard minigel used for about 30 minutes run, this initial screening allows the identification of several scorable bands (Fig. 1). A more accurate binning of bands may be performed by running longer gels of agarose visualising more bands and the scoring becoming much more precise, preventing a rough homoplasy of bands. A final genotyping of the product provides the exact divergence of two apparent homoplasic bands not well enough separated by more standard methods. In relation to the cross-amplified microsatellites tested, 11 out of 19 were polymorphic in the lesser kestrel and the rest either did not amplify or were monomorphic (Table 1). Polymorphic loci were tested for Hardy–Weinberg equilibrium and genotypic disequilibrium in both subpopulations following Weir (1996) (Table 1). Tests for pair-wise linkage disequilibrium between loci were all non-significant ($P > 0.05$ after Bonferroni cor-

rection). None of the loci were found sex linked. Mendelian inheritance was confirmed by comparing the genotypes of three-five known families with three chicks each.

The three novel microsatellite loci were also tested for polymorphism in other related species (European kestrel, $n = 3$ individuals; peregrine falcon, $n = 11$ individuals). Fn2-5 and Fn2-14 were monomorphic and yielded inconsistent products respectively in both species, whereas Fn1-11 did not amplify in peregrine falcon but was polymorphic in European kestrel (6 different alleles). Because of the possible transcendence of Fn2-14 characteristics in other bird species like poultry, amplification was tested in chicken (*Gallus domesticus*), obtaining a negative result.

The probability of exclusion was estimated following Jamieson and Taylor (1997). The probability of exclusion of the two novel loci was 97.20 % for Fn2-14, 57.74 % for Fn1-11, and 98.82 % for the combination of both. On the other hand, the probability of exclusion of paternity for the combination of eleven cross-amplified microsatellites was 0.99. This indicates that using the two novel loci presented here is advantageous and requires less genotyping effort than using the larger panel of cross-amplified microsatellites. The high variability of these two new loci in combination makes them very useful for population and paternity studies. Information is provided for 13 microsatellites with 311 alleles in total, most of them from the two novel loci, available for studies of genetic diversity, dispersal, population substructure and genetics correlates of mating and breeding success in the lesser kestrel.

RESUMEN.—En el presente trabajo hemos desarrollado dos nuevos loci de microsatélite altamente polimórficos para el cernícalo primilla Falco naumanni. *Uno de ellos (locus Fn2-14) fue particularmente variable, presentando 178 alelos con un amplio rango de tamaños (179 - 1300), lo que lo hace muy útil para estudios sobre heterocigosidad individual, genética de poblaciones y paternidad. También se probaron 19 microsatélites previamente desarrollados para otras especies de aves, de los cuales 11 resultaron ser variables en el cernícalo primilla. En conjunto, se han obtenido un total de 311 alelos en un panel de 13 loci para estudios genéticos en esta especie.*

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