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TECHNICAL NOTE

Isolation and characterisation of hazel dormouse (*Muscardinus avellanarius*) microsatellite loci

Cheryl A. Mills · Deborah A. Dawson · Gavin J. Horsburgh · Brendan J. Godley · David J. Hodgson

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Abstract Hazel dormice, *Muscardinus avellanarius* (Rodentia: Gliridae) are vulnerable to habitat loss and fragmentation, and thus protected by European Directives. We isolated hazel dormouse microsatellite sequences from enriched genomic libraries to facilitate conservation-focussed research, such as population genetics, regarding this threatened species. Fifty-three primer sets were designed from 51 newly isolated loci. Additionally, we redesigned and tested nine primer sets from previously-published hazel dormouse microsatellite sequences. These 62 marker sets were initially tested in eight unrelated individuals. Thirty-nine loci, which were polymorphic and amplified in >88 % of these samples (extracted from hair), were then genotyped and characterised in 22–26 individuals. Of these, 26 autosomal loci (18 new and eight published) adhered to Hardy–Weinberg equilibrium ($p \leq 0.05$) and displayed an estimated null allele frequency of <0.10. One loci pair displayed linkage disequilibrium after correction for multiple tests.

Keywords 454 pyrosequencing · Gliridae · Rodent · Hair samples · Simple sequence repeats (STR)

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C. A. Mills · B. J. Godley · D. J. Hodgson (✉)
Centre for Ecology and Conservation, University of Exeter, Tremough, Penryn, Cornwall TR10 9EZ, UK
e-mail: D.J.Hodgson@exeter.ac.uk

C. A. Mills · D. A. Dawson · G. J. Horsburgh
NERC Biomolecular Analysis Facility, Department of Animal and Plant Sciences, University of Sheffield, Western Bank, Sheffield, South Yorkshire S10 2TN, UK

Hazel dormice, *Muscardinus avellanarius,* are listed on Appendix III of the Bern Convention, Annex IV of the EU Habitats and Species Directive, and as Least Concern by the IUCN (Amori et al. 2008). We developed microsatellite markers to facilitate research for this threatened species.

Two microsatellite-enriched libraries were constructed from one dormouse (ID79) found dead in Cornwall, UK in 2009. Genomic DNA was extracted from liver tissue using ammonium acetate precipitation (Nicholls et al. 2000). Libraries were constructed following Armour et al. (1994), enriched for dinucleotide, $(AC)_n$, $(AG)_n$, or tetranucleotide microsatellite motifs, $(GTAA)_n$, $(CTAA)_n$, $(TTTC)_n$, $(GATA)_n$. Motifs were denatured and bound to magnetic beads (Glenn and Schable 2005). Dinucelotide- and tetranucleotide-enriched fragments were PCR amplified separately in parallel, three times each to obtain sufficient DNA (c5 µg) for next generation sequencing (PCR program as in Glenn and Schable (2005), omitting the final hold). Each 25 µl PCR comprised 2.0 µl enriched DNA, 1× reaction buffer (Bioline), 25 µg/ml BSA, 150 µM dNTPs, 0.5 µM Sau-L-A linker/primer (Royle et al. 1992), 2.0 mM $MgCl_2$ and 1 unit of DNA *Taq* polymerase (Bioline). Dinucleotide and tetranucleotide PCRs were pooled and purified using a QIAquick PCR purification column (Qiagen) and eluted in 40 µl, generating a concentration of c125 ng/µl (measured using Nanodrop 8000 (Thermo Scientific)). Pooled PCR-amplified enriched fragments were 454-pyrosequenced without pre-shearing (Roche, FLX).

Additionally, amplification failure led to identification of errors in several published primers (Naim et al. 2009). Reverse primer sequences of *MavB5*, *MavG3* and *MavH3* were not present in the cited corresponding full sequences, and two bases were missing from the forward primer of *MavE3*. Therefore, we redesigned nine loci, to create a set of amplifiable markers, possessing similar melting temperatures to

Table 1 Characterisation of 39 hazel dormouse (*M. avellanarius*) microsatellite loci

Locus	EMBL accession number	Repeat motif	Primer sequence (5′–3′) and fluorescent label	Tm[a] (C)	Exp. allele size (bp)[b]	Obs. allele size range (bp) (ID79 Genotype)[c]	Pop[d]	n[e]	A[f]	H_O^g	H_E^h	P_{HWE}^i	Est. null allele freq.[j]
Mav002	HE819184	$(GA)_{15}$	F:[HEX]CTACCATGTGCTTGGCTGAG	59.5	108	103–111	COR	26	6	0.50	0.56	**0.01**	0.05
			R:CAGCCTGAACCACTCCAAG	59.4		(103, 103)							
Mav003	HE819185	$(GTT)_{16}$	F:[HEX]TTCTCAATTGCCTTCAGCTC	58.2	226	228–231	COR	26	2	0.42	0.38	1.00	−0.06
			R:TTAGTGAGGCCTTCTGCAAC	58.1		(228, 228)							
Mav005	HE819187	$(CTTT)_{12}$ CCTTCTTTCTT $(CTTT)_3$	F:[6-FAM]AGGCATATGGCAGCAGAGC	61.5	314	318–342	COR	25	7	0.72	0.76	0.35	0.02
			R:TTCGTGGACAGCCTCAGC	61.2		(322,338)							
Mav009	HE819190	$(CA)_{14}$	F:[6-FAM]GGCTGGTATGTAGCTCAGTGG	59.8	167	162–164	COR	26	2	0.00	0.15	**≤0.01**	**0.85**
			R:GACCAGCCATTGACACCTG	60.1		(162, 162)							
Mav011	HE819192	$(CTTT)_2$ $(CT)_2$ $(CTTT)_2$ $(CT)_2$ $(CTTT)_{13}$	F:[HEX]CCCGAGTACTGGGATTACAGG	60.7	184	186–214	COR	26	5	0.50	0.55	0.19	0.04
			R:AAGGCTGAGGGTTTAGTTCAGAG	60.3		(190, 190)							
Mav015	HE819196	$(GT)_5$ $(GC)_3$ $(AC)_2$ $(GT)_{12}$	F:[HEX]ACACCAGCCTCTGCAACTTAG	59.6	248	244–250	COR	25	3	0.52	0.54	0.61	0.01
			R:GAGAATGGCCTCTGACGAAG	60.0		(244, 244)							
Mav017	HE819198	$(CTTT)_2$ CT $(CCTT)_2$ CTTTT $(CTTT)_{10}$ $(CTT)_2$ C $(CTT)_2$ GT $(CTTT)_3$	F:[6-FAM]ACCATAAGCGAAGGGTGAG	57.8	246	244–260	COR	26	4	0.54	0.72	**0.05**	**0.13**
			R:TCTCCGTCTTGCTTTACAGG	58.1		(248, 248)							
Mav020	HE819201	$(CTTT)_{12}$	F:[6-FAM]TCAAGCCCTGGGATTACAAG	60.1	281	285–293	COR	25	4	0.52	0.54	**0.03**	0.02
			R:ATAGCCCGGAGGTAGAAAGC	59.7		(289, 289)							
Mav021	HE819202	$(CTTT)_{13}$ CTTCTTTCCTT $(CTTT)_3$	F:[HEX]AACTTGCTAGGCCAGACCAC	59.4	168	162–174	COR	26	4	0.58	0.65	0.64	0.06
			R:CCTGAGCAACTTAGCAAGTCC	59.1		(162, 170)							
Mav023	HE819204	$(GT)_{18}$ GC $(GT)_3$	F:[HEX]GGGAGTATAGCCCGGAGGT	60.3	149	145–162	COR	26	9	0.65	0.77	0.09	0.06
			R:GGCCCTACTTCAAACACATGA	60.0		(145, 151)							
Mav024	HE819205	$(CA)_{12}$	F:[HEX]GGGTGCAGCTGTGAGGTAG	59.4	128	117–123	COR	25	4	0.60	0.57	**0.03**	−0.06
			R:ACCGGGTGATGAAAATTGAG	59.8		(121, 121)							
Mav026	HE819207	$(GT)_6$ GCGTGC $(GT)_{11}$	F:[6-FAM]AGCTTCAGCACCTTAATCCAC	58.5	242	244–246	COR	25	2	0.00	0.08	**0.02**	**0.67**
			R:GCAAGTGAGCTGAGGAAGG	58.7		(246, 246)							
Mav027	HE819208	$(CA)_{15}$	F:[6-FAM]CACGACCATCCCAACCTC	59.9	146	137–141	COR	26	3	0.04	0.11	**0.02**	**0.44**
			R:GGGCGATATATGTCTCAGGTG	59.4		(139, 139)							
Mav028	HE819209	$(CT)_4$ $(CTTT)_{13}$	F:[HEX]CCTGCTCTGGCTGTAGGC	59.7	215	213–245	COR	26	6	0.77	0.76	**0.03**	−0.02
			R:GGAGGTAGAAAGCCCTGGAC	60.1		(213, 217)							
Mav030	HE819211	$(CTTT)_{16}$	F:[6-FAM]GGGAACCATCCAACCTATTG	59.1	157	138–157	COR	26	2	0.54	0.48	0.69	−0.06
			R:AAATGTCTCTGGGTTCCATACC	59.2		(157, 157)							
Mav032	HE819213	$(GATA)_2$ $(GATA)_9$	F:[6-FAM]AGGGTTTCCCACAGAAACAG	59.0	147	145–149	COR	26	2	0.42	0.47	0.68	0.05

Table 1 Characterisation of 39 hazel dormouse (*M. avellanarius*) microsatellite loci

Locus	EMBL accession number	Repeat motif	Primer sequence (5′–3′) and fluorescent label	Tm[a] (C)	Exp. allele size (bp)[b]	Obs. allele size range (bp) (ID79 Genotype)[c]	Pop[d]	n[e]	A[f]	H_O^g	H_E^h	P_{HWE}^i	Est. null allele freq.[j]
			R:GAATCTATAACCAGTTAGCAAATCTCC	59.1		(145, 149)							
Mav033	HE819214	$(CA)_4$ $(CG)_5$ G $(CA)_{14}$	F:[HEX]GTGAAGCCCAGAGACTTTGC	60.0	230	221–223	COR	26	2	0.15	0.15	1.00	−0.03
			R:AGGGAGAATTGTCTCCTTAGTGG	60.0		(221, 223)							
Mav034	HE819215	$(GT)_{15}$	F:[6-FAM]ATGTACACAACGCGGAAGTG	59.6	236	239–241	DEV	22	2	0.08	0.08	1.00	−0.01
			R:GGCAGCTCAGAAGTAGAATGC	59.2		(239, 239)							
Mav036	HE819217	$(CG)_5$ $(CA)_{15}$	F:[HEX]GGTTCTGTGAAAGCCTGAGC	60.0	209	203–207	COR	24	3	0.67	0.51	0.13	−0.18
			R:TGGTCAGAAGCTGTCAATGC	60.0		(205, 207)							
Mav038	HE819219	$(GT)_{18}$ $(GC)_2$ $(GT)_7$	F:[HEX]TCTTGTCTCAGCTTCCCAAGT	59.1	267	263–267	COR	26	2	0.27	0.34	0.29	**0.11**
			R:AGCACCTCAGAGGGAGTTGT	58.9		(263, 267)							
Mav039	HE819220	$(GATA)_5$ GAT $(GATA)_2$	F:[HEX]AATTTACCGAGGCCCACAG	59.9	174	174–178	COR	26	2	0.69	0.48	**0.04**	−0.19
		GAT $(GATA)_{12}$	R:TTGAACCAAGACATTCTACCTCTG	59.7		(174, 178)							
Mav040	HE819221	$(GTAAT)_4$ $(GT)_{13}$ $(GC)_4$	F:[6-FAM]GTGCTGAGTGGTGGAGTGAG	59.4	183	176–178	COR	26	2	0.31	0.32	1.00	0.00
			R:TTTATAGCTAGAATCATGCTGTCTTTG	59.5		(176, 178)							
Mav042	HE819223	$(CTTT)_{11}$	F:[HEX]TCGCAGCAACATAGCAAGAC	60.2	234	231–239	COR	26	3	0.42	0.60	0.11	**0.18**
			R:GAGAAACGCGATTCATGGAC	60.6		(231, 235)							
Mav043	HE819224	$(CTTT)_{11}$	F:[6-FAM]GGCTCTGGTCTCTGTCACTATG	59.0	165	164–180	COR	26	5	0.50	0.58	0.16	0.08
			R:AAGTAGGACCAGGAGGTAGAAGG	59.2		(168, 176)							
Mav044	HE819225	$(CTTT)_{15}$	F:[HEX]CAGCACCCACAGCCTCAT	60.9	142	134–142	COR	26	3	0.62	0.56	0.31	−0.06
			R:GGCAGGCTACTGCTGCAC	60.7		(134, 142)							
Mav047	HE819228	$(CA)_5$ $(A)_3$ $(CA)_{10}$	F:[HEX]ACTGGGGTGTAGCTCAGAGG	59.3	133	133–135	COR	25	2	0.08	0.08	1.00	−0.01
			R:AATTTTACACAGTATGGGGACTGTT	59.2		(133, 133)							
Mav048	HE819229	$(CTTT)_{14}$ TCTTCCTTTCT	F:[HEX[CACAATCCTCCTGTGTCAGC	59.3	221	222–230	COR	26	3	0.54	0.62	0.41	0.07
		TCCTTTT $(CTTT)_3$	R:AGGACTCTCCATGCCAAGAG	59.4		(226, 230)							
Mav049	HE819230	$(CTAT)_{11}$ CTGTCAT	F:[6-FAM]GGGTTTGCAAAGAACCCAAT	61.1	212	208–212	COR	26	2	0.12	0.18	0.19	**0.20**
		$(CTAT)_3$	R:CCTGGGTTCCGTCCCTAGTA	61.2		(212, 212)							
Mav050	HE819231	$(GATA)_9$	F:[6-FAM]CCAAGCACTCAGCTCTCTTG	58.9	281	279–288	COR	26	3	0.23	0.43	**0.01**	**0.31**
			R:TCCATAGAAACTAGGTAATTTGAGTGA	58.8		(279, 288)							
Mav051	HE819232	$(CA)_{18}$	F:[6-FAM]AGTGGTGGCATGTACCTGTG	59.5	235	233–245	COR	26	4	0.69	0.59	0.59	−0.09
			R:ACGATTATTCTGCCACTGAGC	59.4		(233, 239)							

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Mav053	HE819234	$(CA)_3$ GA $(CA)_3$ GA $(CA)_2$	F:[6-FAM]GGCCTTCTATAACTTAGCAAGAACC	60.1	224	219–221	COR	26	2	0.65	0.49	0.12	−0.15
		GA CA CG $(CA)_{17}$	R:ACGGACGAGTGAGCTGTACC	60.3		(221, 221)							
MavSB5 [l]	GF089516	$(CA)_{40}$ C $(CA)_2$	F:[HEX]GTAGAATGCTTCGAGTTCAATTTC	58.5	145 [m]	97–107 [m]	COR	24	5	0.63	0.59	0.16	−0.05
			R:TGGAGAAGGGTACTAGGATGC	58.3		(101, 107)							
MavSE3 [l]	GF089515	$(CA)_{49}$	F:[HEX]TGGGAGTATAGCCCAGAGGTAG	59.6	211 [m]	151–155 [m]	COR	26	3	0.08	0.08	1.00	−0.01
			R:CAGGAGTTAGCATCCCGTTC	59.7		(155, 155)							
MavSF10 [l]	GF089509	$(CA)_{43}$	F:[6-FAM]GCTGAGGGTATAACTTGGAGGTAG	59.6	266 [m]	224–230 [m]	COR	26	3	0.54	0.59	0.73	0.05
			R:GTCTGAAATGGCTGGTATTGC	59.6		(224, 230)							
MavSF12 [l]	GF089514	$(GT)_{16}$ GG $(GA)_{20}$ $(GT)_{17}$	F:[HEX]CAGGCAAGGAGTTGGATG	57.7	300 [m]	224–231 [m]	COR	24	4	0.42	0.42	0.10	−0.03
		$(GA)_{23}$	R:TTGAAAGGCAGGAGAATCC	57.8		(224, 229)							
MavSG3 [l]	GF089517	$(GT)_{54}$	F:[6-FAM]TGTTGACTGATTGAGTGGTGAC	58.6	195 [m]	131–159 [m]	COR	25	4	0.24	0.26	0.22	0.06
			R:GGCTGAAGATGTAGCTCATAGG	58.1		(131, 131)							
MavSG6 [l]	GF089511	$(GT)_{38}$	F:[HEX]AGCCCTTCACTTTCCCTGTATC	60.8	194 [m]	175–177 [m]	COR	26	2	0.54	0.40	0.13	−0.16
			R:TCCTCAGCAACTTAGCAAGACC	60.9		(175, 177)							
MavSG9 [l]	GF089510	$(GT)_{34}$	F:[6-FAM]AGCCACATCCCAACTACTGG	60.0	208 [m]	192–194 [m]	COR	26	2	0.50	0.51	1.00	0.00
			R:AGTGCTGCTGGGACAACC	59.8		(192, 192)							
MavSH3 [l]	GF089518	$(GT)_{29}$ CnT $(GT)_{11}$	F:[HEX]CTGGGAGTGTAGCTTGAAGG	57.6	246 [m]	205–209 [m]	COR	25	2	0.16	0.15	1.00	−0.03
			R:CAAGGATAGGGATACACCTCAG	57.7		(209, 209)							

[a] Tm, primer melting temperature (calculated using PRIMER3)

[b] Exp. allele size (bp), Expected allele size, based on the sequenced allele isolated from the individual used to create the genomic library, for newly isolated loci this comprises individual ID79 and for redesigned primers the expected size was calculated from the sequence data, as provided by Naim et al. (2009)

[c] Obs. allele size range (bp) (ID79 Genotype), Observed allele size range in characterised individuals, with individual ID79's observed genotype shown in brackets

[d] Pop, location of population origin of tested individuals—Cornwall (COR) and Devon (DEV)

[e] n, number of hazel dormouse individuals amplified from a total of 26 (Cornwall) and 22 (Devon) individuals tested

[f] A, number of alleles observed

[g] H_O, observed heterozygosity

[h] H_E, expected heterozygosity

[i] P_{HWE}, p value from testing Hardy–Weinberg equilibrium (HWE) and significant values after FDR correction are shown as underlined and bold

[j] Estimated null allele frequency >0.10 is shown as bold with >0.20 underlined (calculated using CERVUS)

[l] New primer sets redesigned from the hazel dormouse microsatellite sequences isolated by Naim et al. (2009)

[m] The difference between the expected and observed allele size is presumably a result of the different population origins of the individuals

facilitate multiplexing. Locus *MavC42* was not redesigned as it was monomorphic in our samples.

We identified 51 microsatellite sequences as unique, using BlastN 2.2.4 (Altschul et al. 1997). From these, fifty-three primer sets were designed from single-read sequences using PRIMER3 (Rozen and Skaletsky 2000). The 53 markers, plus the nine redesigned primer sets, were used to genotype dormice from two woodland sites, located in Cornwall and Devon, UK. Plucked hair was taken from dormice and stored dry at −20 °C. Genomic DNA was extracted using a Chelex method (Walsh et al. 1991). Primer sets were initially assessed for amplification by genotyping eight unrelated dormice from the Cornish woodland. Additional individuals from the Cornish site were genotyped for polymorphic loci only, totalling 26 unrelated individuals (males = 9, females = 10, unknown sex = 7). Loci monomorphic in the Cornish samples were tested in eight unrelated individuals from the Devon woodland and if polymorphic, characterised in further individuals from the Devon site, to total 22 genotypes per locus (males = 9, females = 12, unknown sex = 1).

Genotyping was performed in 2-µl PCR reactions; <10 ng lyophilised DNA, 0.2 µM of each primer and 1 µl Multiplex PCR Master Mix (QIAGEN Inc.; Kenta et al. 2008). Amplification was performed using a DNA Engine Tetrad PTC-225 thermal cycler (MJ Research, Bio-Rad, Hertfordshire, UK) with the following touch-down program: 95 °C for 15 min; followed by 13 cycles of 95 °C for 30 s, primer annealing for 30 s (decreasing by 1 °C every cycle from 67 °C to 55°) and 72 °C for 45 s; then 25 cycles of 95 °C for 30 s, 55 °C for 30 s and, 72 °C for 45 s, and a final step of 60 °C for 10 min. Amplified products were loaded on an ABI 3730 48-well capillary DNA Analyser and assigned allele sizes using GENEMAPPERv3.7 (Applied Biosystems, California, USA). Observed and expected heterozygosities, and estimated null allele frequencies were calculated in CERVUSv3.0.3 (Kalinowski et al. 2007). Tests for departures from Hardy–Weinberg equilibrium and assessment of linkage disequilibrium were conducted in GENEPOPv4.0.10 (Raymond and Rousset 1995; Rousset 2008). The False Discovery Rate correction for multiple tests (Verhoeven et al. 2005) was applied to p values obtained from Hardy–Weinberg equilibrium and linkage disequilibrium tests.

Of 62 loci tested in eight individuals, 23 amplified in <88 % of samples and/or were monomorphic in both sites. Of the remaining 39 loci (31 new plus eight redesigned), all except *Mav034* were polymorphic in the Cornish woodland; however *Mav034* was polymorphic in the Devon woodland. All 39 loci were confirmed to be autosomal, based on the presence of heterozygotes in both sexes (data not shown). EMBL accession numbers, primer sequences and characterisation data are provided in Table 1. In the Cornish samples, the number of alleles per locus ranged between two and nine (mean = 3.33, SD = 1.63), and mean observed and expected heterozygosities were 0.42 (SD = 0.23) and 0.44 (SD = 0.21), respectively. Nine loci deviated from Hardy–Weinberg equilibrium ($p \geq 0.05$) and eight showed high estimated null allele frequency (>0.1). After FDR correction, locus *Mav009* continued to show significant departure from Hardy–Weinberg equilibrium ($p > 0.05$). Twenty-six pairs of loci displayed linkage disequilibrium in the Cornwall population prior to correction for multiple tests. However, following FDR correction only one loci pair, *Mav017-Mav042*, displayed evidence of linkage disequilibrium.

Nineteen assembled contig microsatellite sequences (SeqMan NGen 1.2; DNAstar, Madison, WI) were also submitted to EMBL (Accession numbers: HE981150–HE981168). Additionally, 460 unused single-read sequences are supplied in Online Resource 1. Numerous sequences displayed regions of homology to parts of other hazel dormouse sequences or SINEs/retroposons in forest dormice (Dryomys nitedula; Kramerov et al. 1999; Veniaminova et al. 2007). Therefore, before use, sequences should be checked for full-length duplicates and any displaying homology with regions present in the genome in multiple copies (e.g. LINEs, SINEs) should be avoided as primer-bind regions.

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