

Isolation and characterisation of microsatellite loci in the papillose woolly bat, *Kerivoula papillosa* (Chiroptera: Vespertilionidae)

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Abstract The papillose woolly bat (*Kerivoula papillosa*) is one of the most well known species of an understudied bat genus that may be particularly vulnerable to disturbance and fragmentation events. We describe 22 novel microsatellite loci, 17 of which are polymorphic in *K. papillosa*, and one of which is polymorphic in a related species *K. pellucida*. When tested in a single population, none of the markers significantly deviated from Hardy-Weinberg expectations, showed the presence of null alleles or exhibited linkage disequilibrium. These markers will be useful in determining impacts of forest fragmentation on this species.

Keywords *Kerivoula* · Microchiroptera · Microsatellite · Population genetics · Vespertilionidae

Introduction

The woolly bats of the genus *Kerivoula* are an understudied animal group distributed within the Asian and African tropics, and are represented by ten Asiatic forest-dwelling species (Vanitharani et al. 2003). Taxonomic and ecological knowledge of the genus is limited because these species have been infrequently encountered historically.

The papillose woolly bat, *Kerivoula papillosa*, is the largest and most well known of the Asiatic *Kerivoula* and is distributed from India to Sulawesi (Vanitharani et al. 2003). Although it is moderately common in undisturbed forests (Kingston et al. 2003; Struebig et al. 2006), its adaptations to foraging and roosting in these habitats (Kingston et al. 2003) suggest that forest disturbance and fragmentation may pose serious threats. Microsatellite markers will prove useful for studying the conservation genetics of this species.

Genomic DNA was extracted from liver of a male *K. papillosa* individual collected from Krau Wildlife Reserve, Pahang, Peninsular Malaysia, using a salt precipitation method (Bruford et al. 1998). DNA was digested with *MboI* (QBIogene) and 200–800 bp restriction fragments used to produce two genomic libraries—one unenriched, and one enriched for dinucleotides and tetranucleotides ((AG)_n, (AC)_n, (AAAG)_n, (GTAA)_n, (GATA)_n, (CTAA)_n, (TAAA)_n and their complements), using the protocol of Armour et al. (1994) as modified by Gibbs et al. (1997). For both libraries, *MboI* DNA fragments were ligated into pUC19BamHI/BAP (QBIogene), and transformed into XL1-Blue competent cells (Stratagene).

From the unenriched library, two thousand recombinant clones were screened by hybridisation to the dinucleotide and tetranucleotide probes as listed above, along with (TTTC)_n. All probes were radiolabelled with [α ³²P]-dCTP

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Table 1 Characterisation of 22 papillose woolly bat, *Kerivoula papillosa*, microsatellite loci (17 polymorphic and 5 monomorphic loci)^a

Locus	EMBL accession number and clone name	Repeat motif and (5'-3')	Primers sequence (5'-3') with fluoro-label underlined, and 'pigtail' modifications, where used, in bold ^c	T _a (°C)	MgCl ₂ (mM)	Homo sapiens chromosome location ^c	E-value of chr. hit	n (known female & males)	A	Expected allele size (bp) ^d	Observed allele size range (bp)	H _o	H _e
Kpa02	AM157681, Ilc12	(GA) ₁₇ (GT) ₉ (GC) ₂	F: TAMRA-GAAAATCTTTTGGGGCTACTTT R: GTTT-GCAAGGCAACACAGGATTTTCA	59	2	No strong hits	-	65	10	195 (+4)	196-217	0.77	0.78
Kpa04	AM157683, 20g06	(CT) ₁₁	F: FAM-GATCCTCTCCCATCATTG R: GTTT-ATCCAGCTGTACCTGAAAGTC	55	2	No strong hits	-	69	9	158 (+4)	161-179	0.51	0.47
Kpa05	AM157684, 28h03	(CA) ₁₃ & (CA) ₄	F: HEX-GATCATCAAAAACGACACACA R: GTTCTT-TCCTTTAACTTTTGACTGTTAGAATC	65	3	Multiple hits	-	39 (11 & 28)	5	166 (+6)	161-173	0.67	0.65
Kpa08	AM157687, Flf05	(G) ₃ (A) ₃ (GACA) ₂ (GA) ₁₀	F: HEX-TGCCTATGGTAGTGGGAGTGAGG R: GTTT-GTCGGAGCTTCCTCGCATC	51	3	Chr. 6, 156761072	2.70E-15	64	12	218 (+4)	217-245	0.86	0.82
Kpa11	AM157690, Ilc09	(TC) ₄ & (T) ₅ (CT) ₃ CC (CT) ₃ & (T) ₅ (AT) ₃ CAT (A) ₇	F: FAM-TGTAAGCCAGGCAGTGTGG R: GTTT-AAGATTGCTGCTCCTTGGCATCC	50-65	2.0-3.0	Chr. 7, 100728190	6.30E-16	17 (3 & 14)	1	247 (+4)	257	0	0
Kpa13	AM157692, I3d01	(T) ₃ (CT) ₃ CT (C) ₃ (T) ₃ (CT) ₅ (A) ₃	F: HEX-CCTAATAGGGGGTGTACAGGAG R: GTTT-GGAGCCAGTCACAGAAAAGG	56	2	No strong hits	-	10 (4 & 6)	2	203 (+4)	173 & 177	0	0.19
Kpa16	AM157695, J3e04	(GA) ₃ (G) ₃ (A) ₃ GAAC (GA) ₅ GG (GA) ₇	F: FAM-TCAGTGCCATCAGGATTCAG R: ATCCCTGCCAGTTCAATCAG	50	2	No strong hits	-	68	5	221	215-239	0.5	0.55
Kpa17	AM157696, K1b10	G (A) ₂ (GA) ₃ (A) ₄ (GA) ₁₅ (A) ₂	F: HEX-CCCTAGTGAGGAATATTTGGGTTGC R: GTTT-CAGCAGGCCAATGCTCTATCC	57	2	No strong hits	-	10 (6 & 4)	1	248 (+4)	242	0	0
Kpa18	AM157697, L1a12	(CT) ₂ CACTCA (CT) ₁₄ TTT (CT) ₄ CC (CT) ₃	F: FAM-ACCCTCAGAACTGCTAAGTGC R: ATGACCTAGGTGGCTTGCAAT	50	2	Chr. 2, 145474382	6.70E-137	68	3	276	244-252	0.54	0.51

Table 1 continued

Locus	EMBL accession number and clone name	Repeat motif (5'-3')	Primers sequence (5'-3') with fluoro-label underlined, and 'pigtail' modifications, where used, in bold ^e	T _a (°C)	MgCl ₂ (mM)	Homo sapiens chromosome location ^c	E-value of chr. hit	n (known female & males)	A	Expected allele size (bp) ^d	Observed allele size range (bp)	H _o	H _e
Kpa19	AM157698, L1b10	(CT) ₂ & (CT) ₆ (C) ₃ (T) ₂ C (CT) ₂	F: TAMRA-CAATGATTCTCTCATCGCTGCTG R: GTTT-GGCACAGAGGAGCAGGTCAG	55	2	No strong hits	-	16 (4 & 11)	2	167 (+4)	169 & 171	0.06	0.18
Kpa20	AM157699, I3d05	CT (C) ₃ TCCT (GGT) ₃ GT (GA) ₄	F: TAMRA-GCAGCTGTACCCGACGTTGAT R: GTTT- ACTTAAGAGTGTACCCAGGAAGCTC	50-65	2.0-3.0	Chr. 17, 34606067	3.80E-61	19 (9 & 10)	1	230 (+4)	234	0	0
Kpa22	AM157701, Q0c01	(GT) ₁₈	F: HEX- AGAAAAATCCTATTTCAGAAAGGCAAG R: GCAGCTGATTGATGTTTCTCATCG	56	3	No strong hits	-	67	8	244	234-248	0.81	0.78
Kpa24	AM180141, Q0c03	(GA) ₁₉	F: FAM- AAGCCAGGATGCACAGGAG R: GGCCCATGTGCTCCCAATC	56	2	Chr. 7, 155028126	5.40E-11	69	9	228	217-237	0.88	0.78
Kpa26	AM180143, T0h03	(CT) ₁₅	F: FAM-TGGTCTCTTCACATGGATGG R: TTTACCCAAAGGACATGTTAAAGAG	55	2.5	No strong hits	-	69	11	123	108-164	0.59	0.66
Kpa27 ^e	AM180144, R0g09	(TTCC) ₁₃ & (TTCC) ₁₅	F: FAM-GGACACTCCTCAGAGCCACTGC R: CATTCTTTCACCCCTGAAGATGAAC	60	3	Chr. 3, 139212745 (& weaker hit to Chr. 21, 38428319)	3.3e-36 (5.7e-23)	66	13	292	244-300	0.91	0.91
Kpa29	AM180146, I5a06	(TC) ₄ & (TC) ₂ A (TC) ₄ & (TC) ₃	F: HEX-CGGGTTTGATTCGGTCAAG R: TTCTGTGTGATTCCTCACAAACAGGTTTC	65	2	Chr. 9, 8536168	1.20E-05	22 (10 & 11)	1	247	247	0	0
Kpa30	AM180147, I5a09	(GA) ₂ CA (GA) ₄ CA (GA) ₃ CA (GA) ₂₈	F: HEX-GATCCTAAGAAAGTCTTGAGACA R: GAGCAGAAAGGAAAAGGAATC	52	2	Chr. 14, 96309266	2.60E-51	62	19	182	149-187	0.82	0.92
Kpa32	AM180149, K3b11	(CA) ₂₁	F: TAMRA-ATCTGGTTCTCCCTACAGGTTG R: TCTTGAAACAGAACCCAGAGC	52	2	No strong hits	-	65	14	199	162-214	0.88	0.89
Kpa35	AM180152, R0c04	(GT) ₁₇	F: FAM-GCGGCAGGACTACCAACCAC R: CCACAGTTCCTTCCTGTACCAAC	55	2	No strong hits	-	66	12	193	189-212 ^f	0.64	0.73
Kpa36 ^{b, g}	AM408241, 122B07	(CA) ₁₁	F: HEX-CCCAACCAACTAAGCCACAT R: GTTT-CATGCAAAAGGACTGACTGA	54	3	No hits	-	13 (4 & 9)	1	109 (+4)	115	0	0
Kpa46 ^{b, g}	AM408251, 125A11	(GATA) ₄ GGTA (GATA) ₇	F: HEX-CCCAAAAGGACACTTTGCAT R: GTTT-CCTTTGAAAAGGGCAGAGC	65	2	No strong hits	-	64	5	169 (+4)	158-172	0.67	0.65

Table 1 continued

Locus	EMBL accession number and clone name	Repeat motif (5'–3')	Primers sequence (5'–3') with fluoro-label underlined, and 'pigtail' modifications, where used, in bold ^e	T _a (°C)	MgCl ₂ (mM)	Homo sapiens chromosome location ^c	E-value of chr. hit	n female & males	A	Expected allele size (bp) ^d	Observed allele size range (bp)	H _o	H _e
Kpa47 _g	AM408252, 126D12	(TCTA) ₄ (TA) ₂ (TCTA) ₃ & (CA) ₁₄	F: FAM-GGACCCCTTAAGTGCATGA R: GTTCTT-TGGATGGGATTGACATGAGA	64	2	No strong hits	–	67	8	163 (+6)	145–171	0.82	0.77

T_a, Optimum annealing temperature and MgCl₂ concentrations were initially identified by genotyping 24 individuals at 50–65°C and with 1.5–3.0 mM of MgCl₂

A, Number of alleles observed; H_o, observed heterozygosity, H_e, expected heterozygosity

n, Number of genotyped papillose woolly bat, *Kerivoula papillosa* individuals from a single population inhabiting Krau Wildlife Reserve, Malaysia, that gave a scorable product (a minimum of 10 known sex females and 10 known sex males were genotyped unless stated). Note: *Kpa20*, *Kpa46*, *Kpa47* were genotyped in a different selection of *K. papillosa* individuals from the same population

^a Untested primers pairs designed for four additional clones that might prove useful for future studies are: ACAGGAGGAGCCCAATCAAT and GTACGTCCCTTGACTGGAA (locus *Kpa14*, clone accession number AM157693; expected size 188 bp), AAAACCTTTTGACCCCTTT and ATCCCAGCGACCTACTCCTT (*Kpa25*; AM180142; 213 bp), GAT-GCATATTGGCCTTGC and GGATGTTGCCAAAACATCAA (*Kpa28*; AM180145; 240 bp) and AACGACACTCAATGGTGGAA and TTTTGGAGCCAGGAGTCTGT (*Kpa33*; AM180150; 228 bp)

^b Loci were developed from the enriched library. All other loci were isolated from the unenriched library

^c human chromosome location obtained by performing a WU-BLAST of the ENSEMBL *Homo sapiens* genome sequence using the distant homologies settings (see <http://www.ensembl.org/>).

Kpa11 and *Kpa24* were both assigned to human chromosome 7

^d Expected allele size based on sequence of the cloned allele not including length of pigtail. The additional length of the pigtail is presented in brackets

^e 'Pigtail' modifications added to the reverse primer to reduce noise from variable adenylation during the PCR (Brownstien et al. 1996). Observed allele sizes therefore include the 4 or 6 bp length of the pigtail

^f For locus *Kpa35*, several alleles vary by only 1-bp differences, therefore care should be taken when scoring this locus. This locus had a high estimated null allele frequency (+0.08)

^g Reverse compliment of sequence submitted to EMBL database

except (TAAA)_n, which was radiolabelled with [α -³²P]-dATP. Sixty-one (3%) positive clones were identified, of which thirty-five were sequenced (EMBL Accession numbers AM157680–157702 and AM180142–180152). Twenty-nine primer pairs were designed. From the enriched library, 384 clones produced 25 positives (7%). Twelve enriched positives were sequenced (AM408241–408252) and seven primer pairs were designed. In total, 36 primer pairs were designed, all using Primer3 software (Rozen and Skaletsky 2000). The 5' end of the forward primer of each pair was fluoro-labelled and for some loci 'pigtail' modifications were added to the reverse primer (Table 1) to reduce noise from variable adenylation during the PCR (Brownstein et al. 1996).

Primers were initially tested for amplification and polymorphism using 24 individuals from a single population at Krau Wildlife Reserve. To identify optimal PCR conditions, loci were tested with a range of twelve different annealing temperatures and four different MgCl₂ concentrations on a T gradient thermocycler (*Biometra*). Each 10- μ L PCR reaction contained 1.5 pmol of each primer, 0.15 mM of each dNTP, X mM MgCl₂ (X was either 1.5, 2.0, 2.5 or 3.0 mM) using 0.5–1.0 unit Gold *Taq* polymerase (PE Applied Biosystems) in the manufacturer's buffer. The following PCR thermo-cycling profile was used on a DNA Engine Tetrad Thermal Cycler (MJ Research): 95°C for 15 min; followed by 36 cycles of 95°C for 30 s, Y°C annealing temperature (Y was between 50–65°C for 30 s, and 72°C for 30 s; followed by 72°C for 10 min.

Allele sizes were assigned on an ABI 3700 Sequencer using GENOTYPER 3.6 NT software (PE Applied Biosystems). Primer pairs that yielded scorable products and amplified at least 3 alleles in a minimum of ten individuals were used to genotype between 39 and 69 *K. papillosa* individuals, most of known sex from Krau Wildlife Reserve, to assess genetic diversity more fully. A minimum of 10 individuals of each known sex were included. PCR reactions were performed as before but using the optimum MgCl₂ and annealing temperatures previously identified (Table 1). Null allele frequencies and observed and expected values of heterozygosity were calculated using CERVUS version 2.0 (Marshall et al. 1998). Tests for linkage disequilibrium and departures from Hardy-Weinberg equilibrium were performed using a Markov-chain method implemented in GENEPOP v3 (Raymond and Rousset 1995).

Of the 36 primer pairs optimised, 22 produced scorable products (Table 1). Seventeen markers were polymorphic and the number of alleles when genotyped in 10–69 individuals ranged from 2 to 19 (Table 1). Null allele frequency estimates were less than 0.05 for all but three loci (*Kpa26*, 0.07; *Kpa30*, 0.06; and *Kpa35*, 0.08) and none of the loci significantly deviated from Hardy-Weinberg

expectations after Bonferroni correction for multiple comparisons. Marker independence was indicated by the absence of significant linkage disequilibrium for any pair of loci after Bonferroni correction but these tests are limited, since some loci were tested in few individuals and *Kpa20*, *Kpa46* and *Kpa47* were genotyped in a different selection of individuals.

Six of the seven loci with ≤ 2 alleles in *K. papillosa* were tested in ten individuals of a related species, *Kerivoula pellucida*. Four loci did not amplify and one locus was monomorphic. However, locus *Kpa17* was polymorphic with 6 alleles (250–285 bp), suggesting that several of the other 16 loci will prove useful for genotyping related species.

All 22 loci amplified in females (XX), so none are Y linked. When the sequences for the 22 loci were compared to the human genome sequence, 8 loci were assigned an autosomal chromosome location (E-val $< 10^{-5}$ or better) but no loci were assigned to either of the human sex chromosomes (Table 1). Of the fifteen polymorphic loci tested in a minimum of 10 male (XY) individuals, all loci amplified some heterozygotes, suggesting none were X-linked (Table 1). It was not possible to test reliably for sex linkage at the other loci.

The polymorphic loci will be used to study the population genetic structure and dispersal of *K. papillosa* bats across continuous and discontinuous forest landscapes in Malaysia. Many loci should also prove useful for genetic studies of the nine other *Kerivoula* species and for perhaps some of the more closely related vespertilionid bat species.

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