Development and characterization of 11 polymorphic compound tetranucleotide microsatellite loci for the Leisler's bat, Nyctalus leisleri (Vespertilionidae, Chiroptera)

Boston, V., Montgomery, I., & Prodohl, P. (2009). Development and characterization of 11 polymorphic compound tetranucleotide microsatellite loci for the Leisler's bat, Nyctalus leisleri (Vespertilionidae, Chiroptera). Conservation Genetics, 10(5), 1501-1504. DOI: 10.1007/s10592-008-9768-x

Published in:
Conservation Genetics

Conserv Genet
DOI 10.1007/s10592-008-9768-x

TECHNICAL NOTE

Development and characterization of 11 polymorphic compound tetranucleotide microsatellite loci for the Leisler's bat, *Nyctalus leisleri* (Vespertilionidae, Chiroptera)

Emma Boston · Ian Montgomery · Paulo A. Prodöhl

Received: 2 November 2008 / Accepted: 19 November 2008

Abstract Eleven polymorphic microsatellite marker loci were developed from a Leisler's bat (*Nyctalus leisleri*) genomic enriched library. Assessment of the usefulness of these markers for population genetics studies of Leisler's bats was carried out by screening 100 specimens sampled from eight locations in Ireland and two in Northeastern France. Both moderately and highly polymorphic marker loci were identified. Five to 28 alleles were found to be segregating per locus with observed heterozygosities values ranging from 28.4 to 94%. Initial evaluation indicates that these microsatellites will be useful for genetic based studies aiming, for instance, at parentage and population structure of Leisler's bats.

Keywords Leisler's bat · *Nyctalus leisleri* · Chiroptera · Polymorphic microsatellite

Introduction

The Leisler's bat (*Nyctalus leisleri*) is a medium sized aerial hawking bat, patchily distributed across Europe and Western Asia, where is found both in woodland and urban areas. Except in Ireland, where it is still comparatively common, Leisler's bats are rare throughout the rest of its distribution range (Hayden and Harrington 2000). Indeed, the species is considered near threatened (IUCN list 2007), and is listed in Annex IV of the Habitats Directive and Appendix II of the Bern Convention as a species requiring strict protection measures. In contrast to other species of the genus (e.g. *N. noctula*), *N. leisleri* has been relatively understudied (Mitchell-Jones et al. 1999). Thus, little is known of the biology, behavioural ecology, and population genetic structure of the species. Given the conspicuous nature of the species, it is difficult to gather such information from standard field ecological studies. The application of microsatellite DNA markers has been revolutionizing the understanding of the biology and population evolutionary dynamics of species (Avise 2004). Although a number of microsatellite markers have been developed for investigations of population and evolutionary genetics of Microchiropteran bats (e.g. Burland et al. 1998; Rossiter et al. 1999), no species specific microsatellite primers are currently available for the Leisler's Bat.

Here we describe the development and initial characterisation of 11 novel informative microsatellite loci for *N. leisleri*. The strategy used for the cloning and identification of microsatellites from an enriched library for the *N. leisleri* was based on the protocol described by Kijas et al. (1994) with modifications. Genomic DNA was extracted from wing tissue from six *N. leisleri* museum specimens following protocol described by Taggart et al. (1992). Three aliquots consisting of ≈10 μg of pooled genomic DNA from these six individuals were fully restricted with *Hae*III, *Rsa*I and *Alu*I (Promega), purified using phenol/chloroform (1× with equal volume of phenol and chloroform), ethanol precipitated, re-suspended in 10 μl TE pH 8.0 buffer, and subsequently pooled together. The resulting 30 μl of pooled restricted fragments was size fractionated in 1.5% 1× TBE agarose gel using a 100 bp (base pairs) ladder for DNA size marker. Following electrophoresis, two pieces containing DNA fragments ranging

E. Boston · I. Montgomery · P. A. Prodöhl (✉)
School of Biological Sciences, Queen's University Belfast, MBC 97 Lisburn Road, Belfast BT9 7BL, N. Ireland
e-mail: p.prodohl@qub.ac.uk

E. Boston
School of Biology & Environmental Science, Science Centre West, University College Dublin, Belfield, Dublin 4, Ireland

Published online: 10 December 2008

from 150 to 300 bp and from 300 to 800 bp were excised from the gel, and the resulting DNA fragments were recovered using the Montage DNA gel extraction kit from Millipore.

The purified size selected fragments were blunt-ligated overnight at 14°C into *Eco*RV digested and dephosphorylated pBluescript phagemid II (Stratagene), using T4 DNA ligase (Pharmacia). Resulting ligations were used as templates for four asymmetric PCR reactions. PCR reactions were carried out in 50 µl volumes containing 1× Promega *Taq* polymerase buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 50 pM T3 universal primer, 5 pM T7 universal primer, 1 U of Promega *Taq* DNA polymerase, and 3 µl of blunt-ended ligated DNA. Aliquots consisting of 25 µl of each one of the four asymmetric PCR reactions were diluted to 75 µl with H_2O, incubated for 10 min at 98°C in a thermoblock, and left to sit on ice for 1 min. Resulting single stranded DNA were allowed to hybridise to 35 µl of a cocktail containing $(GATA)_4$, $(GACA)_4$, $(GGAT)_4$, $(GAA)_5$, and $(GGA)_5$ single stranded biotynilated oligonucleotides bound to streptavidin-coated magnetic beads (Dynal Inc.) suspended in 10× SSC buffer (1.5 M NaCl, 0.15 M Na citrate, pH 7.0). Subsequent low and high stringency washes, and purification of microsatellite enriched fractions followed procedure described by Kijas et al. (1994).

The purified microsatellite enriched fragments were used to seed symmetric PCR reactions to recover double stranded DNA. PCR reactions were carried out in 100 µl volumes containing 1× Promega *Taq* polymerase buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 70 pM of each T3 and T7 universal primers, 2 U of Promega *Taq* DNA polymerase and 25 µl of the microsatellite-enriched DNA fractions. PCR cycling conditions consisted of one cycle at 94°C for 3 min, followed by 32 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Resulting PCR products were purified using Promega Wizard PCR kits and fully digested with *Eco*RI and *Hin*dIII following manufactures recommendation (Promega). Digested products were purified again using the Promega Wizard PCR kits, and ligated overnight at 4°C into the *Eco*RI/*Hin*dIII digested and dephosphorylated pBluescript phagemid II (Stratagene), using T4 DNA ligase (Pharmacia).

Resulting ligation products were transformed into *Epicurian coli* XL1-Blue supercompetent cells (Stratagene). Transformed cells were plated onto LB agar plates containing 60 mg ml^{-1} ampicillin and spread with 80 µl 50 mg ml^{-1} X-GAL and 32 µl 100 mM IPTG. Following overnight growth at 37°C, the resulting white cells were transferred (manual picking) to new LB gridded plates containing 60 mg ml^{-1} ampicillin, allowed to re-grow overnight at 37°C and blotted into Hybond-N+ nylon membranes (Amersham Biosciences) for subsequent screening. Following blotting (according to manufacturer's recomendation), cells were allowed to recover at 37°C for 5 h and stored at 4°C.

Screening of ~7,000 phagemid constructs was carried out following protocol described by Prodöhl et al. (1996), using a probe cocktail containing $(GATA)_4$, $(GACA)_4$, $(GGAT)_4$, $(GAA)_5$, and $(GGA)_5$ ^{32}P end-labelled oligonucleotides. Two hundred eighty-two positives were identified, and sent for commercial sequencing (Macrogen Inc.) on both directions using the T3 and T7 universal sequencing primers. Of these, 151 were found to contain sufficient large unique flanking region for primer design. PCR primer sets were designed for 84 (55%) microsatellites containing flanking regions using PRIMER SELECT (DNASTAR).

All primer sets were initially tested on genomic DNA from five individual Leisler's bats to check out for amplification of products of expected size, and for initial optimisation of PCR conditions. This was carried out in 12 µl reaction volumes comprised of 1× Promega *Taq* polymerase buffer, 1.5 mM $MgCl_2$, 100 µM dNTP, 10 pM of each microsatellite primer, 100 ng template DNA, and 0.5 U of Promega *Taq* DNA polymerase. PCR cycling conditions consisted of one cycle at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, optimum annealing temperature (varying according to specific primer pair) for 1 min, and 72°C for 1 min. Resulting PCR products were visualised on Ethidium Bromide stained (0.5 µg ml^{-1}) 2.5% 1× TBE agarose gels.

From the initial primer sets tested, 11 (7%) were found to consistently amplify PCR products of the correct size, display reduced level of stuttering, and based on the limited screening of samples were shown to be polymorphic. These (Table 1) were subsequently optimised on a Li-Cor™ dual laser automated DNA analyser, and screened for a sample set comprised of 100 bats. These bats been previously sampled using non-destructive wing biopsy following the protocol described by Worthington-Wilmer and Barrett (1996), as part of ongoing investigation on the population genetic structure and phylogeography of the species in Ireland (E. Boston et al. in prep). This sample set consisted of ten individual bats from each of eight geographic locations, within Ireland and from two locations in Northeastern France. Single locus PCR amplifications for genotyping in the Li-Cor system were carried out in 12 µl reaction volumes containing 1× Promega *Taq* polymerase buffer, 1.5 mM $MgCl_2$, 100 µM dNTP, 0.5–6 pM of each microsatellite primer (see Table 1 for specific details for each microsatellite), 100 ng of template DNA, and 0.5 U of Promega *Taq* DNA polymerase. PCR cycling conditions consisted of one cycle at 94°C for 3 min, followed by 24–26 cycles of 94°C for 1 min, 50–54°C for 1 min, and 72°C for 1 min. Following PCR, 4 µl of stop solution (95% formamide, 10 mM NaOH, 10 mM EDTA, 0.01%

Table 1 Details for each of the 11 Irish Leisler's bat microsatellite marker locus developed in this study including: locus ID; repeat motif; primer sequences; primer (PicoMoles) and $MgCl_2$ (mM) concentrations; Tm—annealing temperature (°C); number of PCR cycles; allele size range (base pairs); n*A*—number of alleles; Ho and He—observed and expected heterozygosity

Locus ID	Repeat motif	Primer sequences	Primer (pM)	$MgCl_2$ (mM)	Tm (°C)	No cycles	Allele size range (bp)	n*A*	Ho	He
*Nleis*1	$(GATA)_7$ $(GACA)_3$ GACTAGAGA $(GATA)_{10}$ $(CAGA)_5$	F: CGTATAGGAGGCAGCCAATCAAT	0.5	1.5	57	23	196–244	14	0.804	0.837
		[a]R: ATCTACCGGTCAAACAGTCG								
*Nleis*2	$(GATA)_{10}$	[a]F: TGGTCAGCGCACATCATA	0.4	1.5	50	24	166–148	10	0.684	0.711
		R: GACCTGCAGCTCTACTTG								
*Nleis*3	$(GATAGAT)_3$ $(GATA)_{11}$	F: TCAATCAAGTTCCTTTGTTAGTTA	1.8	1.5	50	24	195–233	29	0.823	0.89
		[a]R: ATAATCATGTGAGCCAGTTCC								
*Nleis*4	$(GA)_3$ $(GACA)_5$	[a]F: TGGTTCGATTCCCGGTCAGACACA	0.5	1.5	52	26	192–210	10	0.521	0.833
		R: GATGGGGCGCAGGTGCTTGTTTT								
*Nleis*5	(GACA)5	[a]F: GTGGCTGCTGGGAGGAAACT	1	1.5	57	24	116–132	5	0.284	0.384
		R: TGAGCGCAGGAAGCCAGAA								
*Nleis*6	$(GATA)_4$ GGTA $(GATA)_9$ GAT $(GATA)_5$ GAT $(GATA)_8$	F: CTCTTAAGCCTTGCCTCCTCTGTG	0.5	1.5	56	20	190–290	27	0.949	0.927
		[a]R: ATCTGCCCCCTAGTGGTCAATGT								
M13 tailed primers										
*Nleis*7	$(GGA)_{10}$	F: M13For-CTCACCAGGCACCAAGTC	1	2.5	50	28	113–137	12	0.343	0.727
		IRD-M13For	1.5							
		R: CCGTCACAACAGGAAATAAGAGAT	1.5							
*Nleis*8	$(GAT)_3$ $(GATA)_7$ AATA $(GATA)_2$	F: M13Rev-TTCCCCAGCTTGCTAACTGC	0.3	2.5	51	27	155–193	28	0.712	0.758
		IRD M13Rev	0.5							
		R: TCCAGGGTGCTCCAGGATAA	0.5							
*Nleis*9	$(GATA)_6$ GATGTTA $(GATA)_{11}$	F: AGGGGGCAGTCGATAATGATT	1	2.5	50	27	224–296	27	0.591	0.847
		R: M13For-ACATGCCAACTTGTTTTTAGAAG	0.7							
		IRD-M13For	1							
*Nleis*10	$(TGAA)_6$ $(TGGATGAA)_2$ TGGG $(TGGA)_6$	F: M13For-TGGTTAGCGTCCTCCAGTGT	0.5	2.5	55	28	145–173	8	0.676	0.74
		IRD-M13For	0.8							
		R: CTGCTTAGAACCCAGCACAG	0.8							
*Nleis*11	$(GCA)_{13}$	F: M13For-TCACTGTGAGTAGCAAGACAATAGAT	0.5	2.5	55	28	160–184	15	0.574	0.816
		IRD-M13For	0.8							
		R: GATCTTTCCAGTTGTCTGGTG	0.8							

[a] Fluorescent labelled IRD primer. In five instances screening was carried out using M13 tailed primers. In these cases, one of the species-specific primers was synthesised with either M13 Forward (For) or M13 Reverse (Rev) universal sequences at the 5′-sequence end. During PCR, three primers were used to amplify the particular locus consisting of: the tailed-species-specific primer, the other of the pair of the species-specific primer, and a fluorescent IRD-labelled M13 For or M13 Rev primer. The respective concentration of these primers used in each case are provided

pararosaniline) was added to each 12 µl reaction. Reactions were denatured at 80°C for 3–4 min, and 1 µl was loaded into 25 cm 6% 1× TBE polyacrylamide gels containing 5.6 M Urea mounted on the Li-Cor system. A commercially available size ladder for the Li-Cor system (i.e. MicroStep-20a from Microzone, UK) was run adjacent to the samples to size allelic fragments. Gels were run on the Li-Cor system at a constant power of 40 W and at a temperature of about 50°C for 1–2 h. Genotypic typing was carried out using GENE PROFILER 4.05 software (Scanalytics Inc., Fairfax, VA, USA). Control samples of known allele size were used on each run to ensure typing consistence. All genotypic data was assembled into an Excel database for subsequent analyses.

All 11 Irish Leisler's bat microsatellite marker loci resolved with exceptional clarity with very little ambiguity in the identification of specific allelic fragments. Most marker loci developed in here can be readily used in multiplex reactions greatly simplifying the screening procedure. Standard genetic indices were calculated using FSTAT (Goudet 1995). All loci were polymorphic, with 5–28 alleles per locus and overall observed and expected heterozygosities ranging from 28.4–94% to 38.4–92.7%, respectively (Table 1). Tests for deviation from Hardy–Weinberg equilibrium (HWE) and non-random association of alleles among different loci (i.e. linkage disequilibrium) were carried out with GENEPOP version 3.4 (Raymond and Rousset 1995). Although no linkage disequilibrium between loci was detected after Bonferroni correction, all loci showed significant departure from HWE ($P < 0.05$). This was not surprising, given the mixed nature of the sample used in here. Departures of HWE can be attributed to several factors including null alleles. Preliminary results of ongoing genetic population survey indicate that null alleles indeed occur in at least two maker loci (*Nleis*4 and *Nleis*7), yet considerable levels of population structure exists within this species. Results highlight the potential usefulness of these markers loci for further investigation on the population genetics, social structure and parentage in the Leisler's bat.

Acknowledgments We thank Ulster Museum, Northern Ireland Bat Group, Ian Forsythe and Pamela Allen, for the donations of samples. Susie Brown and Stephan Rouè and colleagues for their help with extensive field work both in Ireland and France. This research was supported by the Northern Ireland Environmental Agency and the National Parks and Wildlife Service, Republic of Ireland. E. Boston was supported by and PhD studentship from the Department of Employment and Learning of Northern Ireland.

References

Avise JC (2004) Molecular markers, natural history, and evolution, 2nd edn. Sinauer Associates Inc., Sunderland, p 541

Burland TM, Barratt EM, Racey PA (1998) Isolation and characterization of microsatellite loci in the brown long-eared bat, *Plecotus auritus*, and cross-species amplification within the family Vespertilionidae. Mol Ecol 7:136–138

Goudet J (1995) FSTAT (version 1.2): a computer program to calculate F-statistics. J Hered 86(6):485–486

Hayden T, Harrington R (2000) Exploring Irish mammals. Duchas The Heritage Service, Town House Dublin, pp 59–62

Kijas JMH, Fowler JCS, Garbett CA, Thomas MR (1994) Enrichment of microsatellites from citrus genome using biolynilated oligonucleotide sequences bound to streptavidin-coated magnetic particles. Biotechniques 16:657–662

Mitchell-Jones AJ, Amori G, Bodanowicz W, Krištufek B, Reijindus PJH, Psitzenberger F, Stubbe M, Thissen JBM, Vohralik V, Zima J (1999) The atlas of European mammals. Academic Press, London

Prodöhl PA, Loughry WJ, McDonough CM, Nelson WS, Avise JC (1996) Molecular documentation of polyembryony and the micro-spatial dispersion of clonal sibships in the nine-banded armadillo, *Dasypus novemcinctus*. Proc R Soc Lond B Biol Sci 262:1643–1649. doi:10.1098/rspb.1996.0240

Raymond M, Rousset F (1995) Genepop (version 1.2)—population genetics software for exact tests of ecumenicism. J Hered 86:248–249

Rossiter SJ, Burland TM, Jones G, Barratt EM (1999) Characterization of microsatellite loci in the greater horseshoe bat *Rhinolophus ferrumequinum*. Mol Ecol 8:1959–1961. doi:10.1046/j.1365-294x.1999.00778-2.x

Taggart JB, Hynes RA, Prodöhl PA, Ferguson A (1992) A simplified protocol for routine total DNA isolation from salmonid fishes. J Fish Biol 40:963–965. doi:10.1111/j.1095-8649.1992.tb02641.x

Worthington-Wilmer J, Barrett E (1996) A non-lethal method of tissue sampling for genetic studies of chiropterans. Bat Res News 37:1–3