Russian J. Theriol. 15(2): 81–83

New microsatellite markers for *Myotis daubentonii* and *Eptesicus nilssonii* (Vespertilionidae, Chiroptera)

Dmitriy G. Smirnov*, Sergey V. Titov & Farid Z. Baishev

ABSTRACT. New microsatellite markers were developed and characterized for two vespertilionid species: three loci for *Myotis daubentonii* (MmCCA, MdTTA, MdTTTA) and two loci for *Eptesicus nilssonii* (EnTCA, EnTCTA). The usefulness of these markers were assessed by screening a sample of wing membrane biopsy of 136 specimens of *M. daubentonii* and 206 specimens of *E. nilssonii*, collected in Samara Province, Penza Province and Khanty-Mansi Autonomous Area (Russia). All the loci were highly polymorphic. Both moderately and highly polymorphic loci were identified with 3-15 alleles segregating per locus (mean in *M. daubentonii* 3.45±0.29, in *E. nilssonii* 6.07±0.38). The minimal heterozygosity was found in locus MmCCA (H_O=0.284±0.034, H_E=0.305±0.035), while all the rest of loci had moderate and high heterozygosity. These polymorphic markers will provide a valuable tool for assessment of population genetics of these two species.

KEY WORDS: bats, Chiroptera, microsatellite, *Myotis daubentonii, Eptesicus nilssonii*.

Dmitriy G. Smirnov [eptesicus@mail.ru], Sergey V. Titov, Farid Z. Baishev, Department of Zoology and Ecology, Penza State University, Krasnaja str. 40, Penza 440026, Russia.

Новые микросателлитные маркеры для *Myotis daubentonii* и *Eptesicus nilssonii* (Vespertilionidae, Chiroptera)

Д.Г. Смирнов, С.В. Титов, Ф.З. Баишев

Разработаны и апробированы три микросателлитных маркера для *Myotis daubentonii* (MmCCA, MdTTA, MdTTTA) и два для *Eptesicus nilssonii* (EnTCA, EnTCTA). Эффективность маркеров оценивали путем скрининга образцов ткани (биопсия перепонки крыла) от 136 особей *M. daubentonii* и 206 особей *E. nilssonii*, добытых с территорий Самарской, Пензенской областей и Ханты-Мансийского автономного округа России. По всем микросателлитным локусам выявлен высокий полиморфизм. В локусах идентифицировано от 3 до 15 аллелей, где среднее значение для *M. daubentonii* составило 3.45±0.29, а *E. nilssonii* — 6.07±0.38. Минимальная гетерозиготность была обнаружена в локусе MmTGG (H_O=0.284±0.034, H_E=0.305±0.035), тогда как по остальным локусам гетерозиготность оценена как средняя или высокая. Результаты анализа показали, что разработанные маркеры могут быть ценным инструментом и успешно применяться при изучении генетической структуры популяций исследованных видов.

КЛЮЧЕВЫЕ СЛОВА: летучие мыши, Chiroptera, микросателлитные маркеры, *Myotis daubentonii, Eptesicus nilssonii*.

Daubenton's bat *Myotis daubentonii* (Kuhl, 1817) and northern bat *Eptesicus nilssonii* (Keyseling et Blasius, 1839) are widespread in Europe. They are nonmigratory bats and thus are convenient models for studying genetic processes in local populations. During the last decade *M. daubentonii* was one of the most popular subjects of such studies. As a result, several microsatellite loci were isolated from the species (Senior *et al*., 2005; Ngamprasertwong *et al*., 2008; Atterby *et al*., 2010; Smith *et al*., 2011; Laine *et al*., 2013), part of which were initially were developed for different bat species (Petri *et al*., 1997; Burland *et al*., 1998; Castella & Ruedi, 2000; Kerth *et al*., 2002; Jan *et al*., 2012). However, despite the number of available markers, wider range of loci is desirable for a number of reasons, including increased power for studies involving individual and family identification, less problems related to "null alleles" and low genetic diversity (Scott *et al*., 2013). Also, a number of available microsatellite sequences have repetitions, comprised of two nucleotides, or containing palindromic sequences, or non-systemic insertions. The above factors make use of these sequences problematic and unproductive for allele divergence studies. Microsatellite markers for *E. nilssonii* are described for the first time.

Tissue samples for the study were obtained from 136 individuals of *M. daubentonii* from 14 geographically distant locations and 206 individuals of *E. nilssonii* from 23 geographically distant locations. Bats were caught in Samara Province, Penza Province and Khan-

* Corresponding author

ty-Mansi Autonomous Russia (Russia). All the animals were released at the same point where they were caught after measurements and wing membrane biopsy were taken. Sampling in animals was approved by an institutional animal care and use committee (Sikes *et al*., 2011).

DNA was extracted from a wing membrane biopsy punch (d=5mm) stored in 96% ethanol. DNA was isolated on standard methods including homogenization of biomaterial, washing with STE buffer, processing with detergent (SDS, 10%, pH 7.2), incubation with proteinase K at 50°C over a night, further purification with phenol and chloroform-isoamyl alcohol mix (29:1). Soluted DNA was precipitated with cold absolute ethanol in presence of sodium acetate (3M) (Sambrook *et al*., 1989).

Microsatellite primers sequences were developed based on the following genomic sequences obtained from the NCBI database: AF203647, AAPE02000001, AAPE02000007, AAPE02000009, AAPE02000023, ALEH01000006, ALEH01000516, and ALEH01011372. Initially there were 12 primer pairs designed and optimized, only five of which produced acceptable for population studies allele polymorphism (Tab. 1).

Polymerase chain reaction (PCR) was carried out using 0.6 μm of each primer, 50 μm Tris-HCl (pH 8.9), 20 μm ammonium sulfate, 20 μm EDTA, 170 μg/ml bovine serum albumin (BSA), dNTPs cocktail (200 μm of each), 2 μm of magnesium chloride, 0.1–0.2 μg of DNA and 2 units of *Taq* polymerase. The thermal profile for all loci was an initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, annealing at 60°C (primers MmCCA, MdTTA, EnTCA, and EnTCTA) or 62°C (primer MdTTTA) for 30 s, and extension at 72°C for 1 min. Cycling was followed with terminal extension at 72°C for 2 min (Tab. 1).

PCR products were subjected to electrophoresis in 8% polyacrylamide gel using Tris-EDTA-borate buffer system. After electrophoresis gels were stained in ethidium bromide solution and visualized in UV-light, graphic images were obtained using BioRad Universal Hood II Imaging system. DNA of *Escherichia coli* plasmid pBR322, restricted by endonuclease *Hpa*II was used as a molecular weight marker.

Inserts were amplified using PCR followed by sequencing using BigDye v.3.1 dye terminator technology sequenced on an ABI PRISM 3500 DNA Analyser (Applied Biosystems).

Statistical analysis of DNA molecular polymorphism was performed using GenAlEx v.6.0 for MS Excel with identification of observed (H_O) and expected (H_E) heterozygosity and tested for departure from Hardy-Weinberg equilibrium. Populations were tested for deviations from equilibrium at each locus and across all loci in Arlequin v.3.1 (Excoffier *et al*., 2005). Bonferroni corrections were applied for multiple comparisons.

In *M. daubentonii* out of 7 analyzed loci only three were polymorphic, in *E. nilssonii* out of 5 loci only two were polymorphic. The numbers of discovered alleles as well as expected and observed heterozygosity are summarized in the Table 1.

In *M. daubentonii*, polymorphic loci were with 3-6 alleles (average 3.45±0.29) and in *E. nilssonii* with 9-15 alleles (average 6.07±0.38). In *M. daubentonii* observed heterozygosity (H_O=0.222–0.833) was wider than expected (H_E=0.333–0.583). In *E. nilssonii* observed heterozygosity (H_O=0.143–0.893) was relatively close to expected (H_E=0.403–0.847). Obtained values of observed and expected heterozygosity allow to define certain peculiarities in genetic structure of the studied species. The observed heterozygosity equilibrium for loci MmCCA, MdTTTA, EnTCA, and EnTCTA shows the heterozygote deficiency. Heterozygosity values for MdTTA, in contrary, shows their abundance. The discrepancy between expected and observed heterozygosity in studied loci in *M. daubentonii* could be explained by relatively high gene flow between local populations (migrations) and possible intrapopulation subsidiarity. In general, minimal heterozygosity was observed in locus MmCCA, while in other loci it was moderate or high. Similar results that show discrepancy between expected and observed heterozygosity have been described in other bat species (Vonhof *et al*., 2002; Trujillo & Amelon, 2009; Laine *et al*., 2013).

All loci except MmCCA in one sample, MdTTTA and EnTCTA in three samples, and EnTCA in six samples conformed to Hardy-Weinberg expectations. We found no evidence of linkage disequilibrium between loci among all samples in *E. nilssonii*. In *M. daubentonii* only loci MmCCA-MdTTA from one sample violated HWE (χ^2=10.4, 2 df, p=0.006).

The described microsatellite markers allow detailed population genetic studies in *M. daubentonii* and *E. nilssonii*.

ACKNOWLEDGEMENTS. The research was supported by Russian Foundation for Basic Research (grant 15-04-01055a) and by Ministry of Education and Science of the Russian Federation (project 1315 to the Penza State University for scientific research in 2014-2016). We would like to express our gratitude to Dr. K. Tsytsulina for the help in a translation of article into English. We are grateful to Dr. Marina Kholodova and Dr. Sergei Kruskop for their helpful comments on the early version of the manuscript.

References

Atterby H., Aegerter J.N., Smith G.C., Conyers C.M., Allnutt T.R., Ruedi M. & MacNicoll A.D. 2010. Population genetic structure of the Daubenton's bat (*Myotis daubentonii*) in western Europe and the associated occurrence of rabies // European Journal of Wildlife Research. Vol.56. P.67–81.

Burland T.M., Barratt E.M. & Racey P.A. 1998. Isolation and characterization of microsatellite loci in the brown long-eared bat, *Plecotus auritus*, and cross-species am-

Table 1. Characterization of studied polymorphic microsatellite loci. In the H_O/H_E column, the figure above the line indicates the values of the observed heterozygosity (H_O), below the line — value of expected heterozygosity (H_E).

Locus	Primer sequence (5'–3')	Repeat motif	Annealing temperature	Size range	Number of alleles	H_O/H_E	GenBank numbers
Myotis daubentonii							
MmCCA	F: att-aag-gac-gcc-tct-ctg-tg R: act-gct-cag-agc-aat-gct-tc	(CCA)n	60°C	70–80	4	0.284±0.034 0.305±0.035	KX572371–KX572374
MdTTA	F: ggc-act-tcc-aaa-aat-act-gaa-ta R: agg-gat-tga-ccc-tct-cat-taa	(TTA)n	60°C	168–174	3	0.452±0.063 0.416±0.045	KX299063–KX299065
MdTTTA	F: aat-cgg-cta-ggg-ctc-ttc-tc R: aga-tga-aat-gaa-ggg-cat-caa-c	(TTTA)n	62°C	178–200	6	0.683±0.071 0.726±0.021	KX469436–KX469441
Eptesicus nilssonii							
EnTCA	F: aaa-tgc-gat-aac-ttt-ccg-atg R: ggc-tat-aca-atg-ggg-acc-aa	(TCA)n	60°C	190–295	15	0.505±0.045 0.688±0.038	KX469442–KX469456
EnTCTA	F: tca-ctt-gta-gaa-tag-cac-gca R: caa-gct-aat-gtt-taa-aca-gtc-ac	(TCTA)n	60°C	236–276	9	0.704±0.052 0.742±0.019	KX299054–KX299062

plification within the family Vespertilionidae // Molecular Ecology. Vol.7. P.136–138.

Castella V. & Ruedi M. 2000. Characterization of highly variable microsatellite loci in the bat *Myotis myotis* (Chiroptera: Vespertilionidae) // Molecular Ecology. Vol.9. P.1000–1002.

Excoffier L., Laval G. & Schneider S. 2005. Arlequin (version 3.0): an integrated software package for population genetics data analysis // Evolutionary Bioinformatics Online. Vol.1. P.47–50.

Jan C., Dawson D.A., Altringham J.D., Burke T. & Butlin R.K. 2012. Development of conserved microsatellite markers of high cross-species utility in bat species (Vespertilionidae, Chiroptera, Mammalia) // Molecular Ecology Resources. Vol.12. P.532–548.

Kerth G., Mayer F. & Petit E. 2002. Extreme sex-biased dispersal in the communally breeding, nonmigratory Bechstein's bat (*Myotis bechsteinii*) // Molecular Ecology. Vol.11. P.1491–1498.

Laine V.N., Lilley T.M., Norrdahl K. & Primmer C.R. 2013. Population genetics of Daubenton's bat (*Myotis daubentonii*) in the Archipelago Sea, SW Finland // Annales Zoologici Fennici. Vol.50. P.303–315.

Ngamprasertwong T., Mackie I.J., Racey P.A. & Piertney S.B. 2008. Spatial distribution of mitochondrial and microsatellite DNA variation in Daubenton's bat within Scotland // Molecular Ecology. Vol.17. P.3243–3258.

Petri B., Pääbo S., von Haeseler A. & Tautz D. 1997. Paternity assessment and population subdivision in a natural population of the larger mouse-eared bat *Myotis myotis* // Molecular Ecology. Vol.6. P.235–242.

Sambrook J., Fritch E. & Maniatis T. 1989. Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press. 398 p.

Scott D.D., Fitzpatrick S., Bailie D.A., Boston E.S.M., Lundy M.G., Buckley D.J., Teeling E.C., Montgomery W.I. & Prodöhl P.A. 2013. Isolation and characterization of eight polymorphic microsatellite loci for Natterer's bat, *Myotis nattereri* (Vespertilionidae, Chiroptera) // Conservation Genetics Resources. Vol.5. P.643–645.

Senior P., Butlin R.K. & Altringham J.D. 2005. Sex and segregation in temperate bats // Proceedings of the Royal Society, Series B. Vol.272. P.2467–2473.

Sikes R.S., Gannon W.L., & the Animal Care and Use Committee of the American Society of Mammalogists. 2011. Guidelines of the American Society of Mammalogists for the use of wild mammals in research // Journal of Mammalogy. Vol.92. P.235–253.

Smith G.C., Aegerter J.N., Allnutt T.R., Macnicoll A.D., Learmount J., Hutson A.M. & Atterby H. 2011. Bat population genetics and *Lyssavirus* presence in Great Britain // Epidemiology and Infection. Vol.139. P.1463–1469.

Vonhof M.J., Davis C.S., Fenton M.B. & Strobeck C. 2002. Characterization of dinucleotide microsatellite loci in big brown bats (*Eptesicus fuscus*), and their use in other North American vespertilionid bats // Molecular Ecology Resources. Vol.2. P.167–169.

Trujillo R.G. & Amelon S.K. 2009. Development of microsatellite markers in *Myotis sodalis* and cross-species amplification in *M. gricescens*, *M. leibii*, *M. lucifugus*, and *M. septentrionalis* // Conservation Genetics. Vol.10. P.1965–1968.