

Development and characterization of new polymorphic microsatellite markers for Eurasian ground squirrel *Spermophilus fulvus* (Lichtenstein, 1823)

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ABSTRACT. Ground squirrels are ecosystem engineers and keystone species in many open landscapes of Eurasia, America, and Africa. They are model objects for population studies, behavioural ecology, life-history theory, and conservation biology, the research areas where microsatellite analysis is widely applied and fruitful. So far, microsatellite markers have been developed for only few Palearctic ground squirrels. We tested and characterized 14 markers previously developed for ground squirrels and 10 new loci with tri-, tetra-, and five-nucleotide repeats in the yellow ground squirrel, *Spermophilus fulvus*, a species widely distributed in Eurasia and endangered in some regions. We found polymorphism in 10 loci, five of them were highly polymorphic (5–14 alleles). These markers will benefit studies of the population genetic structure, parentage, mating system, reproductive success, and interspecific hybridization as well as conservation efforts in *S. fulvus* and other close-related Eurasian ground squirrels.

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Разработка и характеристика новых полиморфных микросателлитных маркеров для Евразийского вида наземных беличьих *Spermophilus fulvus* (Lichtenstein, 1823)

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РЕЗЮМЕ. Наземные беличьи относятся к числу экосистемных инженеров и ключевых видов открытых ландшафтов Евразии, Америки и Африки. Они служат модельными объектами в популяционной биологии, поведенческой экологии, теории жизненных циклов и природоохранной науке — областях, в которых широко и плодотворно применяют микросателлитный анализ. Однако до сих пор микросателлитные маркеры были разработаны лишь для единичных представителей Палеарктических наземных беличьих. Мы протестировали 14 маркеров, разработанных ранее для наземных беличьих, и 10 новых локусов с три-, тетра- и пентануклеотидными повторами на желтом суслике *Spermophilus fulvus*. Этот вид широко распространен в Евразии, однако в некоторых регионах его численность сокращается, и он находится под угрозой исчезновения. Полиморфизм был обнаружен в 10 локусах, пять из них оказались высоко полиморфными (5–14 аллелей). Эта система маркеров может быть использована в исследованиях генетической структуры популяций, родства и отцовства, брачных отношений, репродуктивного успеха, межвидовой гибридизации, а также в природоохранных целях для желтого суслика и других близко родственных ему видов Евразийских наземных беличьих.

КЛЮЧЕВЫЕ СЛОВА: микросателлитные маркеры, праймеры, желтый суслик, *Spermophilus fulvus*, наземные беличьи, Sciuridae.

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Introduction

Ground squirrels are ecosystem engineers and keystone species in many open landscapes of Eurasia, America, and Africa (Thorington *et al.*, 2012). They are very sensitive to landscape changes and fragmentation caused by human impact and endangered in many regions of the world. Ground squirrels are model objects for population genetics studies, behavioural ecology, life-history theory, and conservation biology, the research areas where microsatellite analysis is widely applied and fruitful. So far, microsatellite markers have been developed mainly for American species and only few for Palearctic ground squirrels: *Spermophilus citellus* (Linnaeus, 1766), *S. suslicus* (Güldenstaedt, 1770), and *Marmota marmota* (Linnaeus, 1758) (Hanslik & Kruckenhauser, 2000; Gondek *et al.*, 2006), all three representing European fauna.

Yellow ground squirrel, *Spermophilus fulvus* (Lichtenstein, 1823), is widely distributed in Asia and Southern European Russia. It inhabits dry steppes, semi-deserts, and deserts of Southern Russia, Central Asia, Kazakhstan, northern Iran and Afghanistan, and north-western China (Kucheruk, 1998). In Russia, it inhabits a narrow zone at the northeastern part of its range in the dry steppes on the left bank of the lower Volga River. Decreased grazing caused by the reduction of the livestock in 1990–2000s combined with ploughing of dry steppes caused landscape fragmentation, habitat loss, population decline, and extinction of some colonies (Oparin *et al.*, 2004; Shilova *et al.*, 2015). The expansion of the sister species *S. major* (Pallas, 1778) to the south has led to the formation of stable hybrid zones with *S. fulvus* expanding with time (Titov *et al.*, 2006). *S. fulvus* is listed as vulnerable or endangered in the regional Red Data Books in Russia (the Republic of Kalmykia and Saratovskaya Oblast'). In our long-term individually-based study of *S. fulvus* we aim at understanding ecological and evolutionary causes of variation in male and female mating tactics and lifetime reproductive success as well as population dynamics and species distribution (Vasilieva & Tchabovsky, 2015; 2018; Shilova *et al.*, 2015). To achieve these goals as well as to contribute to conservation efforts in *S. fulvus* and other Eurasian ground squirrels, in this study, we tested and characterized previously known for other ground squirrel species as well as novel polymorphic microsatellites in *S. fulvus*.

Methods

Genomic DNA was extracted from ethanol-preserved ear tissue of adult presumably unrelated *S. fulvus* (both males and females) from the colony in Saratovskaya Oblast'. Dried tissue was digested overnight at 50°C with 17–20 µl of proteinase K (20 µg/µl), and DNA was extracted using a standard phenol–chloroform extraction method.

To design primers for 10 novel microsatellite loci, we analyzed manually the genome sequence of the

thirteen-lined ground squirrel, *Ictidomys tridecemlineatus* (Mitchill, 1821) (GenBank Acc. No. AC192323) to identify tri-, tetra-, and five-nucleotide microsatellite repeats. We have chosen sequence fragments with eight or more perfect repeats and sufficient flanking sequence for further use. Primers were designed in such a way as to provide a range of product sizes useful to electrophoretic analysis. We modified primers previously designed for other ground squirrel species to shorten microsatellite sequence fragments for better separation of the alleles in gel electrophoresis.

Microsatellite polymerase chain reaction amplifications for all loci were carried out in T100 Thermal Cycler, BioRad in 25-µl reaction volumes containing 0.1–0.2 µg of template DNA, 15 pmol of each primer, the mixture of four dNTP (200 µM of each), 2 mM MgCl₂, 2 units of recombinant Taq polymerase of low concentration, and 1 × PCR buffer (20 mM (NH₄)₂SO₄, 50 mM Tris-HCl (pH 8.9), 20 µM EDTA, and 150 µg/ml bovine serum albumin). The optimal annealing temperature for each marker (the temperature with the highest PCR efficiency) was determined empirically using a gradient PCR protocol with temperatures set between 54 and 64°C with a 2° gradient. PCR cycling conditions were 35 cycles of 94°C for 30 s, 58–60–62°C for 30 s (optimal annealing temperature, Table 1), 72°C for 30 s. Microsatellite PCR products were electrophoresed and separated on a 8% polyacrylamide gel with size marker pBR322/Hpa II to determine the allele sizes. Fragments from polymorphic loci were sequenced on ABI PRISM 3500 (Thermo Fisher Scientific) genetic analyzer with a cycle sequencing kit (The BigDye® Terminator v3.1, ABI) and same primers (used for PCR).

Results and Discussion

Out of 24 tested primer pairs, 17 amplified PCR products (Table 1). For two of 14 previously developed microsatellite markers (10 for three North-American ground squirrels, two for European ground squirrel, *S. citellus*, one for *Urocitellus undulatus* (Pallas, 1778), and one for *S. fulvus*), the product was not amplified, in four no polymorphism was found, while eight other were found polymorphic in *S. fulvus*. In addition to previously developed markers, we tested 10 novel loci with newly developed sets of primers. In five of them, no product was amplified, three were monomorphic, and two polymorphic. Thus, in total we found 10 variable loci with the number of alleles per locus ranging from two to seven. Five of them were highly polymorphic (five-seven alleles).

To characterize a set of 5 highly polymorphic markers in more detail, we tested them at a larger sample of 48 adult males and 38 adult females (Table 2). The product was amplified in all samples. MICRO-CHECKER 2.2.3 (van Oosterhout *et al.*, 2004) revealed no evidence for sluttering, allele dropouts, or presence of a null allele at any locus. Allelic diversity ranged from 5 to 14 alleles/locus (mean = 7.6). Of the five microsatellites, one

Table 1. Characteristics of 24 microsatellite loci in *Spermophilus fulvus* from Saratovskaya Oblast', Russia.

Locus	Primer sequence (5'-3')	Repeat type	Ta (°C)	Allele size range, b.p.	N _d	GenBank Acc. No.	Source species	Reference
IGS bp	F:CTACACGAGAAAGAACTCC R:CTCTACACCCCTTCTGAC	CAG	60	89-125	<u>7</u>	<u>MN119269</u>	<i>S. fulvus</i>	Titov <i>et al.</i> (2007)
IGS 9D	Fm:ACAAACATTTATAACCGTAAAG R:GGATTTGAAAAGAGTGACATC	ACC	60	~140	NoA	-	<i>S. brunneus</i>	May <i>et al.</i> (1997)
IGS 24D	F:CCCTCAGATTAATTAAGTGG R:GCCCTGCATGAAACCTTG	GTT	58	140-162	5	MN107344	<i>S. brunneus</i>	May <i>et al.</i> (1997)
IGS 110b	F:CCATGGAAGCATGTCTGGTG R:TCCTTCCTGATTTCAAGGTG	GCT	62	132-141	2	MN107347	<i>S. brunneus</i>	May <i>et al.</i> (1997)
STR3	F:TGGTCCAGTAGTGGATTAC R:GTTCAITTCACAGACCACA	CTT	60	~300	5	-	<i>Urocyonailurus undulatus</i>	Titov <i>et al.</i> (2009)
Ssu16	F: AACCCCTTCTGTTCCCTTCA Rm:CTGTATGTTAATAAGATGCA	GAT	60	~220	5	-	<i>S. suslicus</i>	Gondek <i>et al.</i> (2006)
Ssu17	Fm: ATATGAAATTCCTAGTAACTCC R: TGATTC AAAGATGCAACATCC	GAT+ GCT	60	106-115	5	MN119270	<i>S. suslicus</i>	Gondek <i>et al.</i> (2006)
XmohC10	Fm: AGCACCCCTGAAATCAATCT Rm: TAGGCAGAAAAGTGAGAGCA	GAAA	58	119-151	<u>7</u>	<u>MN119271</u>	<i>Xerospermophilus mohavensis</i>	Bell & Matocq (2010)
XmohD102	F:CTTGGGAGGCTGAGACAG Rm:CCAGGGAAGCATACCCT	TAGA	58	~185	NoA	-	<i>Xerospermophilus mohavensis</i>	Bell & Matocq (2010)
XmohD116	F: TGGCAGAAAAGGATGGGAGA Rm: TTGGCAGATGGACCTCC	GATG+ GATA	58	116-160	<u>5</u>	<u>MN119272</u>	<i>Xerospermophilus mohavensis</i>	Bell & Matocq (2010)
D1	F:ACCTTTTGTTCATCTCAGC R: TGCCAATGTTTGCCTTCTACT	GATA	58	~185	7	-	<i>Cynomys ludovicianus</i>	Jones <i>et al.</i> (2005)
D2	Fm:TTGAAGAAGTAGCACAGTGGT Rm:GCACACTTGTGCAAAATACATC	CTAT	60	174-186	5	MN107345	<i>Cynomys ludovicianus</i>	Jones <i>et al.</i> (2005)
D12	Fm:TTACCTCCCAACACAAAT Rm:CCATCCAGTGGATATTTAG	GATA	58	234-246	5	MN107346	<i>Cynomys ludovicianus</i>	Jones <i>et al.</i> (2005)
D115	F: CAGCAICTATGGAAGACAG Rm: TGACCTTCCATTCGAGTAACC	GATA	60	~140	5	-	<i>Cynomys ludovicianus</i>	Jones <i>et al.</i> (2005)
STR4	F:CCCCAATGAGACATGCAG R:CTAACCAICTCAGAAAAGCCC	CAAA	60	~170	5	-	<i>S. fulvus</i>	NL
CAG	F:CGTCATCGGTGTTCTTCTCT R:TCGCAATTTCCAGIFCTG	CGT	58	~200	NoA	-	<i>S. fulvus</i>	NL
TR5-1	F:CATCGGTTACACATCCACATT R:ACCTACTACATTTACTATAAGCC	CCCCT	58	~140	7	-	<i>S. fulvus</i>	NL
TR4-1	F:CTTAGTGTCTGGCCCTTGGG R:AGCTACTGCTCAATTTCTCTGTA	GGAA	58	218-242	<u>5</u>	<u>MN119273</u>	<i>S. fulvus</i>	NL
TR4-2	F:ACTTTGTACTAAGCCACATC R:AIGTGTGAGGCACATAGGTTG	TTTA	60	~150	NoA	-	<i>S. fulvus</i>	NL
TR4-3	F:TGGACACAACAICTTTGTAATG R:ACCTACTACATTTACTATAAGCC	GTTT	60	~160	NoA	-	<i>S. fulvus</i>	NL
TR4-4	F:GATCCTCAGGACACATACA R:GTAATTAATGAAGCCTCTTT	AAAT	60	~185	NoA	-	<i>S. fulvus</i>	NL
TR4-5	F:GCATAGGTCAGAAAAGATACA R:AGCACAGAAAAGAGGAGAG	CCCT+ CTTT	60	290-394	<u>5</u>	<u>MN119274</u>	<i>S. fulvus</i>	NL
TR4-6	F:GACGCCCTCAGGAAITTAFTG R:AGGGGTTGAATCCAGGAGTG	CAAA	60	~140	NoA	-	<i>S. fulvus</i>	NL
TR3-1	F:ACCTGATGATGACTGACTAG R:TCCTTGTGACTCCTCAAACT	TGT	58	~190	5	-	<i>S. fulvus</i>	NL

Ta – annealing temperature, N_d – N individuals; N_d – N alleles; NoA – no PCR amplification obtained, NL – novel loci; Fm, Rm – original primers were modified; bold text indicates polymorphic loci, bold and underlined – highly polymorphic; * – PCR amplified two different fragments, same in all tested individuals; i.e., no between-individual variation was observed, and we could not affirm that those two different-sized fragments were alleles of one locus.

Table 2. Characteristics of 5 highly polymorphic microsatellite loci in *Spermophilus fulvus*.

Locus	Allele size range, b.p.	<i>N</i> ind	<i>N_A</i>	<i>H_o</i>	<i>H_e</i>
IGS bp	89-125	86	5	0.733	0.762
XmohC10	119-151	86	8	0.872	0.821
XmohD116	116-136	86	6	0.767*	0.773
TR4-1	218-242	86	5	0.686	0.768
TR4-5	290-398	86	14	0.837	0.879

* Significant deviation from Hardy-Weinberg equilibrium. *N*ind – *N* individuals tested, *N_A* – *N* alleles, *H_o* – observed heterozygosity, *H_e* – unbiased expected heterozygosity.

(XmohD116) showed a slight deviation from Hardy-Weinberg equilibrium after Bonferroni correction for multiple comparisons. Exact *p*-values for deviation from HWE were estimated by the Markov chain method with the chain length of 100,000 in the program GENEPOP 4.7 (web version available at <http://wbiomed.curtin.edu.au/genepop/>; Raymond & Rousset, 1995; Rousset, 2008).

Pair-wise comparisons performed in GENEPOP revealed significant linkage disequilibrium in two pairs of loci, both including TR4-5 (TR4-5/TR4-1 and TR4-5/XmohD116, $p < 0.000001$ and $p = 0.004$, respectively, after standard Bonferroni correction for multiple comparisons, $\alpha = 0.05/10 = 0.005$). This violates the assumption of independence of loci (Selkoe & Toonen, 2006), and, thus, TR4-5 should be excluded from population genetics analysis to avoid pseudo-replication but is useful for parentage analysis due to its high polymorphism. Its resolving power in parentage analysis is increased by one indel between CCTT and CTTT repeat motifs within this compound microsatellite as revealed by sequencing. In XmohD116 with compound motif GATG+GATA, sequencing revealed size homoplasy (i.e. equally sized alleles with different sequences, Estoup *et al.*, 2002). Though, amplicon size correlates well with SSR repeat number, without taking into account sequence variation, this marker should be used with caution in population genetics, and, especially, in phylogenetic and phylogeographic studies (Barthe *et al.*, 2012).

We conducted preliminary cross-amplification tests for IGS 24D, IGS 110b, XmohC10, XmohD116, D2, and D12 with other two Palearctic ground squirrels, *S. major* and *S. suslicus*, with 2 individuals in each species. In both species, the product was amplified for all loci tested. Polymorphism was detected in all loci in *S. suslicus* and in all but one (IGS 110b) in *S. major*.

To conclude, this set of polymorphic microsatellite markers developed for the first time for Eurasian ground squirrel species will benefit studies of the population genetic structure, parentage pattern, mating system, reproductive success, and interspecific hybridization as well as conservation efforts in *S. fulvus* and other close-related Palearctic ground squirrels.

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References

- Barthe S., Gugerli F., Barkley N.A., Maggia L., Cardi C. & Scotti I. 2012. Always look on both sides: phylogenetic information conveyed by simple sequence repeat allele sequences // *PLoS ONE*. No.7. P.e40699.
- Bell K.C. & Matocq M.D. 2010. Development and characterization of polymorphic microsatellite loci in the Mohave ground squirrel (*Xerospermophilus mohavensis*) // *Conservation Genetics Resources*. Vol.2. No.1. P.197–199.
- Estoup A., Jarne P. & Cornuet J.-M. 2002. Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis // *Molecular Ecology*. No.11. P.591–1604.
- Gondek A., Verduijn M. & Wolff K. 2006. Polymorphic microsatellite markers for endangered spotted suslik, *Spermophilus suslicus* // *Molecular Ecology Notes*. Vol.6. No.2. P.359–361.
- Hanslik S. & Kruckenhauser L. 2000. Microsatellite loci for two European sciurid species (*Marmota marmota*, *Spermophilus citellus*) // *Molecular Ecology*. Vol.9. No.9. P.2163–2165.
- Jones R.T., Martin A.P., Mitchell A.J., Collinge S.K. & Ray C. 2005. Characterization of 14 polymorphic microsatellite markers for the black-tailed prairie dog (*Cynomys ludovicianus*) // *Molecular Ecology Notes*. Vol.5. No.1. P.71–73.
- Kucheruk V.V. 1998. [Present views of *Citellus fulvus* range] // *Zoologicheskii Zhurnal*. Vol.77. P.1205–1207 [in Russian].
- May B., Gavin T.A., Sherman P.W. & Korves T.M. 1997. Characterization of microsatellite loci in the Northern Idaho ground squirrel *Spermophilus brunneus brunneus* // *Molecular Ecology*. Vol.6. No.4. P.399–400.
- Oparin M.L., Oparina O.S. & Tsvetkova A.A. 2004. Pasture as a factor of transformations of ground ecosystems in semiarid regions // *Povolzhskii Ecologicheskii Zhurnal*. No.2. P.183–199 [in Russian, with English summary].
- Raymond M. & Rousset F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism // *Journal of Heredity*. Vol.86. P.248–249.
- Rousset F. 2008. Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux // *Molecular Ecology Resources*. No.8. P.103–106.

- Selkoe K.A. & Toonen R. J. 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers // *Ecology Letters*. Vol.9. No.5. P.615–629.
- Shilova S.A., Savinetskaya L.E. & Tchabovsky A.V. 2015. Long-term and recent dynamics of the yellow souslik (*Spermophilus fulvus*, Rodentia, Sciuridae) population from Yeruslan sands of Transvolga region // *Zoologicheskii Zhurnal*. Vol.94. No.8. P.944–954 [in Russian, with English summary].
- Thorington Jr. R.W., Koprowski J.L., Steele M.A. & Wharton J.F. 2012. *Squirrels of the World*. Baltimore: Johns Hopkins University Press. 472 p.
- Titov S.V., Ermakov O.A., Shmyrov A.A., Kuzmin A.A., Surin V.L. & Formozov N.A. 2006. Population features of interspecific hybridization of ground squirrels (*Spermophilus*: Rodentia, Sciuridae) // *Bulleten' Moscovskogo Obshchestva Ispytatelei Prirody. Otdel Biologicheskii*. Vol.111. P.36–41 [in Russian, with English summary].
- Titov S.V., Shmyrov A.A., Kuzmin A.A., Ermakov O.A., Surin V.L. & Formozov N.A. 2007. [Patterns of dynamics of genetic structure of hybrid populations of mammals (with gen. *Spermophilus* as a model)] // Rozhnov V.V. (ed.). *Molekularno-geneticheskiye Osnovy Sohraneniya Bio-raznootbraziya Mlekopitayushchih Golarktiki: Materialy mezhdunarodnoi konferencii*. Moskva: KMK Scientific Press Ltd. P.258–269 [in Russian].
- Titov S.V., Savinetskaya L.E. & Tchabovsky A.V. 2009. High genetic diversity in the long-tailed ground squirrel (*Spermophilus undulatus*) population on Olkhon Island: A natural gene bank or the consequences of long-term isolation? // *Doklady Biological Sciences*. Vol.429. No.1. P.523–535.
- Van Oosterhout C., Hutchinson W.F., Wills D.P.M. & Shipley P. 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data // *Molecular Ecology Notes*. No.4. P.535–538.
- Vasilieva N.A. & Tchabovsky A.V. 2015. A shortage of males causes female reproductive failure in yellow ground squirrels // *Science Advances*. Vol.1. No.9. P.e1500401.
- Vasilieva N.A. & Tchabovsky A.V. 2018. Reproductive decisions in a “fast-living” sciurid: a case study of the yellow ground squirrel (*Spermophilus fulvus*) // *Biology Bulletin Reviews*. Vol.8. No.1. P.12–22.