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# ISOLATION AND CHARACTERISATION OF MICROSATELLITE LOCI FOR TWO SPECIES OF SPINTURNICID BAT WING MITES (*SPINTURNIX MYOTI* AND *SPINTURNIX BECHSTEINI*)

*Primer note*

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ABSTRACT — To investigate the potential for host-parasite coadaptation between bats and their wing mites, we developed microsatellite loci for two species of *Spinturnix* mites. For *Spinturnix myoti*, parasite of *Myotis myotis*, we were able to develop nine polymorphic loci and screened them in 100 mites from five bat colonies. For *S. bechsteini*, parasite of *M. bechsteinii*, we developed five polymorphic loci, which were also screened in 100 mites from five bat colonies. In both species, all markers were highly polymorphic (22-46 and 6-23 alleles per locus respectively). The majority of markers for both species exhibited departure from Hardy-Weinberg proportions (8 of 9 and 3 of 5, respectively). One marker pair in *S. myoti* showed evidence for linkage disequilibrium. As the observed departures from Hardy-Weinberg proportions are most likely a consequence of the biology of the mites, the described microsatellite loci should be useful in studying population genetics and host-parasite dynamics of *Spinturnix myoti* and *Spinturnix bechsteini* in relation to their bat hosts.

KEYWORDS — microsatellites; *Spinturnix*; *Myotis*; wing mite; ectoparasite; bat; coadaptation; population genetics

## INTRODUCTION

Temperate-zone bats harbour a wide variety of parasites, which often exhibit strong patterns of cospeciation (Dick, 2007; Glover, 1962). In European bat species and their ectoparasitic wing mites of the genus *Spinturnix*, substantial evidence for cospeciation between these two taxa has been shown using mtDNA sequences (Bruyndonckx *et al.*, 2009a). *Spinturnix* wing mites are haematophagous, permanent parasites and all life stages are confined to the wing membrane or uropatagium of their host.

Due to this obligate life history these species make excellent parasites to study host-parasite coevolution and coadaptation. Patterns of coadaptation depend greatly on population structure and gene flow among distinct populations (e.g., Gandon *et al.*, 1996), as well as on reproductive mode (Bar-

rett *et al.*, 2008). As these attributes are difficult to determine directly in small organisms, they require the use of indirect methods such as the use of population genetic markers (de Meeus *et al.*, 2007). To date no microsatellite markers have been developed for *Spinturnix*, or any other bat ectoparasite. Furthermore, several population genetic studies have encountered problems when studying the Acari due to a strong underrepresentation of dinucleotide repeat microsatellites (Navajas *et al.*, 1998), as well as non-mendelian transmission of microsatellite alleles (de Meeus *et al.*, 2004). Here, we report the development of polymorphic dinucleotide repeat microsatellites for two species of *Spinturnix* mites.

## MATERIALS, METHODS AND RESULTS

Enriched genomic library construction and microsatellite isolation was performed by Ecogenics GmbH (Zürich, Switzerland) from size selected genomic DNA of *Spinturnix myoti* ligated into SAULA/SAULB-linker (Armour *et al.*, 1994) and enriched by magnetic bead selection with biotin-labeled $CA_{(13)}$ and $GA_{(13)}$ oligonucleotide repeats (as in: Gautschi *et al.*, 2000a; Gautschi *et al.*, 2000b). Of 384 recombinant colonies screened, 160 gave a positive signal after hybridization. Plasmids from 120 positive clones were sequenced and primers were designed for 62 microsatellite inserts. After testing for amplification using pooled DNA from 4 individual mites on a 1.5% agarose gel, 21 were subsequently ordered as fluorescently labelled primers and chosen for further screening. Of these, 18 amplified in *S. myoti* of which three were monomorphic, leaving 15 primer pairs, from which nine were selected for further testing (Table 1a). In *S. bechsteini*, 16 of the 21 loci amplified, of which seven were monomorphic, and four amplified inconsistently, leaving 5 loci (Table 1b). Screening of a third *Spinturnix* species (*S. plecotina*) using all dye-labelled primer pairs, yielded only one locus (SM18), and was therefore not pursued.

After initial screening, 100 mites from five bat colonies (20 per colony) were tested for each species. For both species, mites were collected randomly from multiple bats in a single colony. Colonies of *Myotis myotis,* where *S. myoti* were sampled, were at least 20 km apart, whereas the colonies of *M. bechsteinii*, where *S. bechsteini* were sampled, were occasionally within several kilometers of one another, but not close enough to constitute a single colony. Mites were removed from the wing and tail membrane of host bats using soft forceps and were stored in 90% ethanol until extraction. Samples were rehydrated in sterile water and prepared for extraction by crushing them using a micro-pestle after submersion in liquid nitrogen. Total genomic DNA was extracted using a phenol-chloroform extraction (Sambrook *et al.*, 1989). Where possible, multiplexes of two primer pairs were created. Differences in amplification strength and dye strength were accommodated for by adapting primer concentrations (Table 1a, b). PCR was performed using 5 μL of 2x QIAGEN Multiplex PCR Master-Mix (QIAGEN), 1 μL of 10 μM primer mix, 1 μL of DNA template (±20 ng), and filled to a final volume of 10 μL with distilled water. Attempts at combining more primer pairs in multiplex, as well as attempts at amplification using several other Taq polymerases and buffer solutions were all unsuccessful [Qiagen Taq polymerase (QIAGEN), Amplitaq Gold (Applied Biosystems), HotStarTaq Plus with Q-solution (QIAGEN)]. PCR amplifications were performed using a GeneAmp 9700 thermal cycler (Applied Biosystems). Cycling conditions were: initial activation at 94°C for 15min, 35 cycles of 30s at 94°C, 45s at the annealing temperature and 60s at 72°C, followed by a final extension at 72°C for 10min. 1.5 μL of PCR product was added to a solution of 20 μL Hi-Di formamide (Applied Biosystems) and 0.07 μL GS-500 LIZ size-standard (Applied Biosystems). Samples were resolved in POP4 polymer on an ABI3730 genetic analyzer (Applied Biosystems) and read using Genemapper 4.0. When using high quality DNA template (>20 ng/μL as measured by NanoDrop, Thermo Scientific), PCR amplification of all microsatellite loci was always successful, but most loci produced several stutter and 'plus A' peaks. However, in all cases it was still possible to score loci consistently, as was confirmed using Microchecker 2.2.5 (van Oosterhout *et al.*, 2004), which found no evidence for possible problems with stuttering or allelic dropout.

Data was analyzed using GenePop 4.0.10 on the web (http://genepop.curtin.edu.au/; Raymond and Rousset, 1995) and FSTAT 2.9.4 (Goudet, 2002). All loci were highly polymorphic: 22-46 alleles per locus (average 35.2) in *S. myoti*, and 6-23 (average 17.2) in *S. bechsteini*. Heterozygosity ranged between 0.460 and 0.850 in *S. myoti* and 0.465 and 0.870 in *S. bechsteini* (Table 1). Hardy-Weinberg Exact tests were carried out in Genepop using a probability test per locus and per population with 1000 batches of 1000 iterations. In *S. myoti* only one marker appeared to be in Hardy-Weinberg proportions (SM13), whereas the others showed significant heterozygote deficiencies in all populations. In *S. bechsteini*, 3 of 5 markers showed significant heterozygote deficiencies (SM16, SM18, SM35: $H_e$-$H_o$, Table 1a, b). Although heterozygote deficiencies can be the result of null alleles in the microsatellite loci, we believe this not to be the case in our study for several reasons. Firstly, due to the seasonal cycle of

TABLE 1: Characterisation of microsatellite loci for a) *Spinturnix myoti* b) *Spinturnix bechsteini*. $T_a$, refers to the annealing temperature used during PCR, Primer Mix to the different combinations of primers that were multiplexed during PCR (loci with the same letter were combined), C to the primer concentration in the multiplex primer mix; $H_o$ to the observed heterozygosity, $H_e$ to the expected heterozygosity, and $H_e$-$H_o$ which measures deviations from Hardy-Weinberg proportions (significant deviations are in bold). All sequences have been deposited in the public database GenBank and accession numbers are provided. Primer sequences include information on the fluorescent labels used.

a

| Locus | Repeat motif | $T_a$ | Primer Mix; C (μM) | Size range (bp) | No. of alleles | $H_o$ | $H_e$ | $H_e$-$H_o$ | GenBank Accession no. | Primer Sequences (5'-3') |
|---|---|---|---|---|---|---|---|---|---|---|
| SM7 | $(GT)_9TT(GT)_{12}$ | 58 | A; 0.8 | 180-278 | 38 | 0.819 | 0.909 | **0.09** | JF288840 | F: FAM-CTGTGCGGGTTGGTTGTATGTC<br>R: CCAACCACCTGCTGCGTACTAG |
| SM11 | $(GT)_{17}$ | 50 | D; 1 | 108-224 | 22 | 0.636 | 0.745 | **0.109** | JF288841 | F: HEX-CAATCTGCGTCCAACCATATC<br>R: GGCTCCGATTTGTTGATATTC |
| SM13 | $(CA)_{17}$ | 50 | D; 0.75 | 136-210 | 29 | 0.87 | 0.934 | 0.064 | JF288842 | F: FAM-CATCCGGCTCATTGAACAGAC<br>R: TATGCTGATTTTGTTTCGCTTG |
| SM17 | $(GT)_{31}$ | 58 | B; 2 | 128-224 | 40 | 0.664 | 0.968 | **0.304** | JF288844 | F: FAM-GCTCTGTATGCGTTGCGAATGAC<br>R: GGGCTCGGTGGCGTCAAC |
| SM18 | $(CA)_{17}(CAA)_5$ | 50 | C; 1 | 198-316 | 41 | 0.465 | 0.961 | **0.496** | JF288845 | F: FAM-CAGTCGTGAGCAGATTGTCTTTC<br>R: ACATCACCGCCATCAGCTTC |
| SM19 | $(GT)_{18}$ | 50 | C; 1.25 | 143-207 | 29 | 0.75 | 0.92 | **0.17** | JF288846 | F: HEX-GATCGGAGTGGGACCTCAC<br>R: GGGCGTCCTCGTTTGTAC |
| SM51 | $(CA)_5CG(CA)_{13}GA(CA)_3G(CA)_2$ | 58 | B; 1.5 | 110-238 | 46 | 0.543 | 0.971 | **0.428** | JF288848 | F: HEX-GACATCACCTAGCATTACAG<br>R: TCAACTCTCAGTTTGTACAG |
| SM55 | $(CA)_7GA(CA)_{13}$ | 58 | A; 1.2 | 109-217 | 46 | 0.76 | 0.966 | **0.206** | JF288849 | F: HEX-CCCGGTGCAAACACCACGCAC<br>R: GCGCTTGTATGGATGCGCACTG |
| SM59 | $(CA)_{11}$ | 58 | E; 2 | 115-183 | 26 | 0.682 | 0.863 | **0.181** | JF288850 | F: FAM-CTAGAGGATCAATGTGGAAC<br>R: CAACTTAAATGGGCGAGAAG |

b

| Locus | Repeat motif | $T_a$ | Primer Mix; C (μM) | Size range (bp) | No. of alleles | $H_o$ | $H_e$ | $H_e$-$H_o$ | GenBank Accession no. | Primer Sequences (5'-3') |
|---|---|---|---|---|---|---|---|---|---|---|
| SM11 | $(GT)_{17}$ | 50 | A; 1.2 | 261-331 | 18 | 0.839 | 0.896 | 0.057 | JF288841 | F: HEX-CAATCTGCGTCCAACCATATC<br>R: GGCTCCGATTTGTTGATATTC |
| SM16 | $(CA)_{19}CG(CA)_{11}$ | 45 | B; 2 | 250-338 | 22 | 0.727 | 0.878 | **0.151** | JF288843 | F: HEX-GGGACGGTTTCACACTGAG<br>R: GGTTATCGCATCAATTCTACTC |
| SM17 | $(GT)_{\{31}$ | 58 | C; 2 | 142-212 | 17 | 0.85 | 0.888 | 0.038 | JF288844 | F: FAM-GCTCTGTATGCGTTGCGAATGAC<br>R: GGGCTCGGTGGCGTCAAC |
| SM18 | $(CA)_{17}(CAA)_5$ | 50 | A; 0.8 | 162-178 | 6 | 0.46 | 0.771 | **0.311** | JF288845 | F: FAM-CAGTCGTGAGCAGATTGTCTTTC<br>R: ACATCACCGCCATCAGCTTC |
| SM35 | $(CA)_{11}AA(CA)_{13}$ | 45 | D; 2 | 176-300 | 23 | 0.687 | 0.909 | **0.222** | JF288847 | F: FAM-CTGTTGTGAGAGTTCCTG<br>R: CTGCAGTTTCTAGGGATGTC |

the host, population bottlenecks during the winter are common in the parasites, especially in *S. bechsteini*. Furthermore, bat clustering within the large host colonies and outside of the summer maternity period, may lead to a complex subpopulation structure in *S. myoti*. Both bottlenecks and subpopulation structure could lead to genetic drift and strong heterozygote deficiencies, similar to those observed in these markers. Additionally, estimates of Hardy-Weinberg proportions are strongly influenced by homozygotes for rare alleles. As the number of alleles per locus in relation to the number of sampled mites was high, such influences may be present. Finally, low levels of genotyping error can also cause rare homozygotes (Morin *et al.*, 2009), which would exacerbate the aforementioned problem. Therefore, although the presented markers must be viewed with some caution, we believe them to be informative. Linkage disequilibrium was tested for each pair of loci in each population using the log likelihood ratio statistic in Genepop. We found evidence for linkage between SM13 and SM59 in *S. myoti*, which remained significant after Bonferroni correction for the number of tests within populations. If this is confirmed in future analyses, we recommend removing one of these loci in population genetic analyses. In summary, we have developed nine polymorphic microsatellite loci for *Spinturnix myoti*, and five for *Spinturnix bechsteini*, with which we can study the fine scale genetic structure of these mites in several ways, as described in the review by Criscione *et al.* (2005). Subsequently, we aim to use this information and contrast it to the genetic structure of their hosts, and thereby achieve detailed insight into the evolutionary interactions of bats and their parasites (see Bruyndonckx *et al.*, 2009b for a similar approach using mtDNA).

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