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E. L. Rotheray, M. P. Greminger, A. Nater, M. Krützen, D. Goulson & L. F. Bussière

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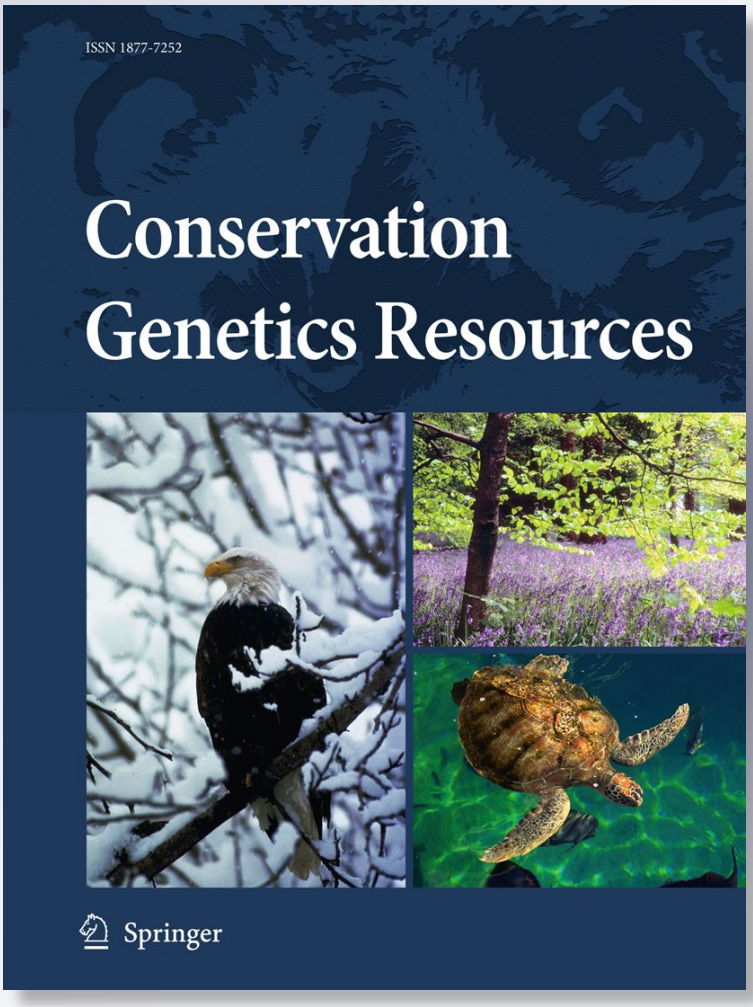

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TECHNICAL NOTE

Polymorphic microsatellite loci for the endangered pine hoverfly *Blera fallax* (Diptera: Syrphidae)

E. L. Rotheray · M. P. Greminger · A. Nater · M. Krützen · D. Goulson · L. F. Bussière

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Abstract We describe eleven polymorphic microsatellite loci developed for *Blera fallax* using 'next generation' 454 whole genome shotgun sequencing, along with conditions for three multiplex PCR reactions. We tested allelic variation on forty *B. fallax* individuals from Scotland and Sweden. Allelic richness and expected heterozygosity were 3.03 (±0.274) and 0.391 (±0.057) respectively. The number of alleles per locus ranged from 2 to 6. We anticipate that these loci will assist conservation management by allowing the monitoring of translocated populations, estimating effective population size, and assessing population structure and dispersal in Scotland and across Europe.

Keywords Syrphid · Pine hoverfly · 454 pyrosequencing · Microsatellite · SSR · Conservation

The pine hoverfly *Blera fallax* has declined in Scotland, UK and is now confined to just two sites (Rotheray and MacGowan 2000). It is listed in the Species Action Framework (2007), a Scottish Natural Heritage initiative for biodiversity significant species. Actions stipulate expanding the range of *B. fallax* from two to five localities by 2012. The small source population has probably gone through a recent genetic bottleneck, so we urgently need data on how translocation might affect genetic variability. Microsatellite markers have been developed for just one syrphid species with limited success (Schönrogge et al. 2006). Here we describe the development of polymorphic microsatellites using 454 pyrosequencing (Santana et al. 2009) to investigate the genetic diversity within *B. fallax*.

We collected 50 larvae from Dulnain Bridge, Scotland, and reared them to eclosion for captive breeding (Rotheray 2010). We froze seventeen adults after death for sequencing and genotyping. We obtained 22 further larvae and one adult from Järfälla, Sweden. These larvae were frozen before storage in 90% alcohol, and the adult was pinned.

We extracted genomic DNA (gDNA) using the DNeasy Blood and Tissue Kit (Qiagen), including optional RNase treatment. DNA yield was quantified using a ND-1000 spectrophotometer (NanoDrop). Pooled gDNA of two male and two female Scottish flies was used for shotgun sequencing of 1/8 plate on a 454 Genome Sequencer FLX with Titanium chemistry (454 Life Sciences, Roche). We obtained 71,804 reads (25,184,020 total bases), of which 74% were longer than 300 base pairs (average read length = 351 bp). We de novo assembled the reads using Newbler 2.3 (454 Life Sciences), which resulted in 1,150 contigs with an average size of 714 bp (21.58% of the reads assembled). Both unassembled reads and contigs were used to identify microsatellite loci using the programs iQDD 0.9 (Meglecz et al. 2007) and Msatcommander 0.8.2 (Faircloth 2008). In total, we obtained 330 uninterrupted microsatellite loci with repeat motifs between 2 and 6 bp and at least five repeats for tetra and penta motifs, six for trinucleotide and eight for dinucleotide motifs. For 75 microsatellite loci we could design PCR primers with the PRIMER 3 package (Rozen and Skaletsky 2000) implemented in iQDD and Msatcommander ($T_M > 60°C$, otherwise default settings).

E. L. Rotheray (✉) · D. Goulson · L. F. Bussière
Biological and Environmental Sciences, University of Stirling, Stirling FK9 4LA, UK
e-mail: e.l.rotheray@stir.ac.uk

M. P. Greminger · A. Nater · M. Krützen
Anthropological Institute and Museum, University of Zürich, Winterthurerstr 190, 8057 Zürich, Switzerland

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Table 1 Characteristics of 12 microsatellite loci in *Blera fallax*

Locus GenBank no.	Repeat motif		Primer sequence	Conc. (μM)	Allele size range (bp)	Fallacplex	Ta (°C)	Pop	Na	H_E	H_o	*P*
HF_8RB	$(CT)_{10}$	F	*6FAM*-TCGCCCATCTACGTTCACC	0.1	169–188	1	64	1	4	0.747	0.941	0.273
JN206627		R	GTTTCCACCGAAAGCAGTACACG	0.15				2	4	0.758	0.957	0.003*
HF_S56	$(AC)_{11}$	F	*NED*-CTCTCGCGCAAACTTTAAATCC	0.15	263–279	1	64	1	3	0.683	0.882	0.017
JN206628		R	GTTTATCGCGTGATGTTGCGAAG	0.2				2	3	0.590	0.435	0.312
HF_WMK	$(ACAT)_7$	F	*VIC*-AGGACAGTGCAGAGGTTGC	0.1	187–199	1	64	1	2	0.059	0.059	–
JN206629		R	TAGGCCGTTCACTATCCGC	0.15				2	3	0.503	0.435	0.170
HF_JRW	$(ACC)_9$	F	*6FAM*-GCATTCAGCAACACAAAAACAGATAAA	0.3	310–322	1	64	1	3	0.508	0.471	0.567
JN206630		R	AGGGGTGCACGACGACTACAG	0.4				2	2	0.449	0.478	1.000
HF_C4A	$(AATG)_6$	F	*PET*-TGATGCAACAGATGCTGGG	0.1	255–267	1	64	1	2	0.337	0.294	0.537
JN206631		R	GTCCTCGGCGGTGAAATAC	0.15				2	2	0.511	0.565	0.689
HF_OH2	$(AC)_{11}$	F	*PET*-ATTAAACTATGAGCGATGTCTGG	0.15	189–205	2	64	1	1	0.000	0.000	–
JN206632		R	GTTTGAATGCACTGCGTCACTCC	0.2				2	6	0.706	0.652	0.980
HF_0IY	$(AGC)_{10}$	F	*NED*-CGATCGGCAACTCATGTGG	0.15	242–257	2	64	1	3	0.570	0.588	0.536
JN206633		R	TACACAGGGTAAGCTCGGC	0.2				2	6	0.763	0.870	0.379
HF_AN4	$(ACG)_{12}$	F	*VIC*-AGGCACTGAGAACGAAAAGAATG	0.1	170–185	2	64	1	2	0.487	0.647	0.303
JN206634		R	GCAGCGAGGCAGACGATAG	0.15				2	4	0.675	0.826	0.253
HF_5VB	$(ACAT)_6$	F	*6FAM*-AGGGCCCAGTATTTGGTTG	0.15	291–303	2	64	1	1	0.000	0.000	–
JN206635		R	GAATTTGGGCCGGTAACGAG	0.2				2	3	0.237	0.261	1.000
HF_AMQ	$(GT)_{10}$	F	*NED*-GGCAGTCGGGATTTCTTCC	0.4	188	3	64–56 TD	1	1	0.000	0.000	–
JN206636		R	GTTTCTCTCCCGCCAGGATACTC	0.5				2	1	0.000	0.000	–
HF_FCT	$(AC)_{10}$	F	*PET*-ACCCCTTTTGTCGTTCGTTTAGT	0.08	277–283	3	64–56 TD	1	1	0.000	0.000	–
JN206637		R	GTTTCATTCAGGTGAGATTCGCTTTTG	0.12				2	2	0.125	0.130	1.000
HF_RKX	$(ACAT)_7$	F	*6FAM*-CAGGAAGAAAGAATCGGCAA	0.08	211–215	3	64–56 TD	1	2	0.337	0.294	0.537
JN206638		R	GAGTAGTTCCCTGTTGGGCA	0.12				2	2	0.348	0.435	0.538

F forward primer sequence, *R* reverse primer sequence, *Conc* primer concentration in the PCR, *Ta* annealing temperature, *TD* touchdown PCR, *Pop* population (1: Scotland, 2: Sweden), *Na* number of alleles, H_E expected heterozygosity, H_o observed heterozygosity, *P* P-value of probability test for deviation from Hardy–Weinberg equilibrium (HWE). Fluorescent dye labels are shown in italics at the 5' end of each forward primer. PIG-tail bases (Brownstein et al. 1996) are presented underscored

* Significant deviation from HWE after sequential Bonferroni correction (Rice 1989), (–) monomorphic loci

Table 2 PCR cycling conditions for the initial testing (PCR1 and PCR2), and the three final multiplexes (Fallac 1, 2 and 3)

	PCR1	PCR2	Fallacplex 1 and 2	Fallacplex 3
Initial denaturation	98°C, 30 s	98°C, 30 s	95°C, 15 min	95°C, 15 min
TD denaturation		98°C, 5 s		94°C, 30 s
TD annealing		64–0.5°C/cycle, 10 s		64–0.5°C/cycle, 1 min 30 s
TD extension		72°C, 20 s		72°C, 1 min
		15 Cycles		15 Cycles
Denaturation	98°C, 5 s	98°C, 5 s	94°C, 30 s	94°C, 30 s
Annealing	58/61/64°C, 10 s	56°C, 10 s	64°C, 1 min 30 s	56°C, 1 min 30 s
Extension	72°C, 20 s	72°C, 20 s	72°C, 1 min	72°C, 1 min
	35 Cycles	25 Cycles	35 Cycles	25 Cycles
Final extension	72°C, 1 min	72°C, 1 min	60°C, 30 min	60°C, 30 min

TD touchdown PCR profile

Of these, we selected 28 loci to test for PCR amplification using gDNA of one male and female from the Scottish population. The PCR reactions contained 10 ng template DNA, 1× Phire Hot Start Reaction Buffer including 1.5 mM $MgCl_2$ (Finnzymes), 0.25 μM forward and reverse primer, 0.2 mM dNTPs, 0.2 μl Phire Hot Start DNA Polymerase (Finnzymes) and ddH_2O to 10 μl total volume. PCR amplifications were performed in a Veriti™ Thermal Cycler (Applied Biosystems) with two different cycling profiles. First, we tested a standard profile with three different annealing temperatures (PCR1, Table 2). In cases where this profile did not result in a specific PCR product, we applied a touchdown profile (PCR2, Table 2). Of the twenty-eight primer pairs tested, sixteen amplified a specific product of the expected size, as verified on 1.5% agarose gels. These sixteen loci were tested for polymorphisms on eight Scottish and six Swedish *B. fallax* using 2.5% agarose gels. Of these, thirteen primers pairs were selected based on band patterns within and between populations. Forward primers were fluorescently labeled and combined into three multiplex PCR reactions, named Fallacplex 1, 2 and 3. PCR reactions contained 10 ng template DNA, 1× Multiplex PCR Master Mix (Qiagen), between 0.08 and 0.5 μM of each primer (Table 1) and ddH_2O to 10 μl. Fallacplex 1, 2 and 3 PCR conditions are described in Table 2. We then genotyped 17 Scottish (7 adult males, 10 adult females) and 23 Swedish (1 adult female, 22 larvae) *B. fallax* individuals. Fragment length analysis was conducted using a 3,730 DNA Analyzer and GENEMAPPER 4.0 software (both Applied Biosystems). The statistical analyses were performed with Fstat 2.9.3 (Goudet 1995), GenALEx 6.1 (Peakall and Smouse 2006) and GENEPOP 4.0 (Raymond and Rousset 1995).

We achieved consistent PCR amplification for all forty individuals. Of thirteen microsatellite loci, one locus failed to amplify in the multiplex reaction, and one was monomorphic for both populations. No significant linkage disequilibrium could be found at any pair of loci in either population, and no evidence for null alleles was detected. The number of alleles per locus ranged from 2 to 6 with a total allelic richness of 3.03 (±0.274). Expected and observed heterozygosities ranged from 0.059 to 0.763 (mean 0.391 ± 0.057) and from 0.059 to 0.957 (mean 0.426 ± 0.067), respectively (Table 1). One locus (HF_S56) showed significant deviation from Hardy–Weinberg equilibrium after sequential Bonferroni correction in one of the two populations (Table 1) (Rice 1989).

The novel microsatellite markers presented here not only assist conservation for this species in Scotland, but have wider applications for describing the distribution and dispersion of *B. fallax* across Europe. Our study confirms the effectiveness of long read, high-throughput sequencing for developing polymorphic microsatellites in non-model invertebrate species.

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