PGR NOTE

Microsatellite markers for identifying hybrids of the damselflies *Calopteryx splendens* and *C. virgo*

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Running title: Detecting hybrids of *Calopteryx* species

Abstract

The damselflies *Calopteryx splendens* and *C. virgo* hybridize in nature. We developed nineteen microsatellite loci for molecular identification of hybrids. Lack of shared alleles at several loci allowed unquestionable identification. Seventeen loci are polymorphic in at least one of the target species, broadening the utility of the loci for population genetic studies.

Calopteryx splendens (banded demoiselle) and *C. virgo* (beautiful demoiselle) are riverine species with largely overlapping distributions in Europe (Askew 1988). Males are distinguished by their wing coloration, but females are cryptic. The two species hybridize in nature (Tynkkynen et al. 2008, Keränen 2009), but identifying hybrid individuals based on morphology is difficult. We developed molecular tools to identify F_1-hybrids and possible introgression between these species.

Microsatellite-enriched genomic libraries were prepared for each species, as described for *Podarcis gaigeae* and *Ischnura elegans* by Wellenreuther et al. (2009) and Molecular Ecology Resources Primer Development Consortium (2010). In short, genomic DNA was digested, ligated and then amplified with specific primers. PCR products were hybridized to simple sequence repeat oligonucleotides with 100 pmol of the following biotinylated probes: $(AG)_{21}$, $(ATT)_6$, $(AT)_7$, $(AAG)_8$ and $(AAAT)_7$. Microsatellite containing fragments were isolated using magnetic beads (Roche Diagnostics), cloned, and finally sequenced. Fifty six inserts of *C. splendens* and 40 of *C. virgo* were sequenced. Of these, 37 and 18, respectively, contained microsatellite motifs.

Primers were designed to flanking regions of the loci using Primer3 (Rozen & Skaletsky 2000). All loci were tested with samples of *C. splendens* and *C. virgo* collected from two localities where the species coexist: Pitkäjoki, Central Finland (62°7´N, 26°3´E) and Turkkijoki, Eastern Finland (62°2´N, 20°10'E). DNA was extracted from *Calopteryx* legs using Qiagen chemistry and a KingFisher (ThermoScientific) magnetic particle processor. Amplification was tested with two $MgCl_2$ concentrations (3 or 5mM) and two annealing temperatures (T_a, 56 or 58°C) using the labeling and thermocycling protocol of Schuelke (2000). Preferred T_a and $MgCl_2$ concentrations for each locus are listed in Table 1. Other

reagent concentrations were: 1X Biotools $MgCl_2$ Free Reaction Buffer, 0.2 mM dNTPs (Fermentas), 8 pmol of each reverse and labeled M13(-21) primer, 2 pmol of the tailed forward primer (see Table 1 for exception), 1 U Biotools Taq DNA polymerase and approximately 50 ng template DNA. All reactions were performed with a BioRad c1000 thermocycler. Products were separated using an ABI 3130xl BioAnalyzer and visualized with GeneMapper software v.3.7 (Both Applied Biosystems).

Twenty-one potential loci isolated from *C. splendens* and ten potential loci isolated from *C. virgo* were tested with 71% and 40% success, respectively. For four of the loci (Cs31, Cs34, Cs60, Cs181) amplification was successful in only *C. splendens*. The others amplified in both species, and DNA sequencing confirmed that loci were indeed homologous (data on request).

Polymorphism per locus was low (2-5 alleles) for both species. Loci were more often polymorphic in *C. splendens* than in *C. virgo* (12 vs. 9 loci), but even monomorphic loci were useful for hybrid identification, since allele sizes of the two species were often distinctively different and shared alleles were either absent or very rare (Table 1).

Twenty to 28 individuals of each species from both localities were surveyed, including two individuals (one from each locality) suspected to be hybrids (Keränen 2009). Observed and expected heterozygosities were calculated in GenAlEx 6.4 (Peakall & Smouse 2006) (Table 1). Deviation from Hardy-Weinberg proportions and genotypic equilibrium were tested in Fstat (Goudet 2001). Within samples, all loci displayed Hardy-Weinberg proportions. Loci Cs19 and Cv95 showed significant disequilibrium in one sample (*C. splendens* from Turkkijoki), otherwise all loci displayed genotypic equilibrium. Null alleles were not suspected, and null allele probabilities calculated in Cervus 3 (Kalinowski et al. 2007) were either negative or very low (less than 0.2).

Due to distinct alleles at several loci (e.g. Cs10, Cs104, Cv60), F_1-hybrid individuals were easily visualized as heterozygotes possessing both "splendens-" and "virgo-specific" alleles. Other individuals appeared to be "pure" *C. splendens* or *C. virgo*. Although we have not identified any introgressed individuals, the possibility of hybrid backcrossing cannot be ruled out, due to the small sample surveyed.

The microsatellite loci described here are useful tools for molecular identification of F_1-hybrids of *Calopteryx splendens* and *C. virgo.* Although the loci are not extremely variable, they can also provide data for studies of population structure within each species.

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Table 1. Microsatellite loci for identifying hybrids of *Calopteryx splendens* and *C. virgo*. For each, GenBank Accession number, Forward (F) and Reverse (R) primer sequences, repeat motif, annealing temperature (T_a), and $MgCl_2$ concentration used in the labeling PCR reaction are listed. For each species, number of alleles detected, their size range and observed (H_o) and expected (H_e) heterozygosity from two sample populations are also reported (NA: not applicable; ----: unsuccessful amplification).

				C. splendens			*C. virgo*		
Locus GenBank Accession number	*Primer sequences 5' → 3'	Repeat Motif	T_a °C $MgCl_2$ conc.	No. All.	**size range (bp)	H_o, H_e, P: Pitkäjoki T:Turkkijoki	No. All.	**size range (bp)	Ho, He, P: Pitkäjoki T:Turkkijoki
Cs5 JF320921	F: TACTCCTTTGCCCCTTCCTT R: GGATTGGATGCACCGATATT	$(TG)_6$	56 °C 5mM	2	126-134	P: 0.148, 0.137 T:0.043, 0.043	1	132	P: NA T: NA
Cs7 JF320922	F: GACGATGAGAGGCACGAGAT R: CATTCCGGTGACACAGTTTTT	$(TG)_6$	58°C 5mM	2	173-175	P: NA T: 0.261, 0.227	5	181-191	P: 0.722, 0.674 T: 0.667, 0.659
Cs10 JF320923	F: TGCGTAATCAGTTAAAGCCAAGTCGT R: *TCAATCGCAATCGACCGGCTG	$(GA)_6$	58 °C 3mM	1	190	P: NA T: NA	1	188	P: NA T: NA

Cs19 JF320924	F: CGAAGAAGGAATCGGTTGTG R: TCCTCAAATCCCCACCAATA	$(AC)_8(TC)_5$	56 °C 3mM	4	194- 200	P: 0.462, 0.429 T: 0.304, 0.258	4	180- 196	P: 0.222, 0.205 T: 0.048, 0.046
Cs31 JF320925	F: GTTCTGTCGGTGGGACACTT R. TTCTCTAACGCCCCAGTTTG	$(AG)_{10}$	58 °C 3mM	3	198- 202	P: 0.417, 0.538 T: 0.500, 0.375	--	--	----
Cs34 JF320926	F: GGGTGAAGTCCACGCAGCGG R: TTGTGCCTTCCCACTGCTCCA	$(CT)_8$	58 °C 3mM	3	151- 157	P: 0.231, 0.204 T: 0.091, 0.088	--	--	----
Cs52 JF320927	F: TGCGTTAATGAAACGCAGAG R: AGAAGAGACGCAGGCAGTGT	$(CT)_2CA(CT)_7$	56 °C 3mM	3	232- 238	P: 0.556, 0.419 T: 0.304, 0.267	3	228- 234	P: 0.211, 0.188 T: 0.476, 0.468
Cs54 JF320928	F: AAGACGCTGTAGCCTTGGAA R. TGGATAAGGTCTCGGGTACG	$(CT)_5TT(CT)_2$	56 °C 3mM	3	209- 225	P: 0.231, 0.208 T: 0.087, 0.083	2	215- 227	P: 0.222, 0.198 T: 0.095, 0.091
Cs60 JF320929	F: AAAGAAGTACGCTGAAGAGTAGACG R: GCGTCCCTTGCTTTTCCT	$(AG)_7$	58 °C 5mM	2	151- 153	P: 0.200, 0.241 T: 0.100, 0.180	--	--	----
Cs66 JF320930	F: GCAGAAATGCTCAAAGAACT R: AATGGAGGCTACCGAAGTAT	$(CA)_3G(CA)_4$	56 °C 3mM	2	219- 231	P: 0.154, 0.142 T: 0.043, 0.043	2	209- 221	P: 0.188, 0.174 T: NA
Cs104 JF320931	F: TCCGCCTCCATCTCCCGGAT	$(CA)_7$	58 °C	3	180-	P: 0.192, 0.178	1	176	P: NA

	R: TGAGGGTTGCTTGCTGCGGG		3mM		184	T: NA			T: NA
Cs160 JF320932	F: ACCCCGGCTCGGATTGGCTT R: CCGGTTCATGTCACTAGCAGAACG	$(GA)_8$	58°C 3mM	2	151-153	P: 0.111, 0.105 T: 0.043, 0.043	1	151	P: NA T: NA
Cs179 JF320933	F: TTGGGAGCGAGGGGGATTGC R: CCCAGTTGCCCCCGAAATGGT	$(GA)_5TA(GA)_2$	58 °C 3mM	3	212-226	P: 0.037, 0.036 T: 0.043, 0.043	2	218-226	P: 0.053, 0.051 T: NA
Cs181 JF320934	F: CCTCGCGATGCCTTTACTT R: TCACTGGGAATTTGTTGCAC	$(GA)_8$	56 °C 3mM	1	228	P: NA T: NA	--	--	----
Cs186 JF320935	F: CCCCAACCCACCTTTATCTT R: TGGACGACCTGAAAATGGAT	$(CT)_{11}$	56 °C 3mM	3	219-231	P: 0.593, 0.586 T: 0.500, 0.505	1	225	P: NA T: NA
Cv7 JF320936	F: TTATGGGGTGGAATGAGAGC R: GAATGGCACTGCACAAAGAA	$(GT)_7$	58 °C 5mM	4	348-372	P: 0.538, 0.554 T: 0.565, 0.417	4	374-380	P: 0.632, 0.608 T: 0.550, 0.476
Cv48 JF320937	F: ATGCTTTGCCCTGATTTTTG R: TTTTAGGCGTGCTCATGTTG	$(CA)_7$	58 °C 5mM	2	203-209	P: NA T: 0.043, 0.043	3	197-203	P: 0.824, 0.507 T: 0.529, 0.389
Cv60 JF320938	F: AGGGTCTGTCAGGGTAAGCA R: TCGCCACTGTCAAATATTGATT	$(GT)_5GC(GT)_5$	58 °C 5mM	2	223-235	P: 0.038, 0.038 T: 0.043, 0.043	5	219-233	P: 0.444, 0.489 T: 0.476, 0.465

Cv95 JF320939	F: CCGGGGTTAGCCGTTTAG R: TTTTTCAAAGCCGCGATAAG	$(CT)_2CC(CT)_8$	58 °C 5mM	4	223-235	P: 0.593, 0.612 T: 0.261, 0.467	4	225-239	P: 0.368, 0.323 T: 0.190, 0.254

127 *following Schuelke (2000), M13(-21) sequence was added to the forward primer for all loci except Cs10

128 **length of M13(-21) tail not included