Development of 12 polymorphic microsatellite loci in *Ischnura elegans* (Odonata: Coenagrionidae)

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Abstract

We isolated and characterised 12 polymorphic microsatellite loci for the blue-tailed damselfly *Ischnura elegans* by screening a genomic library enriched for microsatellite motifs. The loci showed high variability for the number of alleles, and the expected and observed heterozygosities, and thus will be useful for future molecular studies. Cross-amplification in *I. graellsii, I. ramburii* and *I. pumilio* showed that the majority of the microsatellites also produced polymorphic products in these species.

Introduction

Ischnura elegans is a widespread damselfly in Europe, but is replaced by its sister species *I. graellsii* on the Iberian peninsula (Askew, 2004). Males of *I. elegans* and *I. graellsii* are monomorphic, but females of both species fall into one of three phenotypic morphs (Cordero 1990; Sánchez-Guillén *et al.* 2005). One of the female morphs has a similar coloration to conspecific males, while the other two morphs are cryptically coloured. The two species commonly hybridise when they geographically overlap (Monetti *et al*. 2002). Recently, *I. elegans* has been expanding its range in Spain, and consequently, has been replacing *I. graellsii* in several areas (Sánchez-Guillén *et al.*, 2005). The ongoing range expansion and the similarity of the species make them an interesting model for studying morph related gene flow, introgression and population level processes that promote divergence (Kay *et al.*, 2007). However, molecular studies have been hampered by a lack of suitable genetic markers. We developed 12 microsatellite loci from a microsatellite-enriched library for *I. elegans*, and tested these markers in *I. graellsii, I. ramburii and I. pumilio* to enable future molecular studies of these species.

DNA was extracted from individuals stored in 70% ethanol following a standard phenol/chloroform–isoamylalcohol protocol (Sambrook *et al.*, 1989). Microsatellites were isolated using the protocols of Zane *et al*. (2002) and Wellenreuther *et al*. (2009) with slight modifications. Briefly, 500 ng of DNA was digested (37°C 1 h) with 10 U of *Bsp 1431* (Fermentas) following the manufacturer's directions. To ligate adaptors, five microlitre containing 5 μM adaptor (5'-GTGGTAGACTGCGTACC-3'/5'-GATCGGTACGCAGTCTAC-3'), 1 × ligation buffer (USB) and 0.5 U of T4 DNA ligase (USB) was added and incubated at 37°C 3 h and then diluted 1:10.Ten μL was amplified under the following conditions: 0.5 μM specific primer (5'-GACTGCGTACCGATC-3'), 2.5

mM $MgCl_2$, 200 µM dNTPs, 1 × AmpliTaq PCR buffer (Applied Biosystems), 0.4 U AmpliTaq DNA polymerase (Applied Biosystems). Polymerase chain reaction (PCR) conditions: 94°C 30 s, 56°C 1 min, 72°C 1 min, for 25 and 30 cycles. DNA was subsequently denatured and hybridized to biotinylated oligonucleotides: $(AG)_{21}$, $(ATT)_6$, $(AT)_7$, $(AAG)_8$ and $(AAAT)_7$. Streptavidin-coated beads (Roche Diagnostics GmbH, Germany) were added to capture DNA fragments. Beads were magnetically separated using a Magnetic Particle Separator (Roche Diagnostics GmbH, Germany). Following three low and high stringency washes, the bound DNA was recovered by first incubating the beads in TLE (10 mM Tris, 0.1 mM EDTA) at 95°C 5 min, and then by adding 12 µl of 0.15M NaOH (for additional details see Zane *et al.*, 2002).

DNA fragments were cloned following the protocols outlined in the TOPO TA cloning kit (Qiagen, PCR cloning kit). Positive transformants were sequenced using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems), following the manufacturer's instructions. 192 inserts were sequenced, and 61 contained microsatellite motifs. 25 primer pairs were designed using Primer-3 (Rozen, Skaletsky, 2000), and 12 loci were polymorphic when tested on five individuals. The remaining loci were either monomorphic, did not amplify, or had non-specific products.

The polymorphic primers were thoroughly tested on one populations of *I. elegans* from Galicia, Spain (n=20). The following PCR-conditions were used: 0.4 µM of each primer, 1.5 mM $MgCl_2$, 125 µM dNTPs, 0.5 U Platinium Taq polymerase (Invitrogen), 1 × Platinum High Fidelity Buffer (Invitrogen) and 10-20 ng DNA template in a 10 µl reaction. The PCRs were run in 94°C for 2 mins, then 35 cycles at 94°C for 30 s, touchdown 62-58°C for 30 s, 72°C for 30 s, followed by 72°C for 10 mins. PCR products were separated in an ABI PRISM 3730 capillary sequencer and analyzed with GeneMapper 3.0 (Applied Biosystems). Further technical details and GenBank Accession numbers are given in Table 1.

Diversity measures and deviations from Hardy–Weinberg expectations were computed using FSTAT version 2.9.3 (Goudet, 1995), and tests for null-alleles were conducted using Micro-Checker (van Oosterhout *et al.*, 2004). Number of alleles per locus ranged from 3 to 15 with a mean of 8.16, and fragment size varied from 117 to 364 bp (Table 1). Observed and expected heterozygosity ranged from 0.46 to 0.88 and 0.47 to 0.93, respectively (Table 1). Deviations from Hardy–Weinberg equilibrium were found for I026 and I177, and results from Micro-Checker also suggested the presence of null-alleles in these loci (frequency 22.2% and 21.4%, respectively, van Oosterhout *et al.*, 2004). Tests of

linkage equilibrium between all pairs of loci were performed in Arlequin (Schneider *et al.*, 2000) and showed that none of the loci deviated significantly after Bonferroni correction (adjusted nominal level P = 0.0008). Primers were subsequently tested for cross-amplification in *I. graellsii* (n=36)*, I. pumilio* (n=4) and *I. ramburii* (n=8; details given in Table 2).

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Table 1. The number of alleles (A) and the number of *Ischnura elegans* individuals that were successfully genotyped (N), heterozygosities (H_O, observed; H_E, expected), p-value for assessing deviation from Hardy–Weinberg equilibrium (HWE), fragment size range and allele number. GenBank Accession nos: GU434344- GU434355

Locus	GenBank Accession no.	Repeat motif	Primer sequences (5'-3')*	A	N	H_O	H_E	HWE	Size range (bp)
I002	GU434344	$(GT)_7$	F: CCCGGAAATATCTCGTCGAT-FAM	5	20	0.75	0.68	0.352	168-182
			R: GGCTGAGGGTAATGCAGGAG						
I015	GU434347	$(CA)_7$	F: AATCCCAAGGCCCCAGTATC-FAM	6	20	0.75	0.72	0.153	305-324
			R: GTGCCTTTTCCGTGTTGTGA						
I026	GU434346	$(GA)_{11}$	F: ATTAGTTGGCGCACAACACG-M13*	10	15	0.47	0.84	<0.001	117-143
			R: GCGTACCGATCCCAGTGAAT						
I041	GU434348	$(T)_5G(T)_8(ATT)_5(T)_4$	F: GCAGGAGTGGTGGTGGTACA-HEX	6	17	0.88	0.77	0.577	261-269
			R: ATGTCACCCATCGCAACAAA						
I053	GU434345	$(T)_{12}$	F: TTGAAAAGACGGGAGAAGTGC-HEX	12	20	0.80	0.82	0.205	344-364
			R: CACACAAGCCAGTCCACCAT						
I058	GU434352	$(AC)_8$	F: TCCAGGGTTTGCTCTCGACT-M13*	4	18	0.47	0.53	0.356	168-177
			R: TAAGGGGTTGAAGCGGAGTG						
I090	GU434353	$[(GA)_3(A)_6]_3$	F: CGGAATGCATGTACGAGGAA-M13*	10	20	0.65	0.79	0.008	188-213
			R: TCCGCCCCCTATCTAAATCA						
I095	GU434349	$(CT)_6(CT)_7$	F: GCACAGCATCCGTTTGAGAG-FAM	9	18	0.61	0.72	0.249	224-244
			R: AAGGCGTAGCAAAGGCGTTA						
I134	GU434350	$(AC)_6$	F: TCGGCAATACACCCTTACCC-M13*	3	19	0.47	0.49	0.31	170-172
			R: CCGACTACATCCCCTTGCTC						
I177	GU434351	$(CT)_8$	F: TGAATGGGTCGTAGTGGCTTT-M13*	15	20	0.50	0.93	<0.001	235-264
			R: TGAATGGGTCGTAGTGGCTTT						
I210	GU434354	$(AC)_{10}$	F: CCCCGACTCTTTCAACAGGA-HEX	8	19	0.58	0.59	0.795	185-198
			R: TGAGTCAGTCCATGGCATAGTG						
I216	GU434355	$(GA)_{10}$	F: GCGTTCATAGGTTTGGAGTGG-FAM	10	20	0.65	0.75	0.316	166-196
			R: TGAGCCACACGGACTATTGG						

*M-13 (CACGACGTTGTAAAACGAC) tail attached to the 5' end of the forward primer (Schuelke, 2000).

Table 2: Cross-amplification success for *Ischnura graellsii, I. ramburii* and *I. pumilio*. Number of alleles for each locus is given, and the number in brackets denotes number of successful amplifications. NA denotes primers that did not amplify and x primers that were not tested in a species.

Locus name	***I. graellsii* (n=36)**	***I. pumilio* (n=4)**	***I. ramburii* (n=8)**
I002	**6(32) 174-188**	**NA**	**6(8) 174-182**
I015	**12(36) 303-324**	**3(2)**	**3(6)**
I026	**12(15) 119-130**	**x**	**x**
I041	**8(23) 265-296**	**NA**	**4(4) 260-266**
I053	**19(33) 336-366**	**1(1) 348-366**	**1(6) 342-344**
I058	**8(11) 161-176**	**x**	**x**
I090	**7(14) 189-199**	**x**	**x**
I095	**7(13) 222-238**	**1(2) 228**	**2(4) 218-246**
I134	**2(29) 172-174**	**NA**	**4(3) 166-172**
I177	**11(11) 232-256**	**x**	**x**
I210	**9(32) 185-199**	**x**	**x**
I216	**17(32) 164-190**	**x**	**x**

145