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Cross-species amplification of microsatellite loci in some European zygopteran species (Odonata: Coenagrionidae)

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Abstract

Microsatellites have been infrequently used for genetic analysis of odonate species. Here, we report four microsatellite loci that are polymorphic in *Coenagrion mercuriale*. Furthermore, we examine the success of cross-species amplification of a panel of 19 microsatellite loci that were developed from *C. mercuriale* in seven other European odonate species. PCR with microsatellite primers is more likely to be achieved in species that are closely related to the species used for marker isolation. Overall, 10 microsatellite loci amplified interpretable PCR products (seven loci were variable) in both *C. puella* and *C. pulchellum*, whilst two loci were variable in *Ischnura elegans*. These markers should facilitate genetic research into these zygopteran species.

Introduction

Research into the phylogeny, evolution, ecology and population dynamics of a wide variety of taxa has been facilitated by the use of genetic markers. Many different types of molecular markers have been developed, each of which possesses a particular set of benefits and drawbacks (reviewed by Loxdale & Lushai 1998; Sunnucks 2000). Whilst genetic markers have been used to investigate some biological problems in the Odonata, they appear to have been rather infrequently used in this group compared with other insect orders, such as the Diptera and Hymenoptera.

Much genetic research on odonates has characterised sequence variation at mitochondrial (mt) DNA to examine phylogenetic (Chippindale et al. 1999) or phylogeographic (Freeland & Conrad 2002; Turgeon & McPeek 2002; Freeland et al. 2003) relationships. One area of investigation that is conspicuously lacking, however, is the determination of genetic relationships among individuals of the

same species both within and between populations. This bias probably stems from technical rather than scientific difficulties since odonates demonstrate a variety of ecological and behavioural strategies that would make them interesting model organisms to test hypotheses, for example, about the evolution and consequences of different mating systems. Furthermore, several European zygopteran species, e.g. *Coenagrion mercuriale* (Charpentier), are showing population decline. Genetic data on the relationships between fragmented populations would aid their management and protection. Whilst mtDNA can be used to estimate the genetic relationships between higher taxa and populations it cannot be used alone to determine parentage or study sex-biased gene flow because it is almost exclusively maternally inherited. Studies that have characterised genetic variation between individual odonates, therefore, have used either RAPDs (Hadrys et al. 1993; Hooper & Siva-Jothy 1996) or microsatellite loci (Cooper et al. 1996; Fincke & Hadrys 2001).

The advantage of RAPD markers is that they can be generated without prior DNA sequence information. This technique, however, is limited because it requires high-quality genomic DNA and may produce non-specific bands during PCR (Loxdale & Lushai 1998; Sunnucks 2000). Amplified fragment length polymorphisms (AFLPs) can overcome some of these problems but recent work suggested that AFLPs might be unsuitable for estimating some population genetic parameters in Zygoptera (Wong et al. 2001). By contrast microsatellite loci are now the markers of choice for intraspecific genetic analysis (Jarne & Lagoda 1996; Sunnucks 2000). Microsatellites, or simple sequence repeats (SSRs), consist of short (1-6 base pair) DNA motifs that are arranged in tandem. This class of genetic marker is amplified by PCR using oligonucleotide primers situated on the unique DNA sequence that flanks the repetitive region. Alleles at a microsatellite locus vary in the number of repeat units and hence their length. Different alleles can be identified, therefore, by the size of the PCR fragment after acrylamide electrophoresis. Most microsatellite loci are believed to be selectively neutral. The major benefits of microsatellite loci include their typically high level of polymorphism and that small quantities of (even degraded) DNA can be used, thereby allowing genetic analysis of historical specimens and non-destructive (i.e. insect leg) samples (reviewed by Jarne & Lagoda 1996; Sunnucks 2000). Somewhat surprisingly, few microsatellite loci have been characterised in odonates: two polymorphic loci have been described for the zygopteran *Ischnura elegans* (Vander Linden) (Cooper et al. 1996) and – at least – four microsatellites have been used as part of a study into larval survivorship in the neotropical zygopteran *Megaloprepus caerulatus* (Drury) (Fincke & Hadrys 2001).

The paucity of published odonate microsatellites suggests that developing microsatellite loci in this taxon may be particularly problematic. Since the major drawback of using microsatellites is the initial time and cost required to develop loci de novo it is clearly beneficial if loci developed from one species will amplify alleles in non-target taxa. Watts et al. (2004) recently characterised a panel of 15 microsatellite loci in the endangered *C. mercuriale* as part of an ongoing study into the genetic variation and population structure of this species in the UK. Here, we (1) describe an additional four dinucleotide microsatellite loci that are

polymorphic in *C. mercuriale* and (2) report the usefulness of this panel of genetic markers in seven other odonate species representing six European genera. Some of these loci show cross-specific amplification and thus represent a useful resource for the study of molecular ecology in Coenagrionidae.

Materials and Methods

Microsatellite loci were developed in *Coenagrion mercuriale* using an enrichment technique that has been described elsewhere (Watts et al. 2004). In addition to these 15 loci we sequenced and designed primers for another 4 dinucleotide (CA) loci that subsequently proved to be polymorphic in *C. mercuriale*. Genbank accession numbers and PCR conditions for these new loci are described in Table 1. In the UK, there are eleven resident species of Coenagrionidae from five genera. We obtained samples from representatives of each genus: *Ceriagrion tenellum* (de Villers), *Coenagrion puella* (Linnaeus), *C. pulchellum* (Vander Linden), *Erythromma viridulum* (Charpentier), *Ischnura elegans* and *Pyrrhosoma nymphula* (Sulzer). Specimens of the lestid *Lestes sponsa* (Hansemann) were also obtained. All samples were stored in absolute ethanol for several weeks until DNA extraction could be undertaken.

Genomic DNA was isolated from the thorax muscle tissue from all samples except for *C. mercuriale* (because of its conservation status) and *E. viridulum* where DNA was extracted from a single leg. We extracted DNA using the high-salt protocol of Sunnucks & Hales (1996), but with reduced volumes when leg samples were used. Sample sizes for all species tested for microsatellite variability are presented in Tables 1 and 2. DNA was diluted to approximately 5-50 ng/µl for PCR. We tested all of the 19 available microsatellite loci that are polymorphic in *C. mercuriale* (see Watts et al. 2004 and Table 1) for cross-specific amplification in the seven species of European zygopteran listed above. Primers for PCR are the same as those previously described except at LIST4-072 where the reverse primer AATGGCACTGGCTGTATTCCG was used (expected clone size 129 base pairs [bp]). PCR conditions were optimised for all species by (1) varying the magnesium ion concentration to 1.5, 3.0, 4.0 and 5.0 mM and also (2) the annealing temperature (T_a) along a gradient between 45°C and 65°C at each of the three magnesium ion concentrations. This procedure identified an initial set of conditions that would at least produce PCR products for each locus, where possible. Subsequent PCRs were optimised by narrowing the T_a gradient – at a magnesium ion concentration that consistently produced a bright PCR product – until a single band was attained (if possible). The final PCR conditions reported are those that produced consistent PCR amplification, and are not necessarily the highest or lowest magnesium ion concentrations or annealing temperatures that produced a product for some of the samples.

Microsatellite alleles were amplified by PCR in a 10 µl final reaction volume using ReddyMix PCR Mix (ABgene) on a Dyad DNA engine (MJ Research Inc.). PCR conditions were: (1) 1 min at 95°C, (2) 6 cycles of 30 s at 95°C, 30 s at T_a°C and 45 s at 72°C, (3) 26 cycles of 30 s at 92°C, 30 s at T_a°C and 55 s at 72°C, and (4) 72°C for 30 min. Each PCR contained 75 mM Tris-HCl (pH 8.8), 20 mM

$(NH_4)_2SO_4$, 0.01% (v/v) Tween 20®, 0.2 mM each dNTP, 1.5-5.0 mM $MgCl_2$ (see Tables 1 & 2 for optimal concentrations) 10 µg BSA, 10-50 ng template DNA, 10 pmol forward primer, 15 pmol reverse primer and 0.25 units Taq polymerase (ABgene). The forward primers were 5' labelled with either 6-FAM, NED, PET or VIC fluorescent dyes (Applied Biosystems) (Tables 1 & 2). PCR products were pooled with a 500 bp (LIZ) size standard (Applied Biosystems) and separated by capillary electrophoresis through a denaturing acrylamide gel matrix on an ABI3100 automated sequencer (Applied Biosystems).

Table 1. Characteristics of four polymorphic microsatellite loci for sixteen *Coenagrion mercuriale* from the River Itchen, UK. T_a, annealing temperature of the primer pair (°C); Mg, magnesium chloride concentration of PCR reaction (mM); Clone, size of cloned microsatellite (base pairs); Allele, size range of observed alleles (base pairs); N_a, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity.

Locus name: LIST4-038 **Accession number:** BV069720

Forward primer	Reverse primer	Repeat motif
5'TCCTATGAGCAGTCTTCACC3'	5'ATTTAGCACCCAAATGAAAT3'	$(AC)_9(GCACAC)_4(AC)_7$

Dye	T_a	Mg	Clone	Allele	N_a	H_o	H_e
PET	50	4.0	188	181-187	3	0.125	0.565

Locus name: LIST4-067 **Accession number:** BV079670

Forward primer	Reverse primer	Repeat motif
5'TCAAGGTGCCGAACTGTGC3'	5'CTTTATTGCAGCCATTACCGC3'	$(TG)_{23}$

Dye	T_a	Mg	Clone	Allele	N_a	H_o	H_e
VIC	60	4.0	115	103-135	7	0.600	0.720

Locus name: LIST4-071 **Accession number:** BV079667

Forward primer	Reverse primer	Repeat motif
5'AATAAATCTACACCAAGGGC3'	5'TCCATGGACTTTCATGCAGG3'	$(CA)_{24}$

Dye	T_a	Mg	Clone	Allele	N_a	H_o	H_e
6-FAM	55	4.0	107	75-105	6	0.813	0.762

Locus name: LIST4-072 **Accession number:** BV079668

Forward primer	Reverse primer	Repeat motif
5'TGTGTGCCAAACATTCGG3'	5'CAAAGTGATAGAAGGTTGC3'	$(TG)_{12}$

Dye	T_a	Mg	Clone	Allele	N_a	H_o	H_e
VIC	57	4.0	286	286-292	2	0.143	0.203

Genetic diversity within the *C. mercuriale* sample was estimated from (1) number of alleles (N_a), (2) the observed heterozygosity (H_o) and (3) expected heterozygosity (H_e) at each locus. These statistics were calculated using Arlequin version 2.000 (Schneider et al. 2000). The significance of any deviation from Hardy-Weinberg equilibrium was assessed using a modification of Guo & Thompson's (1992) Markov-chain random walk algorithm (1,000 iterations) as implemented by Arlequin version 2.000 (Schneider et al. 2000). Because of the small sample sizes used to test for polymorphisms where we achieved successful cross-species amplification only the observed number of alleles and their size range are reported.

Results

All of the four newly developed microsatellite loci were polymorphic in our sample of 16 *Coenagrion mercuriale* (Table 1). The number of alleles at these loci varied between 2 and 7, whilst the observed heterozygosity varied between 0.125 and 0.813 and the expected heterozygosity ranged between 0.203 and 0.762. Only one locus - LIST4-038 - showed a strong and significant ($p < 0.05$) heterozygote deficit. LIST4-067 & LIST4-071, the two loci with the longest continuous runs of dinucleotode repeats, were the most variable (Table 2).

None of the 19 microsatellite loci isolated from *C. mercuriale* amplified resolvable products in either *Lestes sponsa* or *Erythromma viridulum*. Four loci (LIST4-038, LIST4-053, LIST4-059 & LIST4-062) either failed to amplify PCR products or produced several extra bands after PCR and, therefore, have not been considered further. This left 15 microsatellite loci (79% of the available markers) that could be amplified in one or more of the non-target zygopteran species and resolved clear and unambiguous alleles after PCR. Nine of these loci were polymorphic in three of the species (from two genera) examined (Table 2).

The specific PCR conditions and allele sizes for each locus-species combination are provided in Table 2. It should be noted that LIST4-024 (*Coenagrion pulchellum*) sometimes produced an extra band at 330 bp and LIST4-060 produced extra bands at 340 bp (*Ischnura elegans*). Since these bands are very different in size to the putative alleles they do not interfere with genotyping. The same microsatellite loci did not amplify in all of the species. In summary, however, successful cross-species amplification was greatest in the congeneric *C. puella* and *C. pulchellum*. Ten loci (53% of loci tested) amplified putative alleles for both of these species, and seven of these (70% of working loci) were variable. Furthermore, it is likely that genetic variability at these loci will be high for both species. For example, the sample of *C. puella* (n = 8) had at least as many alleles as the sample of *C. mercuriale* at LIST4-002 (n = 44, Watts et al. 2004) and LIST4-072 (n = 16, Table 1), whilst the smaller (n = 4) sample of *C. pulchellum* had the same or more alleles at four loci, LIST4-002, LIST4-024, LIST4-037 (n = 44, Watts et al. 2004) and LIST4-073 (n = 16, Table 1). Five microsatellites, LIST4-002, LIST4-063, LIST4-066, LIST4-067 and LIST4-072, were polymorphic in both non-target species of *Coenagrion* (Table 2).

Eight loci (44% of those available) demonstrated successful cross-species amplification in *I. elegans*, with two markers (LIST4-023 and LIST4-060) proving to be polymorphic in our sample of three individuals. Only three loci (16%) produced distinct alleles for the two remaining species, *Ceriagrion tenellum* and *Pyrrhosoma nymphula*, in which cross-specific microsatellite amplification was achieved, although no allelic variability was observed at any locus. Although LIST4-071 produced a distinct band in two non-target species it should be noted that the putative allele was much larger than the cloned locus in *C. mercuriale* (cf. Tables 1 and 2).

Where the microsatellite loci were polymorphic in non-target species, the alleles were significantly ($p < 0.05$ for all comparisons, Mann Whitney *U* test, data not shown) shorter in length compared with corresponding alleles at *C. mercuriale* for all loci except for *C. pulchellum* (LIST4-002, $p = 0.857$; LIST4-063, $p = 0.166$; LIST4-066, $p = 0.104$), *C. puella* (LIST4-066, $p = 0.752$) and *I. elegans* (LIST4-060, $p = 0.051$) where no significant differences were observed.

Discussion

Coenagrion mercuriale is a rare species with a fragmented distribution in England and Wales. It is presently protected under the UK's Wildlife & Countryside Act of 1981 and has a high conservation priority. We have developed a panel of microsatellite loci to facilitate our ongoing research into the genetic variability and effective population sizes of this species where it occurs in Great Britain. The new loci described here have increased the number of polymorphic microsatellite loci available to study this species from 15 up to 19. Two loci (LIST4-067 & LIST4-071) are particularly variable and, over all loci now published, there is now clearly enough 'genetic resolution' available to accurately determine genetic relationships between individuals within populations and also to identify likely migrants and source populations in this species.

The lack of published microsatellite loci for odonate species in general suggests, for example, that repetitive DNA may occur rather infrequently in some odonate genomes; however, the underlying mechanisms behind this apparent phenomenon have yet to be explored systematically. It is also difficult to establish useable microsatellite loci within the Lepidoptera (Nève & Meglécz 2000) and GENBANK presently contains microsatellite sequences for only 11 lepidopteran genera. Because our intensive development of microsatellite loci in *C. mercuriale* yielded a reasonably high number of microsatellite loci (for this insect group) we were keen to explore the possibility of using these loci to facilitate research into the genetic structure of other zygopteran species and bypass the relatively expensive process of isolating microsatellites. The success rate of amplification in non-target zygopteran species was somewhat mixed but generally correlated with taxonomic relationships. This is to be expected given the results of other, large-scale studies into cross-species amplification at microsatellite loci (e.g. Primmer et al. 1996).

No microsatellite locus examined in this study amplified interpretable PCR fragments in the zygopteran species from a different family – Lestidae – to that

Table 2. Summary PCR and allele characteristics of cross-species amplification in five zygopteran species for 19 microsatellite loci isolated from *Coenagrion mercuriale*. T_a, annealing temperature of the primer pair (°C); Mg, magnesium chloride concentration of PCR reaction (mM); N_a, number of alleles; Size, minimum and maximum size range (base pairs) of alleles observed; n = sample size. Polymorphic loci are highlighted by a black dot.

Species	Locus	T_a	Mg	N_a	Size	
Coenagrion puella	• LIST4-002	50	4.0	4	119	128
(n = 8)	• LIST4-023	51	4.0	2	194	196
	LIST4-030	45	4.0	1	248	
	LIST4-034	50	4.0	1	254	
	LIST4-037	50	4.0	1	215	
	• LIST4-060	45	4.0	3	195	209
	• LIST4-063	50	3.0	4	196	202
	• LIST4-066	50	4.0	2	179	185
	• LIST4-067	60	4.0	3	82	86
	• LIST4-072	55	4.0	4	137	151
Coenagrion pulchellum	• LIST4-002	50	4.0	3	119	125
(n = 4)	• LIST4-024	50	3.0	3	209	213
	LIST4-030	45	4.0	1	248	
	LIST4-034	50	4.0	1	255	
	• LIST4-037	50	4.0	3	219	221
	LIST4-060	45	4.0	1	196	
	• LIST4-063	50	3.0	3	214	226
	• LIST4-066	50	4.0	2	178	181
	• LIST4-067	60	4.0	4	82	88
	• LIST4-072	55	4.0	3	138	144
Ischnura elegans	LIST4-002	50	4.0	1	92	
(n = 3)	• LIST4-023	44	4.0	2	116	119
	LIST4-031	45	4.0	1	186	
	LIST4-035	50	4.0	1	105	
	LIST4-042	48	4.0	1	272	
	• LIST4-060	50	4.0	2	190	204
	LIST4-067	60	4.0	1	76	
	LIST4-071	55	4.0	1	454	
Ceriagrion tenellum	LIST4-023	46	4.0	1	119	
(n = 3)	LIST4-034	50	4.0	1	244	
	LIST4-037	48	4.0	1	271	
Pyrrhosoma nymphula	LIST4-030	48	4.0	1	195	
(n = 3)	LIST4-037	45	4.0	1	212	
	LIST4-071	55	4.0	1	460	

Note: LIST4-038, LIST4-053, LIST4-059 and LIST4-062 failed to produce any cross-specific PCR products and have been omitted from this table.

of the focal species. Whilst we only examined one non-coenagrionid species the success rate of cross amplification was poor other than in *Coenagrion* and *Ischnura*. As such we do not consider the loci developed in the C. *mercuriale* to be useful as genetic markers in odonate species from outside the Coenagrionidae. Whilst at least three microsatellite loci were amplified in five of the six coenagrionid species we examined, variable loci were found only in three species, C. *puella*, C. *pulchellum* and *Ischnura elegans*. Clearly a larger sample size may reveal further polymorphisms, however, it is likely that other microsatellite loci will have to be specifically developed for meaningful genetical research on individuals of *Ceriagrion tenellum* and *Pyrrhosoma nymphula*.

Five species of the genus *Coenagrion* currently reside in the UK. Since three of these species have restricted distributions it is important to understand the population dynamics of these species as part of the management strategies to prevent their decline. Genetic variation at microsatellite loci, for example, can provide information on likely migrants (Waser & Strobeck 1998) and be used to monitor effective population size (Wang & Whitlock 2003). Genetic markers clearly require a certain degree of variability to be useful for these purposes. A particularly surprising result is that even with a restricted number of samples, 2 and 4 of the polymorphic loci had more alleles in the non-target taxa than in our larger sample of C. *mercuriale*. Several studies have considered the possibility of a selection bias at microsatellite loci used for cross-species amplification (e.g. see Ellegren et al. 1997; Crawford et al. 1998). If such an 'ascertainment bias' operates then microsatellites are expected to be longer (i.e. contain more repeats) in the target species because they represent a non-random selection of loci that possess many repeats. Since allele length is typically positively correlated with heterozygosity at microsatellite loci (e.g. Weber 1990) the corollary is that loci will usually be more polymorphic in the species used for marker isolation. One concern, therefore, is that whilst markers may amplify in non-target taxa, the loci may not be particularly informative. Whilst our data should be regarded as preliminary, there is evidence that the majority of loci are indeed longer in C. *mercuriale* than in other species; we did not observe, however, a significant lack of allelic variation in the non-target taxa. The small sample sizes used here was expected to limit our ability to detect polymorphisms, however, the presence of (at least) seven variable microsatellite loci in both C. *pulchellum* and C. *puella* should enable genetic research at both population- and individual-level (e.g. see Bernatchez & Duchesne 2000) for both species.

Two microsatellite loci have already been isolated in *I. elegans* (Cooper et al. 1996). With the loci described here this increases the number of available, polymorphic microsatellite loci up to four. Whilst more loci are clearly needed for robust estimates of relationships between populations, these extra loci may be used to increase the confidence of genetic relationships between individuals within a population. Given the successful cross-amplification of microsatellites across different species of *Coenagrion*, it is conceivable that these loci may be used in other species of *Ischnura*. Finally, it is worth mentioning that neither of the *I. elegans* microsatellite loci produced consistent PCR amplification in C. *mercuriale* (P.C. Watts unpubl.).

In conclusion, the microsatellite loci described here represent a valuable resource to investigate intraspecific genetic relationships within the zygopteran genus *Coenagrion* and have increased the genetic resolution available to address biological questions in *I. elegans*.

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