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Individual and population variation in invertebrates revealed by Inter-simple Sequence Repeats (ISSRs).

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Abstract

PCR-based molecular markers are well suited for questions requiring large scale surveys of plant and animal populations. Inter-simple Sequence Repeats or ISSRs are analyzed by a recently developed technique based on the amplification of the regions between inverse-oriented microsatellite loci with oligonucleotides anchored in microsatellites themselves. ISSRs have shown much promise for the study of the population biology of plants, but have not yet been explored for similar studies of animals. The value of ISSRs is demonstrated for the study of animal species with low levels of within-population variation. Sets of primers are identified which reveal variation in two aphid species, *Acyrthosiphon pisum* and *Pemphigus obesinymphae*, in the yellow fever mosquito *Aedes aegypti*, and in a rotifer in the genus *Philodina*.

Keywords: inter-simple sequence repeats, ISSRs, microsatellites, molecular markers

Introduction

Simple sequence repeats (SSRs or 'microsatellites') are short, hypervariable elements distributed throughout the genomes of eukaryotes. They have proven to be invaluable sources of markers for population-level studies (Goldstein and Schlotterer, 1999), but can require substantial investment for each taxon studied, since primers often must be uniquely designed for flanking regions. SSRs can also yield PCR-based multilocus markers amenable to rapid development. These markers are derived from primers that anchor within the elements themselves, rather than in flanking regions (Zietkiewicz et al., 1994). Such primers amplify the regions between closely-spaced, inversely oriented SSRs, resulting in anonymous, typically dominant, di-allelic Mendelian markers when divergence in SSR sites or chromosomal structural rearrangements occur (Wolfe & Liston, 1998; Wolfe et al., 1998).

Although inter-SSRs or ISSRs have been used by plant biologists for a variety of applications (Wolfe & Liston, 1998), they have not been used for animal population studies, and are only rarely used in animals (Kostia et al., 2000; Reddy et al., 1999). I evaluated ISSRs for population-level studies in two species of cyclically parthenogenetic aphids (*Acyrthosiphon pisum* (Hemiptera: Aphididae) and *Pemphigus obesinymphae* (Hemiptera: Pemphigidae)) which, because of their small size and low levels of within-population variation, are particularly well suited for PCR-based markers. To assess the ease with which ISSRs may be applied to other invertebrate populations, I also conducted a small survey for ISSR variation in the yellow fever mosquito *Aedes aegypti* (Diptera: Culicidae), and the asexual rotifer *Philodina* (Rotifera: Philodinidae). For the two aphid species and *Ae. aegypti*, there is currently little consensus on appropriate markers that are informative at the population level (N. Moran and S. Kaplan, pers. com.).

Methods

Initially, one sample from each species was screened for banding patterns from synthetic 17-base oligonucleotides consisting either of a 2-base repeat motif and 1- or 2-base, 3´-end anchors, or of 3-base repeat motifs with no anchors (Table 1; primers obtained from the University of British Columbia Nucleic Acid-Protein Service Unit, UBC Primer Set #9). Where possible, prior knowledge of microsatellite motif frequency or primer efficiency was used to reduce the extent of blind screening. For example, *P. obesinymphae* was screened with 47 primers, which then helped guide primer selection for *A. pisum*, a distantly related aphid. For all four species, primers with 'A + G' and 'A + C' compositions were emphasized, because these are common repeat motifs across animal groups (Schug et al., 1998).

For primers that gave bright, distinguishable bands, polymorphism was tested by amplifying multiple individuals (Table 1). Sequence data for each species was also compiled to give an independent estimate of genetic variation between samples (Table 1). To determine if ISSRs are suitable for larger scale population surveys in aphids, *P. obesinymphae* were typed from seven clones,

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in which an unknown number of individuals were different from the maternal genotype. These results were checked with an independent molecular marker - a single nucleotide polymorphism that, when different from the maternal genotype, unambiguously discriminates *P. obesinymphae* clones, albeit with lower resolution than the multi-locus ISSR markers (Abbot et al., 2001).

Whole genomic DNA was extracted from single individuals for aphids and for mosquitoes and from laboratory clones of multiple *Philodina* initiated from single individuals. All PCR reactions were carried out in a 20 µl reaction mixture containing 20-40 ng total DNA, 1X PCR buffer (200 mM tris-HCL (pH 8.4), 500 mM KCL; Gibco/BRL, Rockville, Maryland, U.S.A.), 0.25 mM of each dNTP, 3.75 pM of each primer, 3.0 mM $MgCl_2$, and 2.4 units of Platinum *Taq* DNA polymerase (Gibco/BRL). PCR cycling conditions for all species on an Eppendorf Mastercycler were: 94° C, 2 min, for 1 cycle; 94° C, 30 s; 68° C, 30 s; 72° C, 1 min, for 1 cycle; the annealing temperature was dropped 0.7° C for each of the subsequent 12 cycles; then, for 25 cycles : 94° C, 30 s; 55° C, 30 s; 72° C, 1 min. All reactions were visualized on 2.5% agarose gels stained with EtBr.

Results

For each species, a number of primers produced distinguishable bands (Table 1). The most successful primers were those consisting of 'A + C' and 'A + G' repeats. For *P. obesinymphae*, the least variable of the four species based on sequence divergence (Table 1), no single oligonucleotide produced diagnostic markers that distinguished all genotypes. For example, the oligo $(AC)^{8}G$ distinguished 6 of 9 different genotypes (Fig. 1A; lanes 6 and 9, and 8 and 9 are ambiguous). However, for genotypes that do differ in banding profiles, ISSRs can distinguish between aphid clones, without banding artifacts or null alleles within clones (Fig. 1B), indicating that they are relatively insensitive to chance variation in PCR performance while providing fragments that sufficiently differ in size to be visualized on agarose gels.

In the larger scale population survey in *P. obesinymphae*, 72 aphids were typed in all, and 22 (31%) of these were scored as different from the maternal genotype. When these results were compared with the single nucleotide polymorphism, there was a

Table 1. Intersimple sequence repeat (ISSRs) markers in different taxa, by taxon name, UBC primer number, sequence, number of bands amplified, number of polymorphic bands, and maximal sequence divergence between samples (uncorrected p).

Taxon	Species	Collection locations	UBC Primer no. (5´-3´)	Sequence (5´-3´)	No. of bands amplified[a,c]	No. of polymorphic bands[b] (%)	Max sequence divergence (%; region, size)
Aphid	*Pemphigus obesinymphae*	Santa Cruz Co., Arizona	809	$(AG)^{8}G$	8	3 (38%)	0.00% (COI/II, 1400 bp)[1]
			811	$(GA)^{8}C$	9	2 (22%)	
			812	$(GA)^{8}A$	5	1 (20%)	
			819	$(GT)^{8}A$	5	0	
			826	$(AC)^{8}C$	9	2 (22%)	
			827	$(AC)^{8}G$	8	6 (75%)	
			841	$(GA)^{8}YC$	1	0	
			857	$(AC)^{8}YC$	4	0	
	Acyrthosiphon pisum	Dane Co, Wisconsin; Pima Co., Az.	809	$(AG)^{8}G$	5	2 (40%)	0.14% (TrpE/G *Buchnera* plasmid)[2]
			811	$(GA)^{8}C$	10	3 (30%)	
			827	$(AC)^{8}G$	5	2 (40%)	
Mosquito	*Aedes aegypti*	Pima Co., Az.; Bexar Co., Texas	811	$(GA)^{8}C$	6	2 (33%)	1.3% (COI, 791 bp)[3]
			818	$(CA)^{8}G$	7	5 (71%)	
			827	$(AC)^{8}G$	6	0	
Rotifer	*Philodina* spp.	El Paso Co., Tx; Santa Cruz Co., Az.; Yuma Co., Az.	808	$(AG)^{8}C$	12	10 (83%)	13% (COI, 591 bp)[4]
			826	$(AC)^{8}C$	12	6 (50%)	
			827	$(AC)^{8}G$	5	2 (40%)	
			868	$(GAA)^{6}$	9	2 (22%)	

[a] The cumulative number of bands distinguishable across different genotypes from the given localities. Weak and smeared bands were not scored.
[b] The approximate number of distinguishable polymorphic bands, given that weak or smeared bands were not scored.
[c] Primers also screened but which gave weak or no interpretable bands were: *P. obesinymphae*, UBC nos. 801-8, 810, 813-8, 820-5, 828-40, 861, 862, 865, 867, 868, 873, 880, 889; *A. pisum*, UBC no. 826; *A. aegypti*, UBC nos. 812-7, 819-25; *Philodina*, UBC no. 819.
[1] P. Abbot, unpublished data.
[2] J. Russell, unpublished data. *Buchnera* is a vertically-transmitted, bacterial endosymbiont of aphids, which has proven useful in population-level studies in aphids. TrpEG typically shows faster evolution than mitochondrial COI/II.
[3] S. Kaplan, unpublished data.
[4] B. Birky, unpublished data.

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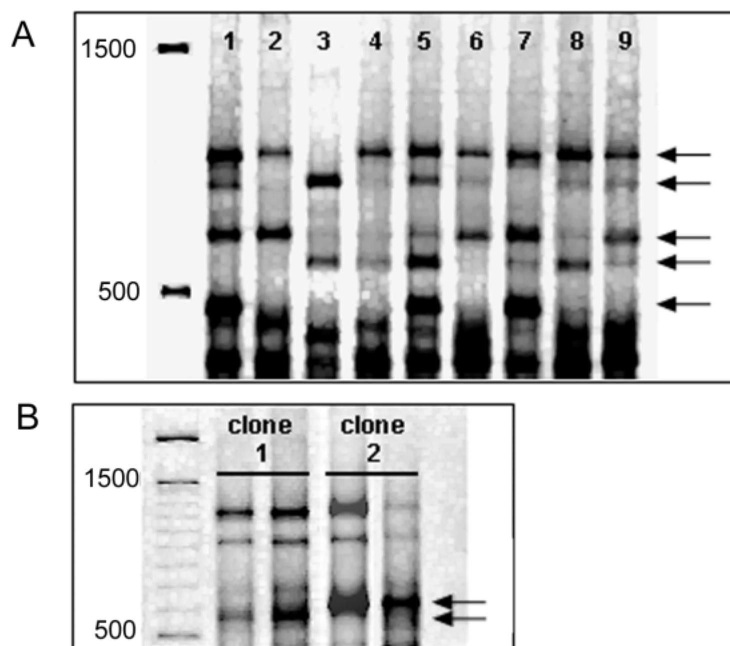

Figure 1. ISSR banding patterns for multiple genotypes in each of three species. Size standards in base pairs are in the left most lanes. **A:** Nine adult aphids (*Pemphigus obesinymphae*) collected from different clones at a single locality, amplified by the 3´-anchored primer (AC)^{8}G (UBC no. 827). Arrows highlight several of the unambiguously variable bands.; **B**: ISSR banding patterns ((AC)^{8}G) for two individual *P. obesinymphae* aphids each from two different clones. Clone-specific bands are indicated by arrows.

significantly positive association between the two markers (Likelihood ratio test, ÷^{2} = 6.693, $p < 0.01$), and they corresponded in 54 of the 72 tabulations (72%). As expected, the ISSR marker distinguished more non-maternal aphids than the single locus nucleotide polymorphism.

ISSRs can readily be applied to other animal species. For *Ae. aegypti*, the oligo (CA)^{8}G exhibited four unique banding profiles for each of the four genotypes, although some bands were not variable between individuals within populations (sample sizes were too small to determine if the bands were diagnostic for the populations sampled). By contrast, for *Philodina*, the oligo (AG)^{8}C provided fewer individually diagnostic bands but, while sample sizes were also small, more bands were subdivided between populations.

Discussion

These results show that, not surprisingly, the performance of ISSR primers varies across taxa, a result that likely reflects the different relative frequencies of microsatellite motifs in the different species. Nevertheless, ISSRs can be informative at various scales of genetic variation, as indicated by numerous such studies in plants (e.g., Clausing et al., 2000; Hess et al., 2000), and here by the contrast between the species sampled in the amount of sequence variation. ISSRs can be advantageous when time and materials costs preclude the development of more robust markers (e.g., locus-specific SSRs). The vagaries of PCR and the chosen method of band detection limit any PCR-based marker (Wolfe and Liston, 1998). But ISSR markers are typically highly reproducible, due to stringent annealing temperatures, long primers, and low primer-template mismatch (that is, the primers are not 'arbitrary', but designed *a priori* to anchor onto anonymous SSR loci; Wolfe et al., 1998). And while detection by more sensitive techniques (autoradiography or silver staining) on polyacrylamide gels might increase the resolution of co-migrating fragments (e.g., Godwin et al., 1997), ISSRs can reveal polymorphisms without more elaborate detection protocols (Nagaoka and Ogihara, 1997; Esselman et al., 1999).

Thus, for biological questions where genomic fingerprinting is appropriate (e.g., Abbot et al., 2001), ISSRs are a valuable addition to the inventory of PCR-based methods for rapid, large-scale screening of genetic variation in animal populations. Dr. Andrea Wolfe at Ohio State University maintains a helpful website on ISSRs: (http://www.biosci.ohio-state.edu/~awolfe/ISSR/ISSR.html).

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