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Molecular markers to differentiate two morphologically-close species of the genus *Sitobion*

Christian C. Figueroa[1], Jean-Christophe Simon[2], Jean-Francois Le Gallic[2] & Hermann M. Niemeyer[1]
[1]*Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile;*
[2]*Laboratoire de Zoologie, INRA, Domaine de la Motte 35650, Le Rheu, France*

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Abstract

A set of molecular markers to differentiate the aphid (Hemiptera: Aphidoidea) species *Sitobion avenae* (Fabricius) from *Sitobion fragariae* (Walker), is presented. These markers correspond to (1) a region of the mitochondrial DNA, (2) five species-specific RAPD banding patterns and (3) four microsatellite loci. Each of the markers was able to clearly distinguish between the species. The utility of each molecular marker is discussed. Mitochondrial DNA is best applicable to species determination and relative abundance, RAPDs to the evaluation of genetic diversity, and microsatellites to the assessment of the population genetic structure; the combined use of mtDNA with the other techniques can be of importance when the presence of hybrids is suspected, and RAPDs with microsatellites are best used together in population genetics and host preference studies.

Introduction

A clear identification of agronomically important species of aphids is necessary in order to establish properly their diversity and population dynamics in a crop. Molecular markers have been used to determine the variability in the field, to distinguish sibling species, and to assess the occurrence of parasitism (Black IV et al., 1992; Cenis et al., 1993; Landry et al., 1993; Puterka et al., 1993; Fukatsu & Ishikawa, 1994; De Barro et al., 1995a), where other methods have shown problems.

Classical morphological criteria for aphid species identification suffer from at least two drawbacks: they can only be applied to adult individuals since in many cases nymphal instars do not lend themselves to an accurate determination, and they may be affected by environmental factors such as climatic conditions and physiological status of the host plant (Cenis et al., 1993). Thus, identification based on morphological traits has been problematic for several closely related aphid species (Blackman & Eastop, 1984; Stroyan, 1984; Loxdale & Brookes, 1989). Biochemical methods based on allozyme polymorphism have been developed which provide diagnostic electrophoretic banding patterns for various aphid species (Loxdale et al., 1983; Loxdale & Brookes, 1989, 1990b). Those enzyme patterns have also been used to study the structure of aphid populations (Loxdale et al., 1985; Loxdale & Brookes, 1990a), but since they have revealed a low level of variability and have a limited use depending on sampling and preservation techniques, they are often not the method of choice to evaluate genetic variation (Puterka et al., 1993; Simon et al., 1995; Simon & Hebert, 1995).

Identification problems still remain in the English grain aphid *Sitobion avenae* (Fabricius), a monoecious and holocyclic/anholocyclic aphid that lives on numerous species of Poaceae, and in the closely related blackberry-grass aphid *Sitobion fragariae* (Walker), a heteroecious and also holocyclic/anholocyclic aphid, which alternates between a primary woody host (usually the blackberry *Rubus fruticosus* agg.) and various Poaceae species (especially cocksfoot grass, *Dactylus*

glomerata L.) (Blackman & Eastop, 1984; Loxdale & Brookes, 1990a).

The co-occurrence of *S. avenae* and *S. fragariae* on many cereal crops could lead to misidentification and hence to an overestimation of variability in *S. avenae*, the predominant species (Starý et al., 1994). Similar species complexes have been described using allozymes for several aphid taxa, including *Aphis fabae* (Blackman et al., 1989), *Aphis pomi* (Singh & Rhomberg, 1984), *Hyalopterus pruni* (Mosco et al., 1997), *Rhopalosiphum maidis* (Steiner et al., 1985; Simon et al., 1995) and *Sitobion* spp. (Blackman et al., 1989; Turak & Hales, 1994).

A novel group of molecular biology-based techniques are being widely used to study aphid population genetics, which include mitochondrial DNA (mtDNA), RAPD-PCR (Random Amplified Polymorphic DNA), and DNA fingerprinting. Mitochondrial DNA analysis has been used to differentiate between closely related aphid species (Foottit & Bonen, 1990), as well as to assess levels of genetic variation in biotypes and clones (Powers et al., 1989; Martínez et al., 1992; Barrette et al., 1994; Martínez-Torres et al., 1996; Simon et al., 1996). RAPD-PCR analysis using arbitrary primers (Welsh & McClelland, 1990; Williams et al., 1990) has also been used to detect interspecific and intraspecific genetic variation, in addition to detection and identification of parasitoid species (Black IV et al., 1992; Cenis et al., 1993; Puterka et al., 1993; Fukatsu & Ishikawa, 1994; De Barro et al., 1995a). DNA fingerprinting has shown to be a very useful method to detect genetic variation among aphid populations and clones where other methods failed to find variability (Carvalho et al., 1991; Shufran et al., 1991; De Barro et al., 1994).

We herein describe a set of molecular tools to distinguish unambiguously *S. avenae* from *S. fragariae*, including cytoplasmic (mtDNA), nuclear (microsatellite loci) and RAPD markers. Furthermore, since the RAPD and microsatellite markers generated a large number of polymorphisms in a small sample of *S. avenae*, their applications to the study of clonal diversity and genetic structure in Chilean populations in relation to the host plant (Via, 1991; De Barro et al., 1995b) is also briefly discussed.

Materials and methods

Aphid sampling and clone culture

Aphids were collected in fields at INIA-La Platina in Santiago, from three different hosts, wheat (*Triticum aestivum* L. and *Triticum durum* L.), oat (*Avena sativa* L.) and wild oat (*Avena fatua* L.) from November to December 1996.

The aphids were kept quarantined for ten days in order to remove parasitised or infected individuals. Surviving aphids were then caged separately on wheat (*T. aestivum*, cv. Millaleu) to reproduce under controlled conditions (20 °C and L16:D8). In this way, 49 putative aphid clonal lines were established from wheat, 35 from oat and 12 from wild oat. These were used to compare the abilities of different molecular markers to identify two aphid species and to assess genetic diversity and structure.

Morphological identification of S. avenae *and* S. fragariae. *Sitobion fragariae* may be distinguished morphologically from *S. avenae* by comparing the ratio between the length of the siphunculi and the length of the cauda in wingless adults. In *S. fragariae* the ratio is greater than 1.9, while in *S. avenae* it is lower than 1.5 (Blackman & Eastop, 1984; Remaudière et al., 1993). One adult wingless aphid from 49 of the putative clones collected, which included samples from the three hosts plants, was mounted to measure the lengths of the siphunculi and the cauda.

DNA isolation. DNA was obtained from three or four individuals of each putative aphid clone. The DNA was extracted by using the Isoquick kit (Microprobe Corporation™) according to the manufacturer's recommendations, and resuspended in 40 μL of RNase-free deionised water. The DNA obtained from each sample was analysed in a 0.8% agarose gel and photographed. The pictures were scanned and analysed with a computer to quantify concentration by comparison with a ladder (phage λDNA digested with *Hin*dIII) of known concentration. The yield of the DNA isolation protocol for *S. avenae* was 0.635 ± 0.021 μg per aphid ($n = 74$), and for *S. fragariae* 0.530 ± 0.039 μg per aphid ($n = 22$).

Mitochondrial DNA analysis. A 2.2 Kb segment of the mitochondrial DNA was used which contains subunit 1 of the NAD dehydrogenase (ND1) gene and portions of the flanking genes 16S and cytochrome

Table 1. Sequences and polymorphism shown by the random decamer primers used in the RAPD-PCR analysis to separate *Sitobion avenae* and *Sitobion fragariae*. P1 to P5 indicate the number of the primer

Primer	Sequence	No. of well amplified polymorphic bands	Ref.
P1	5′-ACG-GTA-CCA-G-3′	5	DeBarro et al., 1995a
P2	5′-ATG-GAT-CCG-C-3′	2	Black IV et al., 1992
P3	5′-TGA-GTG-GGT-G-3′	5	Landry et al., 1993
P4	5′-TGC-GGC-TGA-G-3′	9	Landry et al., 1993
P5	5′-GAA-CGG-ACT-C-3′	7	Puterka et al., 1993

b (cyt b), as previously reported in *Rhopalosiphum padi* (Simon et al., 1996). This segment was amplified by the Polymerase Chain Reaction (PCR), using the following pair of primers: TP5 primer (16S region): 5′-GAG TTC AAA CCG GCG TAA GCC AGG T-3′; CP1 primer (cyt b region): 5′-ACA TGA ATT GGA GCT CGA CCG GT-3′.

The amplification reaction was performed in a volume of 50 μl containing 5 μl of 10X *Taq* polymerase reaction buffer (Gibco-BRL), 4 mm $MgCl_2$, 200 μM dNTP's (Promega), 0.4 μM of each primer, 1 unit of *Taq* DNA polymerase (Gibco-BRL), 1 μl of template DNA, and ultrapure sterile water. The amplification cycles were run in a Perkin Elmer 9600 Thermal Cycler. In order to ensure the denaturation of DNA an initial denaturation step of 94 °C was included for 3 min, followed by 27 amplification cycles of 1 min at 94 °C, 2 min at 54 °C and 2 min at 72 °C, and supplemented by eight cycles in which the extension step of each successive cycle was increased by 20 s (Simon et al., 1996). An 25 μl aliquot of amplification products was taken and separated by electrophoresis in 1.0% agarose gels in 1X TAE buffer (40 mm Tris-acetate, 1 mm EDTA) pH 8.0 at 100 V, and visualised under UV light after ethidium bromide staining.

RAPD-PCR analysis. A set of five random decamer primers previously reported for different insect species were synthesised by Keystone Labs, Biosource International, and used to discern between *S. avenae* and *S. fragariae.* The sequences of the primers are shown in Table 1.

The RAPD-PCR amplification reactions were performed in a volume of 40 μl containing 4 μl of 10X *Taq* polymerase reaction buffer (Gibco-BRL), 2 mm $MgCl_2$, 200 μM dNTP's, 0.5 μM primer, 1 unit of *Taq* DNA polymerase (Gibco-BRL), 3 μl of 1:10 dilution of resuspended DNA and ultrapure sterile water. Thermal cycles consisted of an initial denaturation step of 94 °C for 3 min, followed by 40 cycles of 1 min at 92 °C, 1 min at 38 °C and 1 min at 72 °C. A final extension step of 6 min at 72 °C completed the reaction (Welsh & McClelland, 1990; Williams et al., 1990). The PCR products were separated by electrophoresis in 1.5% agarose gels in 1X TAE buffer pH 8.0 at 100 V.

Microsatellite loci. Patterns of allelic diversity were examined at four microsatellite loci (Sm10, Sm11, Sm17 and Sa4Σ). Sm11 is X-linked, while the three other loci are autosomal (Wilson et al., 1997; A. Wilson, pers. comm.). Primer sequences for Sm10, Sm11 and Sm17 loci and for Sa4Σ locus are available on request from P. Sunnucks and J. C. Simon, respectively. For the amplification of microsatellite loci the PCR reaction was carried out in 15 μl containing 0.5 units of Goldstar polymerase (Eurogentec), Mg^{2+}-free reaction buffer, 2 mM $MgCl_2$, 200 mM of dCTP, dGTP and dTTP, 20 mM dATP (all Pharmacia), 0.075 μl^{33}P-dATP (10 mCi/ml; DuPont), 10 pmol of each primer (Eurogentec) and less than 10 ng aphid DNA.

The PCR reactions were carried out in a Perkin Elmer 480 Thermal Cycler using the following conditions: after initial denaturation step for 3 min at 94 °C, 40 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 56 °C for Sm10 and Sm11 primers, 55 °C for Sm12 primer and 53 °C for Sm17 primer, and elongation reaction at 72 °C for 45 s. For the last cycle, the elongation time was extended to 4 min. Four μl of the reaction were mixed with 2X loading buffer (Sambrook et al., 1989), denatured for 3 min at 90 °C, loaded on a 6% polyacrylamide urea gel, and subjected to electrophoresis in 0.5X TBE (45 mM Tris-borate, 1 mM EDTA) pH 8.0. An M13mp18 sequencing ladder was used as a size marker. After

Table 2. Morphological identification of *Sitobion avenae* and *Sitobion fragariae*. The range and average values of the ratio between siphunculi and cauda length were used to distinguish species

Range	Mean ± SE	*n*	Species	% determination
1.07–1.48	1.29 ± 0.03	13	*S. avenae*	
				49%
1.90–2.50	2.07 ± 0.05	11	*S. fragariae*	
1.53–1.85	1.69 ± 0.02	25	Ambiguous	51%

electrophoresis the gel was dried and exposed to X-ray film (Kodak) for up to four days at room temperature.

Data analysis. Allelic frequencies and unbiased estimates of heterozygosities (Nei, 1978) were calculated using BIOSYS-1 (Swofford & Selander, 1989). Departure from Hardy-Weinberg equilibrium, linkage disequilibrium and genetic heterogeneity among the entire set or pairwise population samples were analysed using the Markov chain procedure in the GENEPOP package version 2.0 (Raymond & Rousset, 1995). Significance of multiple P-values were tested using Fisher's method (GENEPOP package).

Results

Morphological distinction between S. avenae *and* S. fragariae. Table 2 indicates the range and average values of the ratio between siphunculi and cauda length. Individuals with a ratio less than 1.5 were inferred as *S. avenae*; those with a ratio higher than 1.9 were inferred as *S. fragariae*. A clear determination was possible in only 49% of individuals, which was confirmed with molecular markers. The remaining 51% showed an ambiguous morphology, and a subsequent molecular analysis was necessary for their identification.

Mitochondrial DNA analysis. In *S. avenae* a unique segment of 2.2 Kb was amplified (Figure 1, lane 1), while in *S. fragariae* the primers amplified three different PCR products (Figure 1, lane 2), where the major fragment had a size (2.2 Kb) similar to that in *S. avenae*. The two other segments had lower molecular weights of 1.8 Kb and 1.0 Kb, respectively. This clear difference was used to distinguish between the two *Sitobion* species. *Rhopalosiphum padi* was used as a positive control (Figure 1, lane 3), where the primers

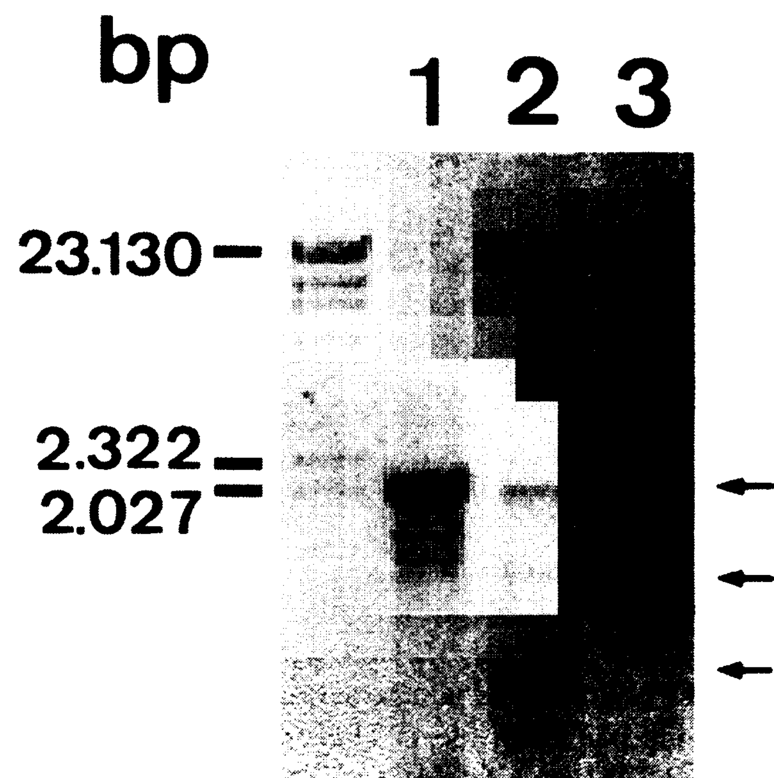

Figure 1. Differential amplification of mtDNA in *Sitobion avenae* and *Sitobion fragariae*. The amplification products were resolved through electrophoresis in a 1% agarose gel. Lane 1: *S. avenae*; Lane 2: *S. fragariae*; Lane 3: *Rhopalosiphum padi*. The amplified fragments are indicated with arrows. Phage lambda DNA digested with *Hind*III was used as molecular weight standard; bp: base pairs. The image was inverted to improve resolution.

amplified the 2.2 Kb segment as described previously (Simon et al., 1996). Other weak bands appeared occasionally which could not be avoided by changing the PCR conditions. Only the above indicated fragments were reproducible.

RAPD-PCR analysis. The primers produced fragments ranging in size from 400 to 1400 bp. Each primer described in this paper was able to produce consistently the same banding pattern when used to amplify DNA from individuals of the same clones, demonstrating the reproducibility of these markers (data not shown). Only well amplified and reproducible polymorphic fragments, ranging in number from 2 to 10 depending on the primer, were used to distinguish between the two species (Table 1). The RAPD method amplified bands that were polymorphic in both *Sitobion* species, generating very distinct species-specific banding patterns which allowed the unequivocal identification of *S. avenae* and *S. fragariae* (Figure 2).

No intraspecific variation was detected with the primers used, with the exception of P2. This primer detected intraspecific variability in *S. avenae*; however, this result did not alter its use in interspecific

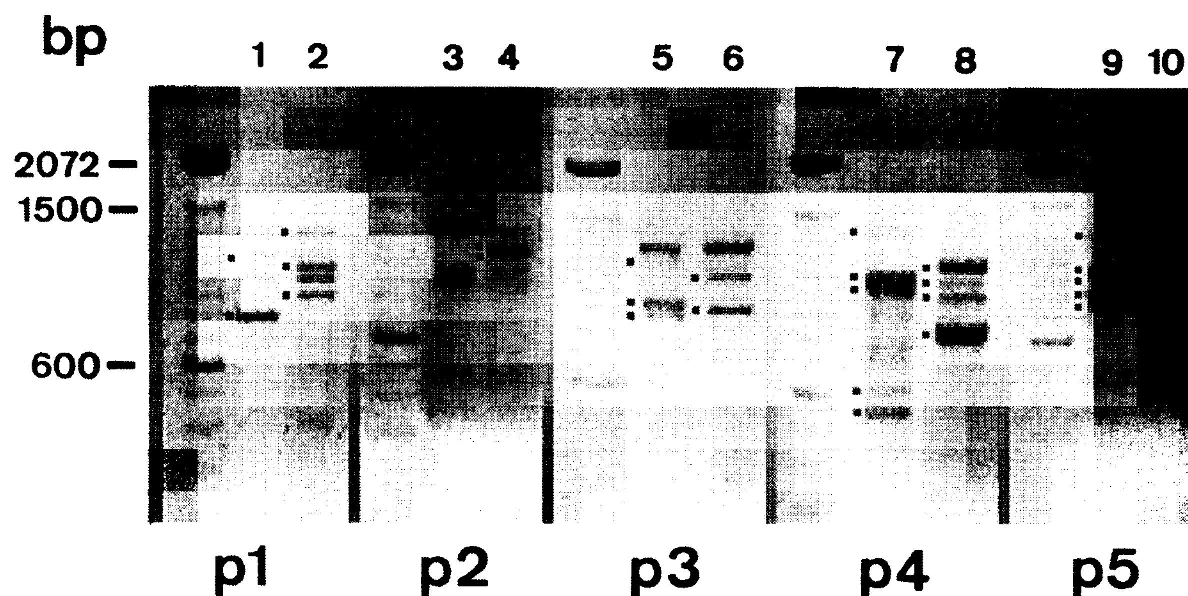

Figure 2. Interspecific identification using RAPD-PCR markers. Lanes 1, 3, 5, 7 and 9 show the band patterns amplified with DNA from *Sitobion avenae*, and lanes 2, 4, 6, 8 and 10 show the bands patterns amplified with DNA from *Sitobion fragariae*. P1 to P5 indicate the primer used. Polymorphic and well amplified bands are indicated with black dots. As molecular weight standard a 100 bp size marker was used. The images were inverted to improve resolution.

identification. The polymorphism found in *S avenae* was used to evaluate variability with respect to the host plant species and cultivar from which the aphids were collected. A dendrogram (not shown) of the putative clones was constructed by the UPGMA method based on the percent of disagreement, which was determined on the basis of presence (1) or absence (0) of the chosen polymorphic bands (see Figure 2). Percent disagreement is particularly useful if the data included in the analysis are categorical in nature. This index was computed as distance $(x, y) = (\text{Number of } x_i \neq y_i)/n$. The analysis allowed the evaluation of genetic diversity (number of clones) of the aphids on two species of wheat, detecting 16 clones in *T. aestivum* cv. Huayún ($n = 42$), 8 clones in *T. durum* cv. Llareta ($n = 34$) and 8 clones in *T. durum* cv. Chagual ($n = 82$). These results show a higher genotypic variability in *T. aestivum* (38%) compared to *T. durum* (24% and 10%, respectively.

Table 3. Differentiation between *Sitobion avenae* and *Sitobion fragariae* using microsatellite loci. DNA from aphid putative clones of both species was subjected to PCR using primers for four different microsatellite loci. The amplified fragments were used as alleles of each locus; their size is given in number of base pairs

Species	Locus			
	Sm10	Sm11	Sm17	Sa4Σ
Alleles in	164	144	178	168
S. avenae	166	149	180	172
	170	155	181	176
Alleles in	157	158	174	142
S. fragariae	161			

Microsatellite analysis. Differentiation between *S. avenae* and *S. fragariae* was also possible through the analysis of four microsatellite loci. The analysis comprised 28 putative aphid clones collected from wheat, 35 from oat and 12 from wild oat. While all individuals of *S. fragariae* shared the same genotype, 9 genotypes were found within the individuals of *S. avenae*. Additionally, the alleles and genotypes of *S. fragariae* were never found in *S. avenae* (Table 3).

To demonstrate the applicability of the technique, we evaluated and compared the allele and genotype frequencies of *S. avenae* collected on different host plant species (Table 4). Analysis of allelefrequencies of all populations showed no significant differences between aphids from different hosts (P=0.64). *Sitobion avenae* clones were almost all heterozygotes at the four loci analysed (data not shown). The number of aphid genotypes in each host plant was determined, eight being found in wheat, six in oat and three in wild oat. A single genotype represented over 70% of aphids independent of the host plant, indicating low diversity of *S. avenae* in the studied area. Genotypes were homogeneously distributed over the populations, since they did not show significant differences (P=0.33) between the three host plants (paired comparisons).

Table 4. Comparison of gene frequencies at four microsatellite loci of *Sitobion avenae* on different host plants. The allele frequencies are given according to where the aphids were collected. The number of samples per host is indicated in parenthesis. W: wheat; O: oat; WO: wild oat

Locus	Sm10			Sm11			Sm17			Sa4Σ		
Alleles	164	166	170	144	149	155	178	180	181	168	172	176
W ($n = 28$)	0.464	0.500	0.036	0.429	0.071	0.500	0.036	0.482	0.482	0.071	0.500	0.429
O ($n = 35$)	0.457	0.500	0.043	0.457	0.000	0.543	0.000	0.500	0.500	0.129	0.500	0.371
WO ($n = 12$)	0.500	0.500	0.000	0.500	0.000	0.500	0.077	0.462	0.462	0.115	0.500	0.385

Relative abundance of S. avenae *and* S. fragariae *on wheat, oat and wild oat.* To determine the relative abundance of these two species, mtDNA and RAPD markers were used to classify aphids collected from three host plants. No disagreement was found using both methods, demonstrating their usefulness in aphid species determination. Table 5 shows that *S. avenae* was the most abundant species on every host.

Discussion

In order to distinguish between *S. fragariae* and *S. avenae*, a set of novel molecular markers was developed. Each and every molecular marker utilised allowed the distinction of the two species studied.

Mitochondrial primers previously developed for *R. padi* and amplifying a 2.2 Kb fragment (Simon et al., 1996; Martinez-Torres et al., 1996), were used to amplify mtDNA from *S. avenae* and *S. fragariae*. Differences were found in the number and size of the amplified products, allowing the immediate distinction between the species. Thus, in *S. avenae* only one 2.2 Kb fragment was amplified, while in *S. fragariae* two additional products of 1.8 Kb and 1.0 Kb were found. Since total DNA was used in the experiments, the presence of the additional fragments in *S. fragariae* could indicate a transfer of mitochondrial segments to the nuclear genome, a phenomenon which is common in the genus *Sitobion* (Sunnucks & Hales, 1996). However, this hypothesis may only be confirmed by cloning and sequencing the PCR products. The distinction between *S. avenae* and *S. fragariae* with the mitochondrial markers appears as the easiest, fastest and least ambiguous of the methods herein presented, although it is limited to the evaluation of relative abundance of the species under study. The use of mitochondrial markers combined with RAPD and microsatellite markers can be justified when hybrids are expected to occur, since the last two mentioned genomic markers are inherited in a different way than mitochondrial markers. In studies of British populations of *Sitobion,* nuclear and mitochondrial markers showed the presence of hybrids between *S. avenae* and *S. fragariae* (Sunnucks et al., 1997); in contrast, a French survey of *Sitobion* failed to show hybridisation (J. C. Simon, pers. comm.). No hybrids between *S. avenae* and *S. fragariae* were found in the present study.

RAPD markers have been used for the identification of genetic polymorphisms in *S. avenae* as well as in other aphid species (Black IV et al., 1992; Cenis et al., 1993; Puterka et al., 1993; Fukatsu & Ishikawa, 1994; De Barro et al., 1995a). In this work, RAPD markers using the primer P2 in a sample of 178 putative aphid clones not only allowed interspecific distinction, but also the identification of intraspecific polymorphism in *S. avenae* aphids collected from different wheat species and cultivars. The clonal diversity found in *T. aestivum* cv. Huayún was twice (16) that in *T. durum* cvs. Llareta and Chagual (8 in each), suggesting possible host-plant based differences (Vanlerberghe-Masutti & Chavigny, 1998).

Polymorphisms at microsatellite loci generated molecular markers consisting of a different set of alleles for each *Sitobion* species, thus allowing also a clear interspecific distinction. Microsatellites also offered the possibility to identify different genotypes in *S. avenae* and to analyse their distribution in populations on different host plant species, but the cost and time requirements are greater than with RAPD markers. The principal advantage of microsatellite markers with respect to RAPDs is the possibility to better assess the genetic structure of the population under study due to their codominant mode of inheritance. In the Chilean population, the genetic diversity was lower and the mean heterozygosity higher than in Old World populations (Sunnucks et al., 1997; Simon et al., 1999).

Table 5. Abundance of *Sitobion avenae* and *Sitobion fragariae* on three different host plants: wheat (*T. aestivum* and *T. durum*), oat (*A. sativa*) and wild oat (*A. fatua*)

	T. aestivum	*T. durum*		*A. sativa*	*A. fatua*
	cv. Huayún	cv. Llareta	cv. Chagual		
S. avenae	42	34	82	35	10
S. fragariae	1	3	16	0	2

These observations could result from a strong founder effect, as a consequence of an introduction of individuals mostly heterozygous for these loci, followed by an asexual reproduction regime. Alternatively, they could result from the relatively recent introduction of this species to Chile coupled with a high mutation rate within the marker. No genotypic or allelic differences were found in *S. avenae* from wheat, oat and wild oat, as was observed in British *S. avenae* populations from cocksfoot and wheat (De Barro et al., 1995a,b). However, these results need to be confirmed by studying a larger aphid sample with increased geographic and host plant diversity.

Finally, RAPD and mtDNA markers proved to be unambiguous when they were used to determine species and quantify the relative abundance of *S. avenae* and *S. fragariae* in different host plant species. Furthermore, RAPD and microsatellite markers allowed a preliminary assessment of clonal diversity and intraspecific genetic variability in relation to the host plant, and hence the evaluation of host-plant related population structure (Vanlerberghe-Masutti & Chavigny, 1998). Thus, the choice of one technique over another will depend first upon the scientific problem at hand, and second upon the equipment and economic possibilities of the laboratory.

In conclusion, the molecular markers employed in this study provide a fast and accurate methodology to differentiate between *S. avenae* and *S. fragariae*, complementing and increasing the reliability of analysis by morphological and allozyme gel electrophoretic methods. In addition, they can be powerful and useful tools in studies of population biology, genetic variability, genetic structure, and distribution and abundance of different species.

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