makes re-amplification unnecessary because DNA is extracted efficiently from the hydrophilic matrix. Its combination of spatial separation, precise excision, efficient extraction, and contamination-free subcloning provides high-quality characterization of DNA fragments.

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Microsatellite Allele Sizing: Difference between Automated Capillary Electrophoresis and Manual Technique

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ABSTRACT

By comparing data collected with different automated sequencers and a manual technique (fragment separation in a silver-stained polyacrylamide gel), we found strong discrepancies in allele size of microsatellite loci. To quantify the sizing bias generated by automated capillary electrophoresis, we typed 51 alleles at seven loci and found that differences between actual (manual) and called (automated) sizing were inversely related to locus size. This result seems independent of the fluorescent dye but might be due to different migration patterns of the size standard and the microsatellite loci. Thus, it is essential to distinguish between actual (that can only be confirmed by sequencing) and called (obtained with automated sequencer) allele sizes. To enable the comparison of data collected by different laboratories on different instruments, the greatest attention should be paid to material and protocol descriptions used for allele sizing, and reference standard DNA genotypes should be shared between collaborating laboratories. Without these precautions, scoring errors in allele size might result in important misleading conclusions.

INTRODUCTION

As the use of microsatellites increases in population biology, more methods for DNA fragment sizing are becoming available: manual electrophoresis in polyacrylamide gel with silver staining or radiolabeled detection and slab or capillary gel electrophoresis of fluorescent DNA fragments in automated sequencers. Besides the need for reliable techniques that give accurate and reproducible sizing, their concordance is essential for making comparable data collected by different equipment and laboratories. Schwengel et al. (5) found no sizing discrepancies between allele sizes of three microsatellite markers provided by a benchmark manual radiation-based method and a fluorescence-based method (ABI PRISM® 373; Applied Biosystems, Foster City, CA, USA). By contrast, when genotyping microsatellites with capillary array electrophoresis, Mansfield et al. (3) found a systematic bias in estimated fragment sizes. In the same way, Haberl and Tautz (2) showed that the fluorescence-based technique on a slab gel electrophoresis instrument (ABI PRISM 377) can lead to inaccurate allele size measures. These conflicting results lead us

to compare the sizing accuracy between capillary gel electrophoresis and a manual polyacrylamide method.

MATERIALS AND METHODS

Alleles from seven microsatellites (6,7) were amplified in the aphid *Rhopalosiphum padi* and typed both with a standard polyacrylamide method and the ABI PRISM 310 automated Genetic Analyzer. Alleles of homozygous individuals were counted only once, making a total of 578 different typings. Following the Applied Biosystems protocol for ABI PRISM 310, we also sequenced one allele for each locus and confirmed that sizes obtained with the manual electrophoresis technique were identical to exact sizes determined by sequencing.

Moreover, a sub-sample of 22 alleles across all loci was also typed with an ABI PRISM 3100 to compare sizing made by these two capillary gel electrophoresis automatic sequencers. Forward primers of microsatellite loci amplifying the strand containing the core repeat were labeled with a fluorescent dye: R1.35 (NED), R2.73 (HEX), R5.10 (NED), R3.171 (6-FAM), R5.29b (HEX), S16b (NED), and S17b (NED). For ABI PRISM 310 and 3100, PCR products from the seven loci were pooled as indicated hereafter. PCR products were diluted at 1:5, and a mixture of these seven products was loaded in 18 µL Template Suppression Reagent (Applied Biosystems) and 0.5 µL Genescan®-400HD size standard (Applied Biosystems). The samples were denatured for 3 min at 94°C, immediately cooled on ice, and placed in an autosampler tray for automatic injection in the ABI PRISM Genetic Analyzers. The samples were run in a 47-cm/50-µm capillary with POP-4 gel (Applied Biosystems). Samples were injected for 10 s and run at 15 kV for 24 min at the constant temperature of 60°C. DNA fragments were automatically sized with ABI PRISM Genescan 3.5 software using the local Southern sizing algorithm for all samples.

At the same time, loci were analyzed by electrophoresis in polyacrylamide gels. Each PCR product was diluted by half with a 4× loading buffer for electrophoresis. Samples were then loaded in a 6% polyacrylamide urea gel electrophoresis, run for 3 h at 1000 V, 75 W in 0.5× TBE, and silver stained following the method of Budowle et al. (1).

Size calling error was calculated at each locus as the mean difference between actual (sizing with manual technique) and called (automated techniques) allele sizes. Linear regressions between allele size error and actual size, as well as an ANOVA testing the locus effect on allele sizing error, were performed with SAS software (4).

RESULTS AND DISCUSSION

Because of the small sample size of the data collected with ABI PRISM 3100, we tested reliability and within locus size variation for the ABI PRISM 310 only. However, between-loci size variation was analyzed for both capillary electrophoresis sequencers.

Overall Reliability

Standard deviations calculated from multiple typings of identical alleles (deduced from manual techniques) on the ABI PRISM 310 were low, ranging from 0.01 to 0.32 nucleotides (Table 1). However, they were higher than those found by Haberl and Tautz (2) with an ABI PRISM 377, which is most likely due to smaller sample sizes in our study rather than to different instruments. Thus, sizing of alleles with capillary DNA sequencer (ABI PRISM 310) is very reproducible.

According to manufacturer's instructions, we confirmed that standard deviations of called size increased with allele size. Of 51 alleles typed, the difference between the smallest and the largest called size for identical alleles was always less than one nucleotide, and size ranges of contiguous alleles never overlapped. These results ensure that, even for alleles differing by a single nucleotide (e.g., locus R1.35), DNA fragment analysis with the ABI PRISM 310 is fully reliable (but not accurate without correction).

Within-Locus Size Variation

For the R5.10 locus, a compression of called size of the loci was observed with capillary electrophoresis. For this locus, the difference between the smallest and the largest allele was smaller with the automatic sequencer (ABI PRISM 310) than with manual electrophoresis: the 18 nucleotides difference between the allele "256" and the allele "274" was actually compressed to a called size of 17.3 nucleotides (Table 1). Notably, the difference between called and actual nucleotide number equally increases with allele size in this locus, confirming results of Haberl and Tautz (2) that called size range of loci can be compressed. However, the same authors showed that called size of loci can also be stretched, which was not found here. In fact, most loci of this study seem to be compressed in the range of some alleles only, and stretched for the others (Table 1). Thus, since size calling error did not vary proportionally to allele size, it was not possible to apply any normalizing factor for allele size correction as did Mansfield et al. (3).

Between-Loci Size Variation

Our most original result is that size calling errors of alleles across all loci decrease with allele size both with the ABI PRISM 310 ($r^2 = 0.85$; $P < 0.0001$; Figure 1 and Table 2) and the ABI PRISM 3100 ($r^2 = 0.76$; $P < 0.0001$; Figure 2). Moreover, size calling error of alleles of close actual sizes, but belonging to different loci, clearly differed. Indeed, we found a strong locus-specific effect on size calling error (ANOVA, $P < 0.0001$). It is worth noting that Mansfield et al. (3) observed similar locus-specific sizing biases when testing the accuracy of microsatellite genotyping using capillary array electrophoresis. However, they found no relation between the magnitude of the sizing error and locus size. Several factors might contribute to errors in fragment sizing: (*i*) mobility shifts caused by the dyes used to label primers, (*ii*) the composition of microsatellite loci, and (*iii*) the composition of the DNA sizing standard.

Dye Mobility Shifts

The bias found with ABI PRISM 310 and 3100 seems independent of the fluorescent dye used since differences be-

tween actual and called size range from 0.56 to 4.15 for loci labeled with the same dye (NED). Moreover, 50 samples were genotyped using forward primer of the locus R5.29 labeled with two different fluorescent dyes (HEX and 6-FAM). This experiment showed that the relative size scale for the locus remained constant even if we noticed a uniform shift of ±0.5 bp between dyes (data not shown).

Composition of Microsatellite Loci

It is unlikely that the locus-specific differences observed were due to biases in nucleotide composition because all loci had similar A+T composition. However, there seems to be a trend that loci with AC repeats show larger sizing error than those with GA or TC repeats (Table 2). Unfortunately, this is correlated with locus length so that no clear statement is possible. There also seems to be a relation between size calling error and the length of the flanking region: alleles of different loci having a similar number of repeats migrate faster, as they have a shorter flanking region (Spearman rank, R = 0.9, *P* =

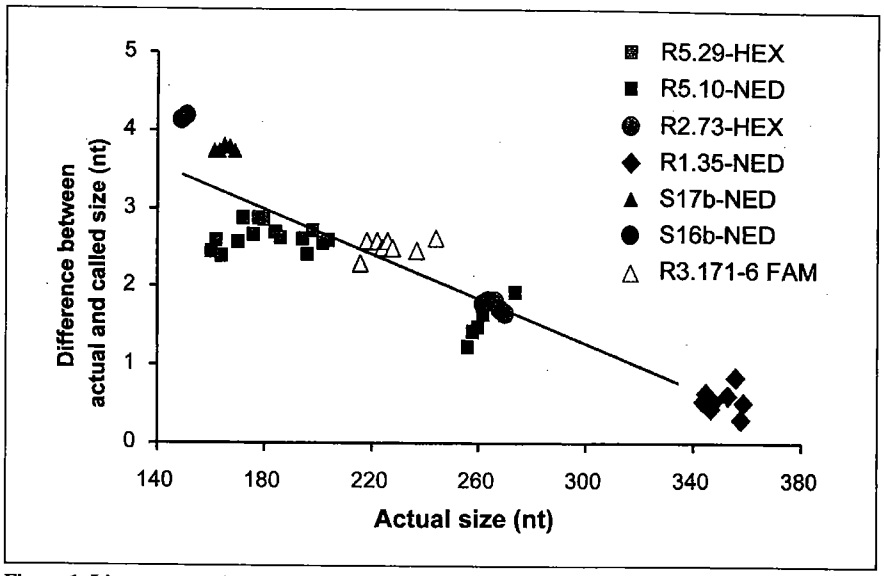

Figure 1. Linear regression analysis of the relation between the actual allele size (X axis) and the mean difference between actual and called size in nucleotides (Y axis) for 51 alleles from seven microsatellite loci of the aphid *Rhopalosiphum padi*. Y = -0.0142 × +5.54; $R^2 = 0.85$; $P < 0.0001$.

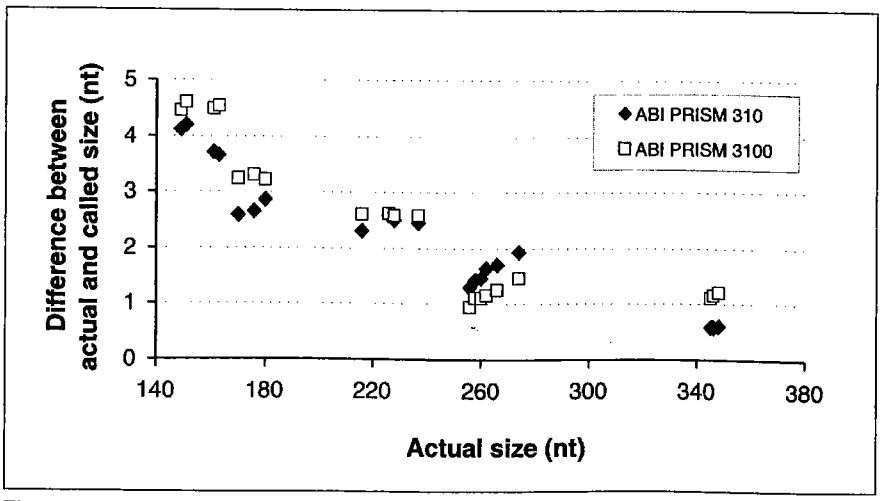

Figure 2. Mean difference between actual and called size in nucleotides obtained with two different automatic sequencers (ABI PRISM 310 and 3100) against actual allele size for 22 alleles from seven microsatellite loci of the aphid *Rhopalosiphum padi*.

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Table 1. Actual and Called Allele Size for Selected Alleles from Seven Microsatellite Loci Amplified in the Aphid *Rhopalosiphum padi*

Locus	Dye	*n*	Actual Size	Mean Called Size	SD	Minimum	Maximum	Called Size Range	Difference: Actual Nucleotide	Difference: Called Nucleotide	Difference: Actual vs. Called Size
S16b	NED	20	149	144.88	0.102	144.76	145.16	0.40	0	0.0	4.12
		16	151	146.82	0.106	146.68	147.02	0.34	2	1.94	4.188
S17b	NED	16	161	157.28	0.117	156.94	157.44	0.50	0	0	3.72
		10	163	159.28	0.117	159.05	159.49	0.44	2	2.00	3.72
		4	165	161.20	0.213	160.89	161.35	0.46	4	3.92	3.80
		2	167	163.21	0.113	163.13	163.29	0.16	6	5.93	3.79
		2	169	165.28	0.014	165.27	165.29	0.02	8	8.00	3.72
R5.29	HEX	8	160	157.57	0.090	157.41	157.69	0.28	0	0.0	2.43
		11	162	159.42	0.097	159.27	159.58	0.31	2	1.85	2.58
		7	164	161.62	0.191	161.37	161.92	0.55	4	4.05	2.38
		20	170	167.43	0.178	167.01	167.62	0.61	10	9.86	2.57
		7	172	169.12	0.183	168.87	169.35	0.48	12	11.55	2.88
		17	176	173.35	0.135	173.14	173.57	0.43	16	15.78	2.65
		3	178	175.14	0.115	175.05	175.27	0.22	18	17.57	2.86
		20	180	177.15	0.151	176.85	177.43	0.58	20	19.58	2.85
		3	184	181.31	0.130	181.18	181.44	0.26	24	23.74	2.69
		3	186	183.39	0.078	183.33	183.44	0.11	26	25.82	2.62
		3	194	191.41	0.329	191.03	191.60	0.57	34	33.84	2.59
		2	196	193.61	0.000	193.57	193.64	0.07	36	36.04	2.40
		2	198	195.29	0.071	195.24	195.34	0.10	38	37.72	2.71
		5	202	199.46	0.115	199.35	199.59	0.24	42	41.89	2.54
		2	204	201.43	0.078	201.37	201.48	0.11	44	43.86	2.57
R3.171	6-FAM	4	216	213.70	0.121	213.62	213.88	0.26	0	0.0	2.30
		10	218	215.42	0.130	215.10	215.57	0.47	2	1.72	2.58
		2	222	219.43	0.007	219.42	219.43	0.01	6	5.72	2.58
		3	224	221.51	0.240	221.37	221.79	0.42	8	7.81	2.49
		13	226	223.42	0.171	223.00	223.60	0.60	10	9.72	2.58
		15	228	225.50	0.084	225.36	225.71	0.35	12	11.80	2.50
		4	237	234.55	0.070	234.48	234.61	0.13	21	20.85	2.46
		2	244	241.39	0.007	241.38	241.39	0.01	28	27.69	2.62
R5.10	NED	23	256	254.78	0.329	254.43	255.22	0.79	0	0.0	1.22
		23	258	256.58	0.159	256.15	256.87	0.72	2	1.80	1.42
		23	260	258.54	0.179	258.28	258.89	0.61	4	3.76	1.46
		22	262	260.37	0.217	260.00	260.78	0.78	6	5.59	1.63
		5	270	268.37	0.200	268.12	268.60	0.48	14	13.58	1.63
		23	274	272.08	0.185	271.78	272.49	0.71	18	17.30	1.92
R2.73	HEX	19	262	260.23	0.097	260.11	260.42	0.31	0	0.0	1.77
		18	264	262.20	0.087	262.02	262.31	0.29	2	1.97	1.80
		20	266	264.18	0.162	263.92	264.38	0.46	4	3.96	1.82
		12	268	266.31	0.170	266.06	266.58	0.52	6	6.08	1.69
		18	270	268.34	0.151	268.14	268.59	0.45	8	8.12	1.66
		20	285	282.86	0.126	282.47	283.01	0.54	23	22.63	2.14
R1.35	NED	20	344	343.47	0.184	343.19	343.76	0.57	0	0.0	0.53
		20	345	344.35	0.160	344.14	344.79	0.65	1	0.88	0.65
		11	346	345.42	0.243	345.01	345.94	0.93	2	1.96	0.58
		9	347	346.55	0.240	346.18	346.87	0.69	3	3.09	0.45
		10	348	347.47	0.237	347.18	347.80	0.62	4	4.00	0.53
		11	353	352.39	0.161	352.07	352.67	0.60	9	8.92	0.61
		8	356	355.17	0.125	355.05	355.42	0.37	12	11.70	0.84
		9	358	357.69	0.252	357.29	357.92	0.63	14	14.23	0.31
		18	359	358.48	0.252	358.11	358.96	0.85	15	15.01	0.52

The actual sizes were determined by manual electrophoresis and called sizes were obtained with an ABI PRISM 310. For each locus, one allele was sequenced that confirmed manual sizing (underlined).

Table 2. Mean Difference Between Actual and Called Size Calculated for Alleles of Seven Microsatellite Loci Amplified in the Aphid *Rhopalosiphum padi*

Locus	Dye	Core Repeat	Size Range	Mean Difference BetweenActual and Called Size
S16.b	NED	$(AC)_{14}$	149-151	4.15
S17.b	NED	$(CA)_{11}TA(CA)_8(TA)_7$	161-169	3.75
R5.29	HEX	$(AC)_{13}$	160-204	2.62
R3.171	6-FAM	$(AC)_{11}$	216-244	2.51
R5.10	NED	$(GA)_{15}$	256-274	1.55
R2.73	HEX	$(GT)_{25}GG(ATT)_4$	260-285	1.82
R1.35	NED	$(TC)_{13}$	344-359	0.56

0.037). To check whether the core repeat of microsatellite loci accounts for sizing bias, PCR fragments with no sequence repeats should be sized. Since such control was not used in this study, we cannot exclude that the type and length of the amplified sequence induce locus-specific effects during electrophoresis, as previously suggested (2).

Size Standards

It is worth noting that different size standards have been used for manual and automated sizing techniques. Since allele sequencing confirmed that the ladder (which was a plasmid sequence) used in manual technique gave the correct allele sizes, we can exclude it as a source of error. However, a major source of size calling error could be attributed to the fragment composition of the size standard used in capillary electrophoresis (3). To check this hypothesis, it would be valuable to compare the magnitude of size calling errors of alleles obtained with different size standards used for capillary electrophoresis.

CONCLUSION

We have shown that sizing alleles with ABI PRISM 310 and 3100 generates differences, especially for small DNA fragments. This may have important consequences when calculating genetic distances or estimates of population genetics parameters based on allele size. Thus, it is essential to distinguish between actual (that can only be confirmed by sequencing) and called (obtained with automated sequencer) allele sizes. To enable comparison of data collected by different laboratories on different instruments, the greatest attention should be paid to material and protocol descriptions used for allele sizing, and reference standard DNA genotypes (i.e., control individuals) should be shared between collaborating laboratories. Without these precautions, scoring errors in allele size might result in misleading conclusions.

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