Chapter 3

Allele Size Miscalling due to the Pull-Up Effect Influencing Size Standard Calibration in Capillary Electrophoresis: A Case Study Using HEX Fluorescent Dye in Microsatellites

Zheng-Feng Wang, Se-Ping Dai, Ju-Yu Lian,
Hong-Feng Chen, Wan-Hui Ye and Hong-Lin Cao

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.73028

Abstract

Microsatellites are important genetic markers and have been broadly employed in many genetic studies. Currently, polymorphisms in microsatellites are often detected by an automated system of capillary electrophoresis with fluorescent dyes. In this situation, different dye combinations may cause pull-up/bleed-through problems, which introduce noise signals from one dye channel into another, causing genotyping errors. Here, we report the detection of such a problem at two microsatellite loci that used the HEX dye. Using three datasets, we tested for noise effects in four allele-scoring programmes: Genemapper, Genemarker, Gelquest and Fragman. We found that, because some allele sizes were identical or close to the size of one of the internal size standards, all four programmes gave allele size calling errors due to wrongly identifying pull-up signals as the internal size standard. In addition, because allele miscalling in this study was caused by the fluorescent dye that the microsatellites used introducing noise of the same colour as the internal size standard used, the pull-up correction function in Genemapper, Genemarker and Fragman failed to deal with this. Considering that pull-up peak scoring errors can occur with any dye colour, the phenomenon is not limited to the current HEX dye. Using different software and visual scoring of each result will allow accurate sizing of microsatellite alleles.

Keywords: Fragman, Gelquest, Genemapper, Genemarker, genetic markers, allele size scoring

1. Introduction

Microsatellites are important genetic markers that are widely used in evolutionary, ecological, animal and plant breeding and forensic studies [1, 2]. Because their polymorphisms are generally caused by the gain or loss of a repeat unit, they can be easily detected using a gel separation method to detect length variations. Therefore, using fluorescent dye-labelled primers with automatic capillary electrophoresis is one of the most popular methods for high-throughput assessment of their polymorphisms. Microsatellite allele fragments are then estimated or calculated by comparison with a co-migrated internal size standard, which uses a different fluorescent dye that displays a different colour from that used for the microsatellite.

In this process, incorrect microsatellite genotype scoring can occur at many steps [3–7]. Stutter, null alleles and allelic dropout are the three major problems, which have been discussed extensively [5, 7]. These problems were generally related to sample quality, polymorphisms in microsatellite priming sites, PCR amplification procedures and others. Unlike errors relating to DNA templates or PCR procedures, pull-up/bleed-through ("pull-up" hereafter) in capillary electrophoresis per se has also been an important problem causing allele miscalling.

The pull-up effect is due to spectral overlap of the fluorescent dyes in capillary electrophoresis producing more than one peak colour for the allele with one colour dominant and the others minor [7]. Pull-up peaks of the minor colours occur when a peak has reached intensity saturation such that the major peak cannot increase in signal intensity due to saturation, but minor peaks that are normally of very low intensity (background) reach appreciable signal intensity. In addition, because allele calling from capillary electrophoresis depends on detecting the emission spectra of fluorescent dyes, a spectral standard is needed to compensate for the emission spectrum overlap between dyes. Therefore, an incorrect spectral standard will also cause the pull-up effect [8].

Because most DNA fragment analysis software (aiding allele size calling, see below) provides functions to deal with the pull-up effect, such a problem can be easily overlooked. Such functions, called pull-up correction, work by removing the extra noise colours and only keeping the dye colour that the microsatellite uses for the allele peak [9]. However, because the dye colour used by the internal size standard will be performed to calibrate allele sizes, the function will not correct this dye colour. Therefore, if the dye that the microsatellite uses leads to noise colour that is the same as the colour the internal size standard uses, the pull-up correction function does not correct such noise colour. In this case, errors can still occur if researchers are unaware of the problem.

Here, we report the cause of such errors that arose when we used the HEX fluorescent dye for microsatellite loci and the ROX dye for the internal size standard and used electrophoresis in an ABI 3730 automated sequencer. Because some allele fragments happened to overlap with or were close to one of the size standard fragments, a pull-up effect caused miscalling of alleles.

2. Materials and methods

We used two microsatellite loci to reveal size scoring errors. One was locus HQ-53 (EMBL Accession No. HG421133) in *Engelhardia roxburghiana*, a diploid species belonging to the family Juglandaceae [10]. The other was locus WJ-39 (GenBank Accession No. KY428838) in Chinese tallow tree (*Triadica sebifera*), a tetraploid species belonging to the family Euphorbiaceae [11]. HQ-53 is a dinucleotide microsatellite locus, and WJ-39 is a trinucleotide microsatellite locus.

Such errors were first found at locus HQ-53 when we used it to genotype 522 *E. roxburghiana* samples in a 20-ha (400 × 500 m) DHS plot in the 1155-ha DHS National Nature Reserve on the southern verge of the Tropic of Cancer in the subtropical part of South China [12]. For this allele size calling procedure, we used HEX dye for the HQ-53 locus and ROX dye for the internal size standard. For each sample, when we double-checked the Genemapper (see below) scored electrophoresis results with other scoring programmes, some results were inconsistent. Then, when we isolated and characterised microsatellites in *T. sebifera*, a similar problem occurred at locus WJ-39 for which we used the same HEX and ROX dye combination. Therefore, we think that such allele miscalling could be a common problem if ignored.

The primers for the two loci were designed by Primer3 software [13, 14]. Therefore, for HQ-53, it occurred by chance that one amplified allele was 200 bp and the other was 198 bp after PCR amplification, sizes that are identical or close to the 200 bp size standard fragment. For WJ-39, instead of directly using the designed primers, the forward primer was 5′-tailed with the 15 bp 5′-CAGTCGGGCGTCATC-3′ sequence (CAG-tagged sequence) to decrease the cost at the microsatellite screening stage [15]. Two PCR amplification steps were then employed for this locus. For the first step, PCR amplification was performed with the CAG-tagged forward primer plus the reverse primer using 12 reaction cycles. One microlitre of amplification product was then used for the second 35-cycle PCR amplification but with the fluorescently labelled CAG-tagged sequence as the forward primer. The allele sizes after PCR amplification were 250 and 253 bp, while a 250 bp fragment also occurred in the internal size standard used.

We provided three datasets (**Table 1**). Datasets 1 and 2 are for locus HQ-53, and they include 48 and 96 samples, respectively. These datasets contain the results using the HEX dye (producing green peaks in the electropherograms; **Figures 1** and **2**), and they were electrophoresed on an ABI 3730 sequencer in 2013 and 2012. Here, we have provided two datasets in HQ-53 just to illustrate that such errors were not once-only electrophoresis problems (in fact, such errors occurred frequently in allele size scoring in locus HQ-53, and we just chose two datasets as examples). Dataset 3 is for locus WJ-39 and contains results electrophoresed on the same sequencer in 2016. Dataset 3 includes only six samples. The reason Dataset 3 only contains six samples was because we used these six samples to identify polymorphisms at locus WJ-39 before deciding to use this locus or not for large-scale genotyping in *T. sebifera*. In addition, small sample sizes were cost-saving and facilitated our use of different treatments (different dye combinations; see **Table 1**) to reveal the way to avoid such allele size miscalling. It is worth mentioning that for Treatment-5 and Treatment-6 in Dataset 3, we run each experiment (including PCR amplification and electrophoresis) twice for them on different days to check the consistency between experiments. This was because we found that the results in

Dataset	Species and its ploidy level	Sample size	Microsatellite locus and its type tested in samples	Fluorescent dye for locus	Fluorescent dye for internal size standard	Treatment
Dataset 1	*Engelhardia roxburghiana* (diploid)	48	HQ-53 (dinucleotide)	HEX	GeneScan™ 500 ROX	
Dataset 2	*Engelhardia roxburghiana* (diploid)	96	HQ-53 (dinucleotide)	HEX	GeneScan™ 500 ROX	
Dataset 3	*Triadica sebifera* (tetraploid)	6	WJ-39 (trinucleotide)	HEX	GeneScan™ 500 ROX	Treatment-1
				FAM	GeneScan™ 500 ROX	Treatment-2
				HEX	GeneScan™ 500 ROX	Treatment-3 (PCR products in Treatment-1 diluted 20-fold)
				HEX	GeneScan™ 500 ROX	Treatment-4 (PCR products in Treatment-1 diluted 50-fold)
				HEX	GeneScan™ 500 LIZ	Treatment-5 (repeated twice, named Treatment-5-1 and Treatment-5-2)
				FAM	GeneScan™ 500 LIZ	Treatment-6 (repeated twice, named Treatment-6-1 and Treatment-6-2)

Table 1. Summary of datasets used to illustrate allele size calling errors.

Treatment-5 gave many size calling errors for the first experiment (see results and **Table 2**). We then ran the second experiments to confirm that. All electrophoreses and data analyses were performed by Thermo Fisher Scientific, Inc. in Guangzhou branch, China.

Datasets 1 and 2 were analysed by Genemapper ID v3.2 software previously, while Dataset 3 was analysed by Genemapper 4.1. To make the results comparable among datasets, Datasets 1 and 2 were re-analysed with Genemapper 4.1. After checking the results in Datasets 1 and 2 with the two software versions, they were found to be identical.

Because Genemapper is expensive, most researchers cannot afford it. However, when samples are sent to companies or laboratories that have ABI sequencers, they will provide microsatellite allele size calling after electrophoresis. Therefore, for most researchers, these results

http://dx.doi.org/10.5772/intechopen.73028

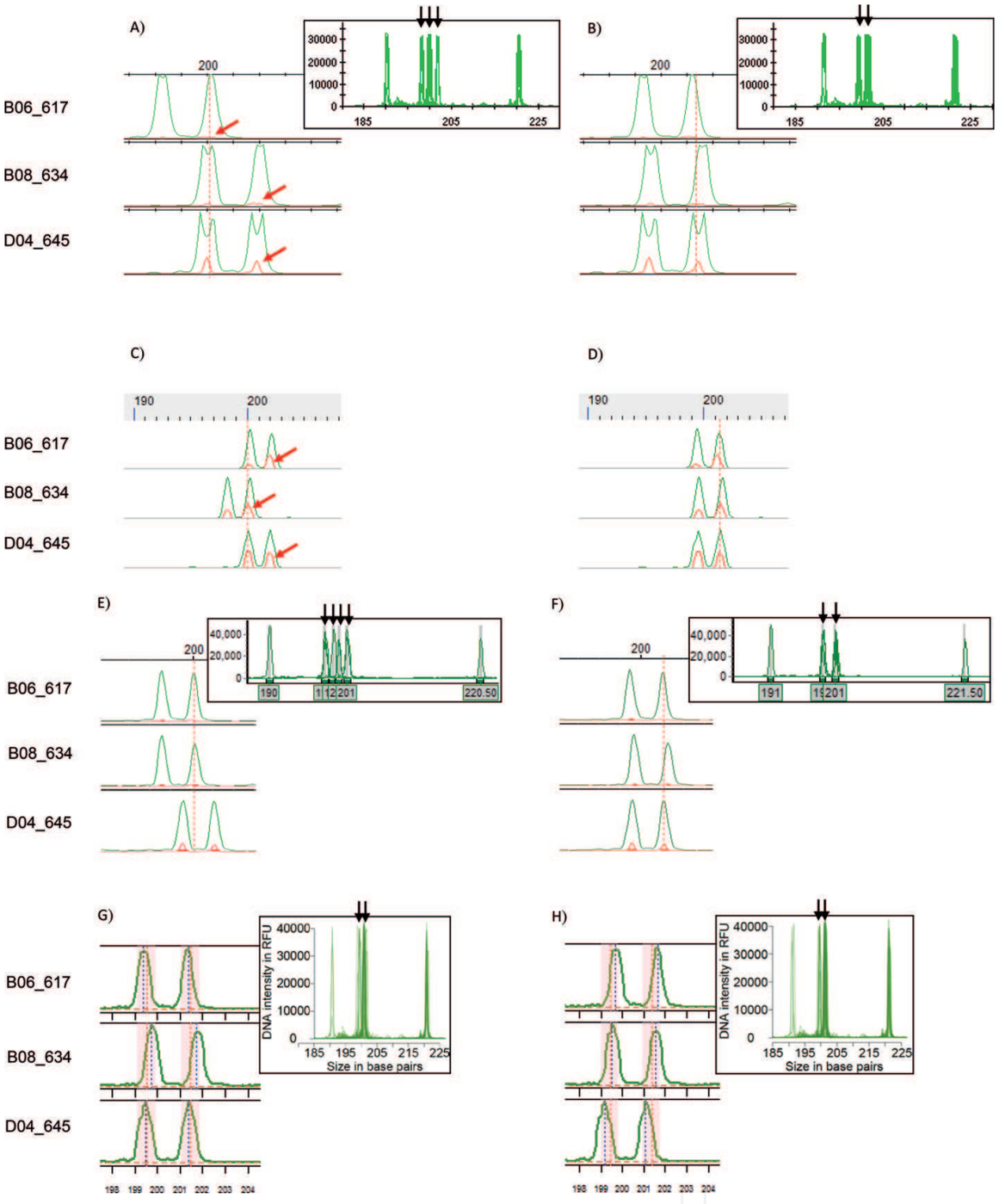

Figure 1. Electropherograms showing alleles (green peaks with HEX fluorescent dye) in example samples from Dataset 1 using Genemapper 5.0 (A–B), Gelquest 3.1.3 (C–D), Genemarker 2.7 (E–F) and Fragman 1.0.7 (G–H). The results in A, C, E and F were derived using the full set of internal size standard fragments (red peaks with ROX fluorescent dye), and B, D, F and H were scored with the 200 bp fragment omitted from the internal size standard. For each electropherogram, except those generated by Gelquest, the upper or lower right is the allele panel constructed by overlapping allele peaks. The black arrows on each allele panel correspond to the alleles shown in each electropherogram. Red-dashed lines in the electropherograms indicate the positions where the 200 bp internal size standard should appear, and the red arrows show the actual position of the 200 bp internal size standard. Sample names and electrophoresis names (in parentheses) are indicated on the left side of each electropherogram. For Genemarker, its allele panels (E and F) were constructed using only six samples because it was a demo version. [Colour figure can be downloaded and viewed at http://molecular-ecologist.com/pd.jsp?id=1#_jcp=2].

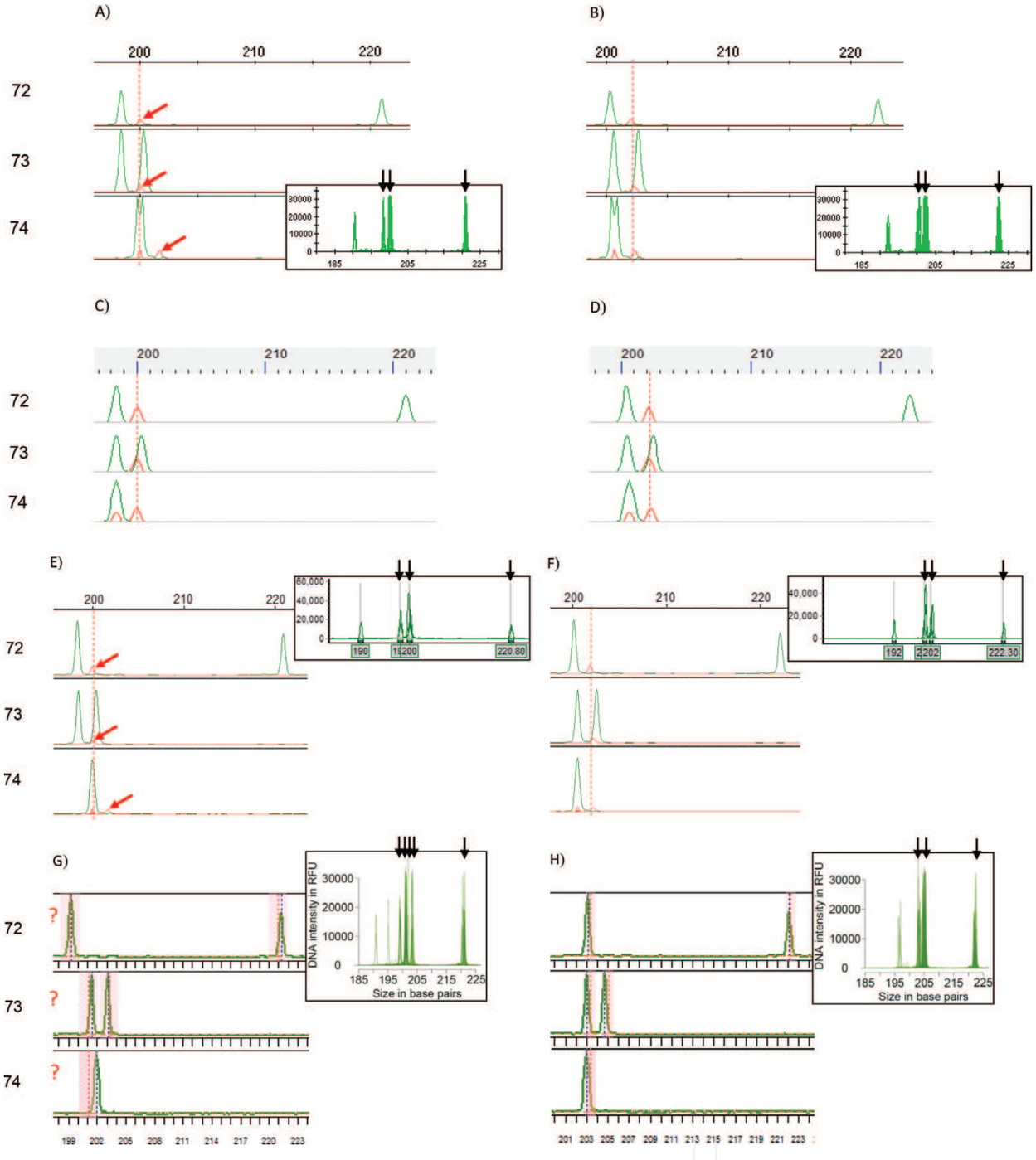

Figure 2. Electropherograms showing alleles (green peaks with HEX fluorescent dye) in example samples from Dataset 2 using Genemapper 5.0 (A–B), Gelquest 3.1.3 (C–D), Genemarker 2.7 (E–F) or Fragman 1.0.7 (G–H). The results in A, C, E and F were derived using the full internal size standard fragment set (red peaks with ROX fluorescent dye), and B, D, F and H were scored with the 200 bp fragment omitted from the internal size standard. For each electropherogram, except those generated by Gelquest, the upper or lower right was the allele panel constructed by overlapping allele peaks. The black arrows on each allele panel correspond to the alleles shown in each electropherogram. Red-dashed lines in the electropherograms indicate the positions where the 200 bp internal size standard should appear, and the red arrows show the actual position of the 200 bp internal size standard. Question marks indicate allele sizes that were not scored because the allele panel was generated improperly. Sample names and electrophoresis names (in the parentheses) are indicated on the left side of each electropherogram. For Genemarker, its allele panels (E and F) were constructed using only six samples because it was a demo version. [Colour figure can be downloaded and viewed at http://molecular-ecologist.com/pd.jsp?id=1#_jcp=2].

Dataset (treatment)	Programme and its version used to call allele size	Allele numbers generated by allele panel with full internal size standard	Allele numbers generated by allele panel with a particular internal size standard fragment omitted[a]	No. of samples with wrong allele calling[b]	Proportion of incorrect calls among samples
Dataset 1	Genemapper 5.0	5	4	4	8.3% (4/48)
	Gelquest 3.1.3	—	—	8	16.7% (8/48)
	Genemarker 2.7	6	4	4	8.3% (4/48)
	Fragman 1.0.7	4	4	0	0.0% (0/48)
Dataset 2	Genemapper 5.0	4	4	1	1.0% (1/96)
	Gelquest 3.1.3	—	—	0	0.0% (0/96)
	Genemarker 2.7	4	4	1	1.0% (1/96)
	Fragman 1.0.7	7	4	Not count	Not count
Dataset 3 (Treatment 1)	Genemapper 5.0	4	3	2	33.3% (2/6)
	Gelquest 3.1.3	—	—	0	0.0% (0/6)
	Genemarker 2.7	4	3	1	16.7% (1/6)
	Fragman 1.0.7	5	3	Not count	Not count
Dataset 3 (Treatment 2)	Genemapper 5.0	3	3	0	0% (0/6)
	Gelquest 3.1.3	—	—	1	16.7% (1/6)
	Genemarker 2.7	3	3	0	0% (0/6)
	Fragman 1.0.7	3	3	0	0% (0/6)
Dataset 3 (Treatment 3)	Genemapper 5.0	3	3	0	0% (0/6)
	Gelquest 3.1.3	—	—	0	0% (0/6)
	Genemarker 2.7	3	3	0	0% (0/6)
	Fragman 1.0.7	3	3	0	0% (0/6)
Dataset 3 (Treatment 4)	Genemapper 5.0	3	3	0	0% (0/6)
	Gelquest 3.1.3	—	—	0	0% (0/6)
	Genemarker 2.7	3	3	0	0% (0/6)
	Fragman 1.0.7	3	3	0	0% (0/6)
Dataset 3 (Treatment-5-1)	Genemapper 5.0	3	3	6	100% (6/6)[c]
	Gelquest 3.1.3	—	—	0	0% (0/6)
	Genemarker 2.7	3	3	0	0% (0/6)
	Fragman 1.0.7	3	3	0	0% (0/6)

Dataset (treatment)	Programme and its version used to call allele size	Allele numbers generated by allele panel with full internal size standard	Allele numbers generated by allele panel with a particular internal size standard fragment omitted[a]	No. of samples with wrong allele calling[b]	Proportion of incorrect calls among samples
Dataset 3 (Treatment-5-2)	Genemapper 5.0	3	3	0	0% (0/6)
	Gelquest 3.1.3	—	—	0	0% (0/6)
	Genemarker 2.7	3	3	0	0% (0/6)
	Fragman 1.0.7	3	3	0	0% (0/6)
Dataset 3 (Treatment-6-1)	Genemapper 5.0	3	3	6	0% (0/6)[c]
	Gelquest 3.1.3	—	—	0	0% (0/6)
	Genemarker 2.7	3	3	0	0% (0/6)
	Fragman 1.0.7	3	3	0	0% (0/6)
Dataset 3 (Treatment-6-2)	Genemapper 5.0	3	3	0	0% (0/6)
	Gelquest 3.1.3	—	—	0	0% (0/6)
	Genemarker 2.7	3	3	0	0% (0/6)
	Fragman 1.0.7	3	3	0	0% (0/6)

[a]Fragments omitted from internal size standard were 200 bp for Datasets 1 and 2 and 250 bp for Dataset 3.

[b]Wrong allele calling refers to the allele size called with full internal size standard in the programme being different from the size called by all the programmes after omitting a particular internal size standard fragment. Because the allele panel generated by the Fragman programme was doubtful, we did not score it, and the error rates were not counted in Dataset 2 and Dataset 3 (Treatment 1).

[c]There were five calling errors using Genemapper 4.1.

Table 2. Genotyping results in different datasets.

are their final "standard" results and are generally not critically assessed. Because of a version update, we could only find a Genemapper 5.0 trial version for comparing to the other allele calling software (see below). It is worth noting that Genemapper 5.0 also gave identical results to Genemapper ID v3.2 and Genemapper 4.1 using the same internal size standard except some inconsistency in Treatment-5 in Dataset 3 (see **Table 2** footnote).

We found size calling problems when we checked the consistency of the company-provided results with the calling results from Gelquest (http://www.sequentix.de/gelquest/), another DNA fragment analysis programme. In addition to these two programmes, we also used two other third-party software programmes to compare the results among them. These were Genemarker 2.7 demo version (http://www.softgenetics.com/GeneMarker.php) and the newly developed R software Fragman 1.0.7 [9].

Because of a significant bug in the latest Gelquest version 3.4.3, which meant that it could not use different size standards or adjust size standards, the previous version 3.1.3 was used. By comparing the results from the same dataset under the same internal size standard, these

two versions produced the same results. The Genemarker 2.7 demo version could only allow inputting six samples at a time, so for datasets that included more than six samples, the samples were broken into several portions and analysed one portion at a time.

All datasets were analysed by the four above-mentioned software programmes, both with the full set of fragments for the internal size standard and with particular fragments (200 bp for HG-53, 250 bp for WJ-39) omitted from the internal size standard. For simplification, we omitted the version numbers of the four programmes. Therefore, if not specifically noted, they were Genemapper 5.0, Gelquest 3.1.3, Genemarker 2.7 and Fragman 1.0.7.

3. Results

For Dataset 1, using the full set of fragments in the internal size standard, Genemapper, Genemarker and Fragman produced different allele panels (**Figure 1A**, **E** and **G** and **Table 2**). Genemarker indicated that there were six alleles, Genemapper five and Fragman four. By omitting the 200 bp fragment from the internal size standard, these three programmes generated the same allele panel (**Figure 1B**, **F** and **H** and **Table 2**). However, unlike Genemapper and Genemarker, Fragman generated roughly the same size panel before and after omitting the 200 bp fragment. Without the 200 bp fragment in the internal size standard, all four programmes (including Gelquest) gave the same allele calling results. Then, considering the allele size calling results using the full set of internal size standard fragments, Genemapper generated four calling errors, Gelquest eight, Genemarker four and Fragman zero (**Table 2**).

For Dataset 2, only Fragman generated very different size panels before and after the 200 bp fragment was omitted (**Figure 2G** and **H** and **Table 2**). The allele panel generated by Fragman, with the full set of internal size standard fragments indicating that seven alleles existed, was also highly different from those generated from the other two programmes, Genemapper and Genemarker. Therefore, the allele size results called for the panel by Fragman with full fragments in the internal size standard were doubtful, and we did not score them. After comparing the results produced without the 200 bp internal size standard fragment by the four programmes, we found that they were consistent. Thus, considering the allele size calling results using the full set of internal size standard fragments, no errors occurred using Gelquest, while both Genemapper and Genemarker gave one size calling error each (**Table 2**) even though their size panels were the same before and after omitting the 200 bp fragment of the internal size standard.

For Treatment-1 in Dataset 3, comparing the size panels generated using the full set of fragments of the internal size standard with those generated using the internal size standard with the 250 bp fragment omitted, Genemapper, Genemarker and Fragman each had different panels (**Figure 3** and **Table 2**). The allele panel generated by Fragman with the full set of fragments of the internal size standard was also different from those generated by the other two programmes, making its size calling results doubtful. After omitting the 250 bp fragment, all four programmes gave consistent results. Therefore, particular size calling errors using the full set of fragments of the internal size standard were two for Genemapper, one for Genemarker and zero for Gelquest (**Table 2**).

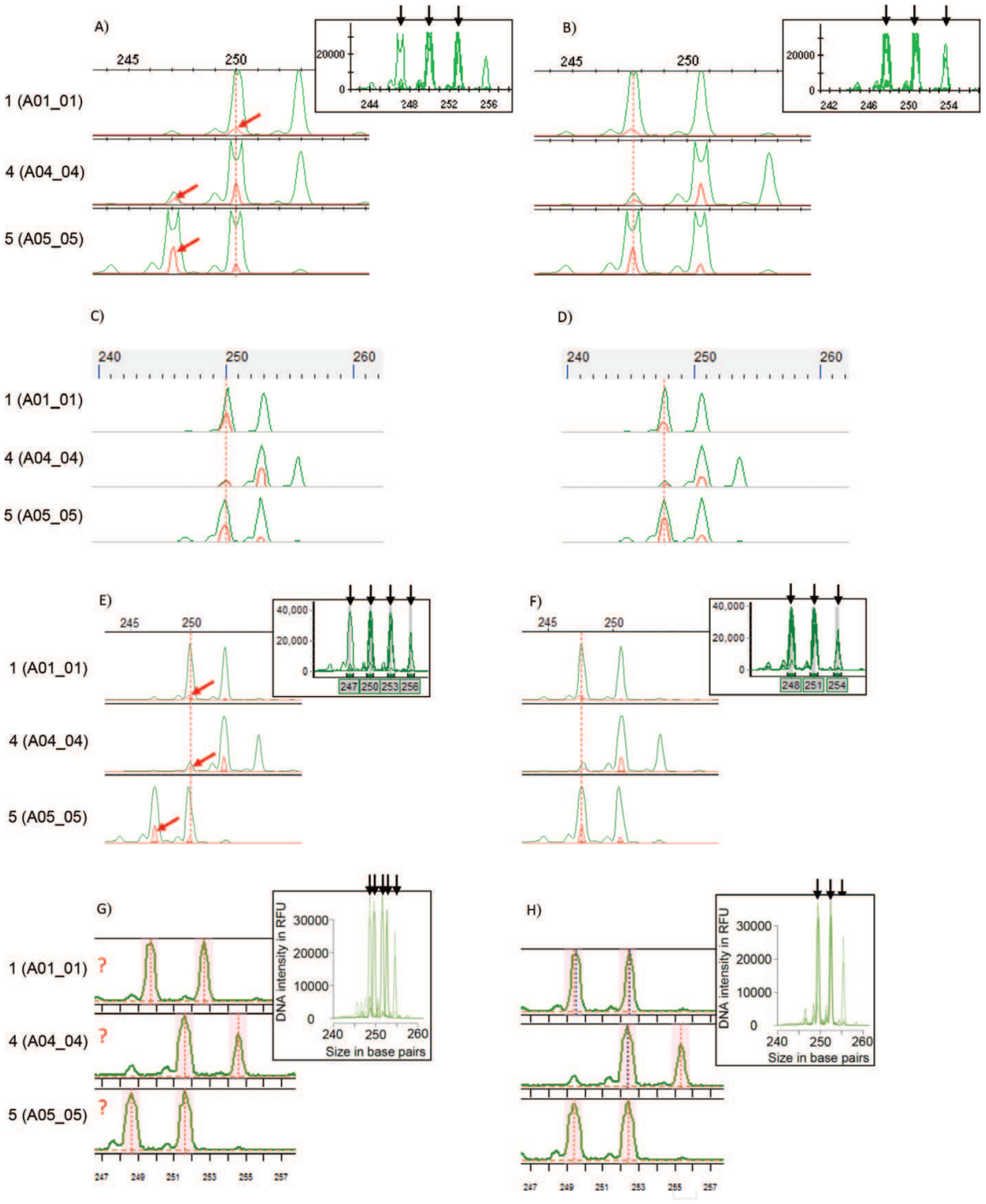

Figure 3. Electropherograms showing alleles (green peaks with HEX fluorescent dye) in example samples from Treatment-1 in Dataset 3 using Genemapper 5.0 (A–B), Gelquest 3.1.3 (C–D), Genemarker 2.7 (E–F) or Fragman 1.0.7 (G–H). The results in A, C, E and F were derived using the full internal size standard fragment set (red peaks with ROX fluorescent dye), and B, D, F and H were scored with the 250 bp fragment omitted from the internal size standard. For each electropherogram, except those generated by Gelquest, the upper or lower right was the allele panel constructed by overlapping allele peaks. The black arrows on each allele panel correspond to the alleles shown in each electropherogram.Red-dashed lines in the electropherograms indicate the position where the 250 bp internal size standard should appear, and the red arrows show the actual position of the 250 bp internal size standard. Question marks indicate allele sizes that were not scored because the allele panel was generated improperly. Sample names and electrophoresis names (in the parentheses) are indicated on the left side of each electropherogram. For Genemarker, its allele panels (E and F) were constructed using only six samples because it was a demo version. [Colour figure can be downloaded and viewed at http://molecular-ecologist.com/pd.jsp?id=1#_jcp=2].

For Treatment-2 in Dataset 3, all four programmes, regardless of whether the 250 bp fragment was omitted from the internal size standard or not, produced consistent results (see allele panels and sample examples in S1 Figure and **Table 2**).

For Treatment-3 and Treatment-4 in Dataset 3, which are the results from diluting the PCR products of Treatment-1 in Dataset 3 20- and 50-fold, consistent results were found for all four programmes regardless of whether the 250 bp fragment was omitted from the internal size standard or not (**Table 2** and S2 and S3 Figures).

For Treatment-5-1 in Dataset 3, which used HEX and LIZ in combination (**Table 1**), Genemapper, Genemarker and Fragman all produced the same allele panel pattern, indicating that three alleles existed whether the 250 bp internal size standard was omitted or not (S4 Figure and **Table 2**). However, close examination indicated that, without omitting the 250 bp internal size standard for Genemapper, its 250 bp internal size standards in all the samples were located in the wrong position, the pull-up peak position (see examples in S4 Figure), causing allele size calling errors in all of the samples (**Table 2**). The other three programmes, Gelquest, Genemarker and Fragman, scored consistent results with or without omitting the 250 bp internal size standard fragment.

For Treatment-5-2 in Dataset 3, which was the second experiment for Dataset 3 using the HEX and LIZ combination, all four programmes obtained consistent results regardless of whether the 250 bp fragment was omitted from the internal size standard or not (**Table 2** and S5 Figure).

For Treatment-6-1 and Treatment-6-2 in Dataset 3, similarly to Treatment-2, all four programmes, regardless of whether the 250 bp fragment was omitted from the internal size standard, produced consistent results (**Table 2** and S6 and S7 Figures).

4. Discussion

We report here one kind of microsatellite genotyping error caused by pull-up in capillary electrophoresis. Certainly, this is not new in microsatellite genotyping errors [7, 16]. However, to our knowledge, this is the first report that microsatellite genotyping is prone to such an effect when pull-up peaks influence the size standard match. In this case, the HEX fluorophore introduced extra signals (peaks) in the ROX fluorophore channel, causing size calibration collapse and allele miscalling. However, such a combination was not deemed to cause improper allele calling. In our case, this only occurred when the allele peak overlapped or was close to one of the internal size standard peaks.

Pull-up problems have received attention in previous studies [7, 16], and functions to diminish their influence on allele calling have been integrated into three programmes used in this study, i.e., Genemapper, Genemarker and Fragman. However, as the allele panels were constructed by these three programmes with the full set of fragments of the internal size standard (**Figures 1–3**), none of them dealt with this problem effectively. Therefore, extra signals caused by pull-up effects (extra red peaks in the internal size standard channel) were still strong and influenced the sizing calibration. For example, for sample 4(A04_04) in Treatment-1 in Dataset 3, because of the false pull-up, the 250 bp peak was much higher than the true 250 bp peak in the internal size

standard channel (**Figure 4**), and Genemapper wrongly identified the false peak as the 250 bp standard and even indicated "Sizing Quality = 1.0", meaning a complete match. Therefore, the true allele pattern in this sample was shifted left due to wrong standard matching (**Figure 3A**). Similarly, for sample 74 in Dataset 2, there were two red peaks side by side around the 200 bp standard position, and Genemarker incorrectly chose the left peak (**Figure 5**). In this situation, Genemarker still gave a calibration score of 95, the highest among six samples. However, for sample 74 (**Figure 2E**, also A and C), compared to the right red peak that was independent, the left red peak occurred under the allele peak and was clearly caused by the pull-up effect.

According to the Genemapper user guide (DNA Fragment Analysis by Capillary Electrophoresis, Publication Number 4474504, Revision B, https://tools.thermofisher.com/content/sfs/manuals/4474504.pdf), for internal size matching, Genemapper used the ratio-matching method, which is based on relative height and distance of neighbouring peaks. This algorithm theoretically ignores anomalous peaks that occur between two size standard peaks (page 99 in the user guide). However, in this case, because the pull-up peaks only had a 2 or 3 bp difference from the particular size standard peak, the ratio-matching method could not differentiate them very effectively.

To address the pull-up effect that occurred above, we provide four solutions:

1. Choosing a different fluorescent dye for the internal size standard or microsatellite loci. For example, from Treatment-2 and Treatment-6 in Dataset 3 (**Table 2** and S1, S6 and S7 Figures), when we used FAM dye for the WJ-39 locus, whether ROX or LIZ dye was used in the internal size standard, the pull-up effect was not problematic, and all software produced consistent results. However, because it was not known in advance that the HEX and ROX combination or HEX and LIZ combination (as Treatment-5-1 displayed; **Table 2** and S4 Figure) would lead to a problem, and this might only have been apparent at the end of the experiment, changing dyes was therefore not cost-efficient.
2. Redesigning the primers to avoid allele sizes identical or close to the internal standard sizes. However, because we generally only used one microsatellite sequence as a template to design the primers, the whole allele pattern in the population or species remains unknown. Therefore, some unknown alleles could still have fragment lengths identical or close to the lengths of the internal size standard.
3. Avoiding overloading the PCR products in capillary electrophoresis. Overloading was the major reason for the pull-up effect. It is generally suggested that the fluorescence signal should be approximately 150–4000 relative fluorescence units (RFU) [7]. In our case, the pull-up effect was clearly caused by overloading PCR products. From the allele panels in **Figures 1–3**, the RFU values in our samples were generally higher than 20,000. Therefore, to meet the instrument requirements, it is necessary to optimise the final PCR product concentration for each locus by, for example, adjusting the DNA template concentration, PCR cycling or using post-PCR dilution (as Treatment-3 and Treatment-4 display; **Table 2** and S2 and S3 Figures). However, these steps certainly increase the cost and time of the analysis [16], especially for a laboratory without an automated sequencer instrument. Indeed, high RFU values are not uncommon. For example, in the literature of Fragman software [9], many samples displayed high RFU values (see **Figures 1**, **4** and **5** in their literature).

4. Omitting particular size standard peaks that overlap or are close to the allele peaks. Compared to the above solutions, this was both cost- and time-efficient. In Genemapper, Genemarker and Gelquest, all provided size standards with a particular size fragment are excluded (such as 250 bp and/or 340 bp) because of their abnormal migration behaviour. In this study, after omitting the 200 or 250 bp fragment, all four programmes resulted in identical allele patterns for Datasets 1–3. Furthermore, for Dataset 3, the allele pattern with 250 bp omitted using HEX dye was identical to the pattern results derived from Treatment-2 in Dataset 3 with the full set of internal size standard fragments using FAM dye. Therefore, creating a custom size standard by omitting particular fragments from the internal size standard could circumvent the pull-up problem.

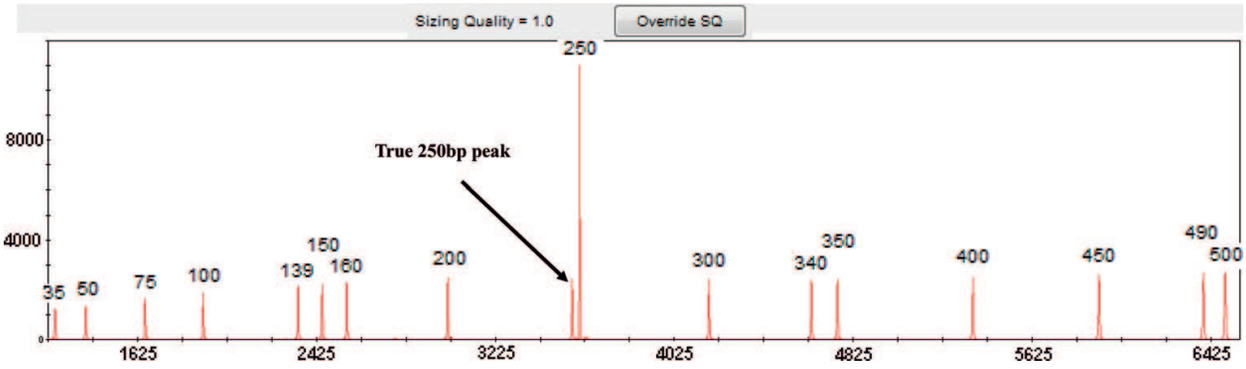

Figure 4. Size standard calibration for sample 4(A04_04) in Treatment-1 in Dataset 3 using Genemapper 5.0. [Colour figure can be downloaded and viewed at http://molecular-ecologist.com/pd.jsp?id=1#_jcp=2].

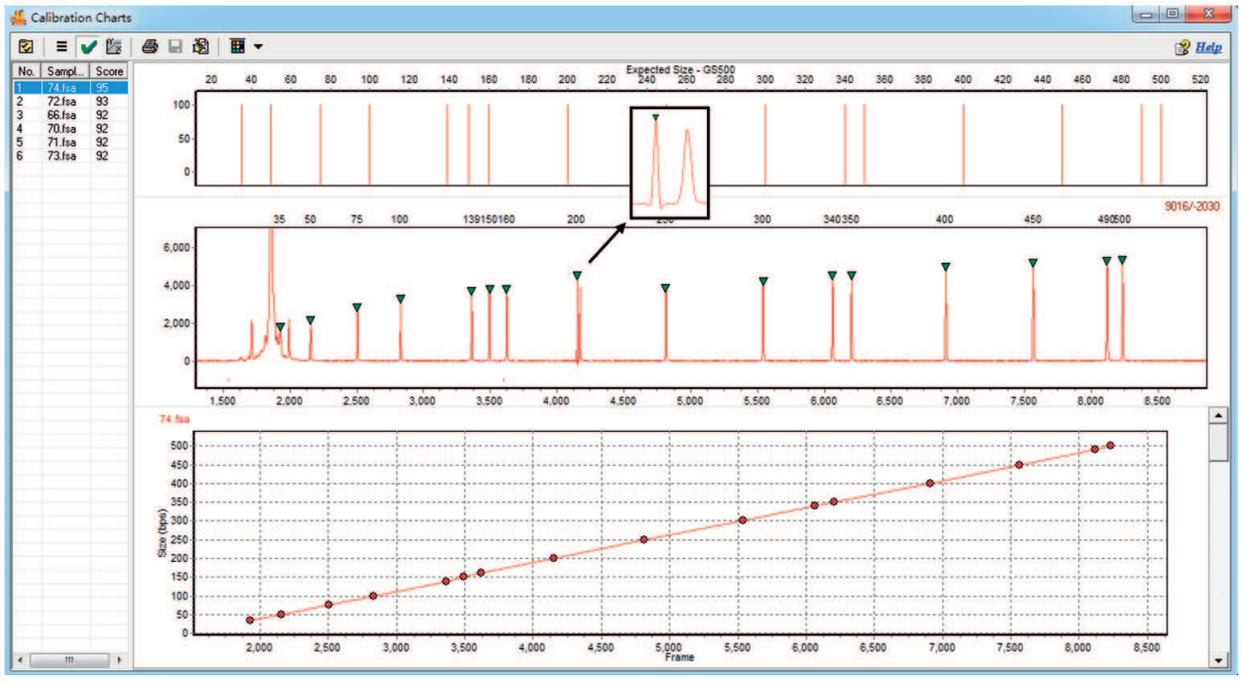

Figure 5. Size standard calibration for sample 74 in Dataset 2 using Genemarker 2.7. [Colour figure can be downloaded and viewed at http://molecular-ecologist.com/pd.jsp?id=1#_jcp=2].

5. Conclusions

This study was the first to describe one particular microsatellite allele size calling error attributed to using HEX dye and ROX dye in capillary electrophoresis. This cautions researchers to carefully assess the results of automatic allele calling. Using different software and visually scoring, each result will allow accurate sizing of microsatellite alleles. Of the four software programmes used here, both Genemapper and Genemarker are commercial, and most laboratories cannot afford them. However, compared to Genemapper, Genemarker is easier to use. Of the two free programmes, Gelquest has a graphical user interface that is easy to use and provides many user-friendly functions to help display sample alleles, while Fragman did not. Since ABI sequencers are most commonly used for analysing microsatellites, polymorphisms are generally identified by Genemapper; thus, we recommend that researchers use Gelquest as an alternative tool to check the consistency of the results.

Acknowledgements

We thank Dan-Dan Zhang, Ying Chen, Lin-Fang Wu, Yi-Fan Zhuang and others who have helped in the field and in the laboratory. This study is supported by the National Natural Science Foundation of China (Grant No. 41371078 and No. 31170196); the Science and Technology Program of Guangzhou, China (Grant No. 201707010339 and No. 201705040006); the Science and Technology Program of Guangdong Province, China (Grant No. 2016A030303003 and Grant No. 2017A030303060); and the Chinese Forest Biodiversity Monitoring Network.

Conflict of interest

The authors declare no conflict of interest.

A. Appendices and nomenclature

S1 Figure Electropherograms showing alleles in example samples of Treatment-2 in Dataset 3.

S2 Figure Electropherograms showing alleles in example samples of Treatment-3 in Dataset 3.

S3 Figure Electropherograms showing alleles in example samples of Treatment-4 in Dataset 3.

S4 Figure Electropherograms showing alleles in example samples of Treatment-5-1 in Dataset 3.

S5 Figure Electropherograms showing alleles in example samples of Treatment-5-2 in Dataset 3.

S6 Figure Electropherograms showing alleles in example samples of Treatment-6-1 in Dataset 3.

S7 Figure Electropherograms showing alleles in example samples of Treatment-6-2 in Dataset 3.

S8 Datasets Datasets 1–3 used for this study.

Appendices (S1–S8) can be downloaded from the website http://www.molecular-ecologist.com/pd.jsp?id=1#_jcp=2.

Author details

Zheng-Feng Wang[1]*, Se-Ping Dai[2], Ju-Yu Lian[1], Hong-Feng Chen[1], Wan-Hui Ye[1] and Hong-Lin Cao[1]

*Address all correspondence to: wzf@scib.ac.cn

1 Guangdong Provincial Key Laboratory of Applied Botany, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, China

2 Guangzhou Institute of Forestry and Landscape Architecture, Guangzhou, China

References

[1] Jarne P, Lagoda PJL. Microsatellites, from molecules to populations and back. Trend in Ecology & Evolution. 1996;**11**(10):424-429. DOI: 10.1016/0169-5347(96)10049-5

[2] Kantartzi SK. Micorsatellite: Methods and Protocols. New York: Humana Press; 2013. DOI: 10.1007/978-1-62703-389-3

[3] Fernando P, Evans BJ, Morales JC, Melnick DJ. Electrophoresis artefacts—A previously unrecognised cause of error in microsatellite analysis. Molecular Ecology Notes. 2001;**1**:325-328. DOI: 10.1046/j.1471-8278.2001.00083.x

[4] Hoffman JI, Amos W. Microsatellite genotyping errors: Detection approaches, common sources and consequences for paternal exclusion. Molecular Ecology. 2005;**14**:599-612. DOI: 10.1111/j.1365-294X.2004.02419.x

[5] Dewoody J, Nason JD, Hipkins VD. Mitigating scoring errors in microsatellite data from wild populations. Molecular Ecology Notes. 2006;**6**:951-957. DOI: 10.1111/j.1471-8286.2006.01449.x

[6] Hess MA, Rhydderch JG, LeClair LL, Buckley RM, Kawase M, Hauser L. Estimation of genotyping error rate from repeat genotyping, unintentional recaptures and known parent–offspring comparisons in 16 microsatellite loci for brown rockfish (*Sebastes auriculatus*). Molecular Ecology Resources. 2012;**12**:1-10. DOI: 10.1111/1755-0998.12002

[7] Flores-Rentería L, Krohn A. Scoring microsatellite loci. In: Kantartzi SK, editor. Micorsatellite: Methods and Protocols. New York: Humana Press; 2013. pp. 319-336. DOI: 10.1007/978-1-62703-389-3.ch21

[8] Cloete K, Ristow WPG, Kasu M, D'Amato ME. Design, installation, and performance evaluation of a custom dye matrix standard for automated capillary. Electrophoresis. 2017;**38**:617-623. DOI: 10.1002/elps.201600257

[9] Covarrubias-Pazaran G, Diaz-Garcia L, Schlautman B, Salazar W, Zalapa J. Fragman: An R package for fragment analysis. BMC Genetics. 2016;**17**:62. DOI: 10.1186/s12863-016-0365-6

[10] Zhang DD, Luo P, Chen Y, Wang ZF, Ye WH, Cao HL. Isolation and characterization of 12 polymorphic microsatellite markers in *Engelhardia roxburghiana* (Juglandaceae). Silvae Genetica. 2014;**63**(3):109-112

[11] Zhuang YF, Wang ZF, Wu LF. New set of microsatellites for Chinese tallow tree, *Triadica sebifera*. Genetics and Molecular Research. 2017;**16**(2):gmr16029624. DOI: http://dx.doi.org/10.4238/gmr16029624

[12] Wang ZF, Lian JY, Ye WH, Cao HL, Zhang QM, Wang ZM. Pollen and seed flow under different predominant winds in wind-pollinated and wind-dispersed species *Engelhardia roxburghiana*. Tree genetics & Genomes. 2016;**12**:19. DOI: 10.1007/s11295-016-0973-3

[13] Koressaar T, Remm M. Enhancements and modifications of primer design program Primer3. Bioinformatics. 2007;**23**(10):1289-1291. DOI: 10.1093/bioinformatics/btm091

[14] Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3—New capabilities and interfaces. Nucleic Acids Research. 2012;**40**(15):e115

[15] Schuelke M. An economic method for the fluorescent labeling of PCR fragments. Nature Biotechnology. 2000;**18**:233-234. DOI: 10.1038/72708

[16] Dimsoski P. Development of a 17-plex microsatellite polymerase chain reaction kit for genotyping horses. Croatian Medical Journal. 2003;**44**(3):332-335