Chapter 21

Scoring Microsatellite Loci

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Abstract

Microsatellites have been utilized for decades for genotyping individuals in various types of research. Automated scoring of microsatellite loci has allowed for rapid interpretation of large datasets. Although the use of software produces an automated process to score or genotype samples, several sources of error have to be taken into account to produce accurate genotypes. A variety of problems (from extracting DNA to entering a genotype into a database) which can arise throughout this process might result in erroneous genotype assignment to one or more samples, potentially confounding the conclusions of your study. Correctly assigning a genotype to a sample requires knowledge of the chemistry you use to generate the data as well as the software you use to analyze these results. In this chapter we describe the critical and more common points that researchers experience when scoring microsatellite loci. More importantly we provide insight from an experienced perspective for these challenges.

Key words Allelic drop-off, Error rate, Fluorescent markers, Genotyping, Null alleles, Polymerase slippage, Scoring microsatellites, Size standard

1 Introduction

Microsatellites, also called simple sequence repeats (SSR) or short tandem repeats (STR), are short repetitive sequences that are prone to rapid mutations that result in sequence length polymorphisms across individuals. The use of microsatellites as polymorphic DNA markers has considerably increased both in the number of studies and in the number of organisms, primarily for population genetics, genetic mapping, studying genomic instability in cancer, forensics, conservation biology, molecular anthropology, and in the studies of human evolutionary history ((5, 55, 59) and references therein). Microsatellite loci can be genotyped by PCR amplification of the microsatellite region, and separation of the products from different samples by electrophoresis. The common detection method is to label one of your PCR primers with a fluorophore that can be detected by laser-induced fluorescence on a capillary electrophoresis system. Amplification produces a pair of fluorescent allelic

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products (for diploid genes) that will vary in size according to the number of microsatellite repeat units. A suitable choice of fluorescent labeling enables analysis of multiple loci in the same capillary injection. Using different color and size to distinguish between fragments, it is possible to multiplex or pseudoplex >20 markers in a single capillary (14, 15, 33), although it is more common to multiplex only five to eight markers at a time (32).

Fluorescently labeled DNA fragments mixed with an internal size standard migrate through polymer-filled capillaries past a laser beam which excites them. Emission spectra from individual fluorophores are separated by a diffraction grating, and a CCD camera converts the fluorescence signal into digital data that is processed by the instrument data collection software. Allele sizing, scoring, and subsequent data analysis are performed using external software. The automated process of allele scoring allows the analysis of a massive amount of data (number of samples and markers). However, several sources of error have to be taken into account to produce accurate genotypes.

Even if you are adept of the use of your preferred analysis software, correct assignment of genotypes to your sample data is contingent upon first performing PCR using correct chemical conditions. Otherwise, your work may suffer from the computer science adage, "garbage in, garbage out." It is therefore essential that you test each locus to be amplified individually prior to initiating data collection for your project. Many researchers find this step to be difficult, time-consuming, and therefore intimidating. A genotyping error rate of even 1 % (i.e., 1 % of the alleles in an entire dataset are misidentified), which is an uncommonly good value for most studies, can lead to a substantial number of incorrect multilocus genotypes in a large dataset, which in turn will lead you to wrong conclusions (34). In addition to poor amplification, sources of error include incorrect interpretation of stutter patterns or artifact peaks, contamination, mislabeling, and data entry errors (6). In many cases, knowing the sources of error in the genotype data can allow one to correct for it, such as re-genotyping homozygous individuals to catch poorly amplifying alleles. With a few tips, we hope that you can identify and reduce the sources of error, thus improving the allele scoring in your future projects.

2 Materials

For microsatellite scoring by capillary electrophoresis you will need a thermal cycler and reagents for amplifying the desired loci (such as fluorescent label primers), formamide, size standard, access to a capillary electrophoresis-based genetic analyzer, and a computer to run your analysis software and for databasing your results. For fragment analysis on an Applied Biosystems Genetic Analyzer, detectable fluorescent labels are available from Life Technologies,

Table 1
Commonly used fluorophores in microsatellite analysis and their excitation and emission spectra

Dye	Excitation (nm)	Emission (nm)	Analysis color
6-FAM	495	520	Blue
HEX	535	553	Green
TAMRA	555	590	Yellow
ROX	575	607	Red
LIZ (ABI)	638	655	Orange (size std)

though several fluorophore analogues are also available at cheaper prices from companies that offer oligonucleotide synthesis (e.g., Operon, IDT, Table 1).

2.1 Software

There are several programs available to perform fragment analysis of microsatellite electropherograms. Unfortunately, most are not open source and require one to purchase expensive licenses for unrestricted use. This is the case for programs such as GeneMapper from Applied Biosystems, CEQ 8000 software from Beckman Coulter, and GeneMarker from SoftGenetics LLC. Applied Biosystems does provide a simple electropherogram viewer (Peak Scanner) for examining individual samples, but it does not perform comprehensive analyses. SoftGenetics will provide a demo version of GeneMarker to the end user upon request. Freely available software is also able to perform the most important tasks of identifying peak sizes relative to your internal size standard. For instance, the software STRand (60), created at University of California Davis, is available for download free of charge (http://www.vgl.ucdavis.edu/informatics/strand.php). Each software package is different, so we will detail a standard procedure for scoring microsatellites using the popular software packages GeneMapper (Applied Biosystems) and GeneMarker (SoftGenetics, LLC).

3 Methods

(1) *PCR amplification*: Find the optimal conditions to amplify your markers. We find the following concentrations of PCR components to be optimal for most microsatellite analyses: 1× PCR buffer (specific to the manufacturer of your polymerase), 200 nM each dNTP, 200 nM each primer, 0.015 U/μL polymerase, 1.5 mM $MgCl_2$ (or $MgSO_4$), and ~25 ng template DNA. The volume of your reaction matters little, but we find that in the interest of conserving reagents, one can reliably perform 10 μL reactions in a 96-well PCR plate or 4 μL reactions in a 384-well

plate. A 4 μL reaction permits one to check the PCR product on a gel using 2 μL and have 2 μL leftover for capillary analysis (in case of a weak amplification), resulting in no wasted reagent. A thermal cycling protocol we use with standard *Taq* polymerase is 90S °C 1 min; 35 cycles of 90S °C 30 s, 65S °C 2 min, and 72S °C 15 s, though cycling conditions may vary somewhat depending upon the locus being tested and the polymerase being used. Longer annealing time and a higher and therefore more stringent annealing temperature seem to dramatically improve amplification efficiency of microsatellite loci. Our primers are typically designed with a T_m of about 60S °C. For studies involving many loci, it may be more cost-effective to use tailed primers for fluorescent labeling of your amplified PCR products (53). We find the set of universal primers reported by Missiaggia and Grattapaglia (44) to function well in studies of plant population genetics when the fluorescent and reverse primers are included in the reaction at 200 nM each and the forward tailed primer is included at 20 nM.

(2) *Preparing and running the samples in the genetic analyzer (sequencer)*: One microliter PCR product may yield ideal signal strength, though the product may need to be diluted up to tenfold. One microliter of the appropriate dilution is mixed with 10 μL deionized formamide (nucleic acid denaturant) and an internal fluorescent size standard which encompasses the range of product sizes expected for your products. Numerous size standards are available from commercial sources or one can synthesize their own standard (see ref. 8). We routinely use between 0.1 and 0.2 μL GeneScan 500 LIZ Size Standard (Applied Biosystems) per sample with excellent results. To ensure that none of your PCR amplicons possess any secondary structure which may interfere with data interpretation, the samples are then denatured for 5 min at 90–95S °C. Using too little or too much sample can cause problems. Your genetic analyzer instrument can convert a limited range of fluorescence signal into digital values. For optimal results, you should keep the fluorescence signal between approximately 150 and 4,000 relative fluorescent units (RFU). Below this range, the signal-to-noise ratio may be too low to discriminate between sample peaks and background fluctuations. When fluorescence is too intense, the peak may not be sufficiently narrow to accurately assess your allele size.

Size standard: A set of 5′ fluorescently labeled fragments of known sizes. The size standard, possessing a fluorophore distinct from those bound to your fragments of interest, is simultaneously read with the PCR product(s) allowing the software to calibrate fragment sizes within a given sample. In some cases the software may misinterpret one or more of the internal size standard peaks, creating the potential for miscalled alleles due to incorrect calibration. When this occurs, the software will indicate

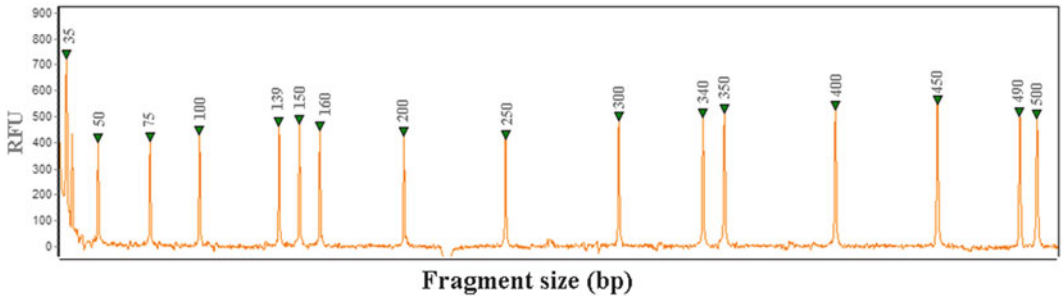


Fig. 1 Pattern of peaks in GeneScan 500 LIZ size standard. The height of each peak corresponds to its relative fluorescence intensity (*y*-axis)

which samples require manual calibration. The manufacturer of the internal size standard will provide information with their product that will enable the researcher to accurately make the necessary corrections. A popular standard is GeneScan 500 LIZ (Applied Biosystems), which ranges from 35 to 500 bp and uses an orange analysis color (Table 1 and Fig. 1).

(3) *Scoring alleles*: Although microsatellite genotyping was first developed in agarose and later in acrylamide gels, capillary electrophoresis (CE) is now the preferred method due to the higher accuracy and increased throughput. Ideally, a peak on the electropherogram (visual representation of a DNA fragment resolved by CE) rises sharply from the baseline, has smooth sides, and is symmetrical in shape.

The steps in data interpretation are the following:

1. Create a panel (see Table 2).
2. Assess peaks of interest versus PCR artifacts.
3. Assess the data from each sample or allele calling.
4. Export a data table.

3.1 Create a Panel

Once your sample files (.fsa, .ab1, .abi, .scf, .rsd, .esd, .smd, or .hid format) have been imported, you need to run your data initially without a panel (use default settings). This permits the software to compare peaks in your sample to those in the internal size standard so that you can begin to build a panel. Once run, enter alleles for each locus using your software's panel editor function. Screening of a locus across several samples should reveal alleles within the expected size range, exhibiting characteristic peak patterns, and any potential scoring problems for that locus. When a large sample size is represented in the analyzed data it is common to observe all expected alleles within the reported size range. For example, if you work with a perfect trinucleotide SSR ranging from 200 to 215 bp, you would expect to observe six alleles at 200, 203, 206, 209, 212, and 215 bp. Such "perfect" results are not always the case however, so one should not get discouraged if an allele remains unrepresented or additional loci are observed within one's dataset (see Subheading 4 for possible explanations).

Table 2
Common terms and definitions

Term	Definition
Bin	For each marker, separate bins (size in bp) are defined by the user for each allele observed. A group of bins is sometimes referred to as a bin set.
Marker	Each marker (or locus) is defined by name, size range (bp), dye color, and repeat length. The size range will include bins for each expected allele.
Panel	A group of markers for simultaneous analysis.
Color channel	Each channel is viewed in the analysis as a separate color defined by the emission spectra of each fluorophore. Different instruments can interpret different dyes, but each will be capable of reading four or five colors simultaneously.
Peak	Visual representation of a DNA fragment resolved by capillary electrophoresis.
Size standard	A set of 5′ fluorescently labeled fragments of known sizes.
RFU	Relative fluorescence units which measure the intensity of a fluorescence signal.

3.2 Get Familiar with Your Loci

Once you have identified all possible alleles in your data you can create bins for each allele (expected size limit for each allele). Bins usually are one bp long to avoid capturing neighboring alleles within the same bin, and to allow for slight variation among called alleles due to sequencer error (approximately ± 0.5 bp). Selection of the fluorophore used is assigned during the panel creation.

When working with only a handful of samples and loci, it may be expedient to simply call each allele one at a time and record the results in a spreadsheet. For larger datasets it will be necessary to instead automate this process by creating a panel of expected allele sizes for each locus against which the software will compare your samples. Though not all software are the same, this process is fairly uniform. Use the panel editor function of your analysis software to identify peaks present in the expected size range across all your samples, and record their positions. Once entered into the panel (specific to an individual project), the software will be able to call peaks observed within each sample into bins which refer to the individual alleles that you designate. Though post-processing editing of automated allele calls may be necessary, this step will greatly facilitate analysis of medium- to large-sized datasets.

3.3 Assess Peaks of Interest Versus PCR Artifacts

The complexity of distinguishing between peaks of interest and PCR artifacts is associated with the complexity of the genome amplified (haploid, diploid, or polyploid) or the number of markers included in a multiplex design. For example, the use of haploid chloroplast or mitochondrial SSRs will yield one allele per sample for each marker, making it relatively simple to perform allele calls. In contrast, working with an organism such as hexaploid wheat will yield between one and six peaks per sample, each of which must be efficiently amplified to be accurately scored.

There are multiple reasons to have confounding peaks in the data. Common causes for such peaks may be PCR artifact(s) created during amplification, incomplete terminal adenylation, mono- or dinucleotide stutter, or pull-up due to spectral overlap of two fluorophores. However, there may be other factors associated with the nature of the marker such as null alleles or the presence of an imperfect repeat. All of these factors can challenge the scoring process. Solutions to eliminate or reduce these confounding peaks are given in Subheading 4.

3.4 Allele Calling

Once your panel has been established you will be able to determine the genotype (sizes of your PCR products) of each sample based on the pattern of peaks or bands on the electropherogram. Rerun your data against your new panel (default settings again), and verify each allele call by hand. You may find that you did not capture all of the alleles present in your sample data; therefore some panel adjustment may be necessary (and subsequent rerunning of your samples) before you finalize your data. Software-automated allele calling will take a few seconds or minutes depending on the size of your dataset.

3.5 Create and Export a Table

The software generates a table with your genotype associated to each sample. For subsequent analysis in a population genetics analysis package such as Arlequin (25) or GenAlEx (48), export your data as a bin table or a genotype table as appropriate.

Though the above protocols are quite similar and may seem straightforward, and the software performs allele calls in an automated fashion, one should never fully trust one's initial data output. Variations among your PCR products may be due to a number of factors including well-to-well variation across your thermal cycler, pipeting errors, and inconsistent quality among your template DNA samples. These factors (and others) can contribute to variation in PCR amplification efficiency that is exhibited as differences in peak height among your electropherograms. Since it is these data that are interpreted by the software and you may restrict allele calling based on peak heights across all samples, the software should be considered fallible. Following the automated allele calling step, scan through the samples and look for obvious errors (e.g., peaks not called, stutter peaks called). You may notice that certain loci yield different characteristic peaks. For instance, a trinucleotide locus may exhibit very clear peaks, each with a small preceding peak while a dinucleotide locus may suffer more from stutter, contributing to a "rooster-comb" appearance (Fig. 2). Mononucleotide repeats will generate peak profiles similar to those of dinucleotide repeats; however there are some strategies that allow reduction of the stutter in these markers difficult to score (see Subheading 4).

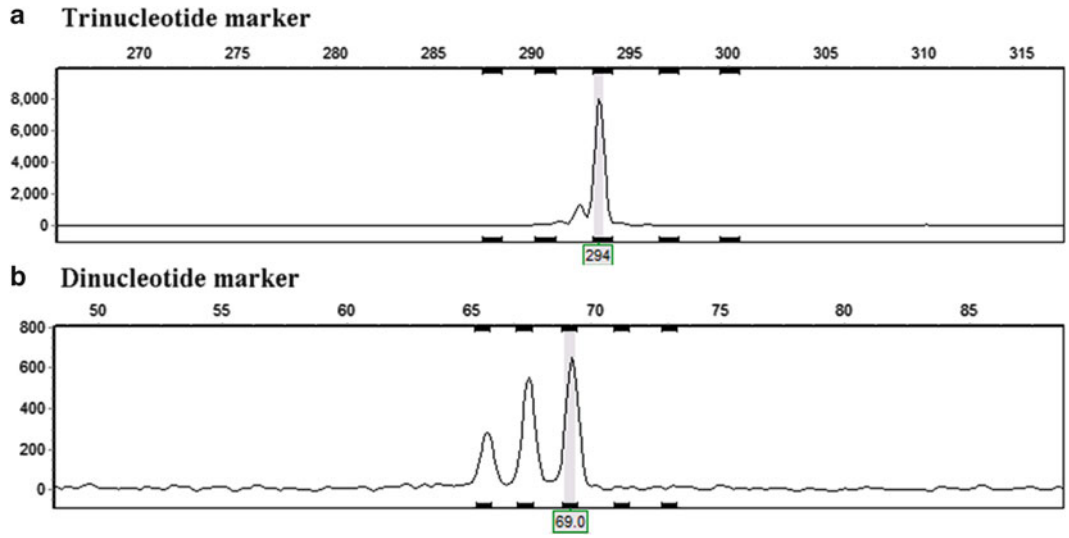


Fig. 2 Peak profiles characteristic of different repeat lengths. Trinucleotide (or longer) repeats (**a**) usually exhibit a very clear major peak with a preceding minor peak. Dinucleotide repeats (**b**) usually exhibit more than one major peak per allele

4 Notes

One should manually check the quality of automated allele calling. Most errors incurred during the allele calling process are derived from poor PCR amplifications which result in low amplification efficiency or production of nonspecific products. You might need to optimize your PCR conditions or even redesign your primers. Re-extracting DNA from difficult samples and re-amplifying questionable genotypes (e.g., heterozygotes with closely sized alleles, faint alleles) is a common practice to increase the accuracy during genotyping. However other factors can cause some troubles during allele calling. In this section we describe common problems that researchers encounter during this procedure and suggest some solutions.

4.1 Previously Unreported Alleles

Occasionally, one may encounter alleles that were not previously reported in the publication from which one derived one's microsatellite markers. This is more common for loci that exhibit many alleles than for loci that exhibit only a few. It will be up to the researcher to determine whether these "new" alleles are valid genotypes or if the result is an error. We suggest that you first look through all your samples to determine if you are observing unreported alleles in more than one sample. If observing a new allele in multiple samples, you can feel more confident that the allele is valid. If instead a new allele is represented in just a single sample, the PCR reaction should be

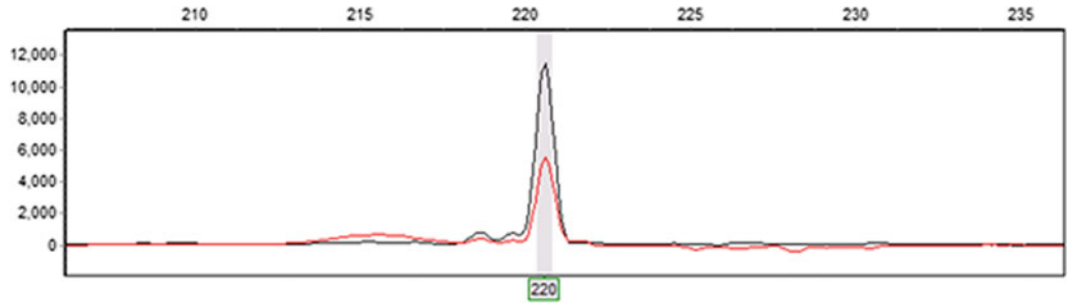


Fig. 3 TAMRA fluorophore (black peak) causing pull-up of the red channel on an ABI 3730xl genetic analyzer

repeated to verify that there was no error in your chemistry. In either case, a subset of samples should be re-amplified to provide a measure of confidence in each allele call.

4.2 "Extra" Alleles

Some loci will exhibit more alleles than expected for a given organism. For example, one would expect a maximum of one allele per locus for a haploid organism or two alleles per locus for a diploid organism. If, when working with a haploid organism and you observe two peaks within your expected allele range, you are likely encountering a locus that has been duplicated within the genome (e.g., ref. 63), yielding amplification of a microsatellite family rather than a single discrete locus. Anderson et al. (2) were the first to report successful utilization of these loci, characterized by their repetitive flanking sequences (ReFS). Though ReFS can be useful for genetic inquiry of populations, such loci are beyond the scope of this chapter and require statistical treatment as dominant markers. Many researchers will choose to simply discard such anonymous loci in favor of properly functioning microsatellites which will better serve to answer their particular research question.

4.3 Pull-Up

If you include too much PCR product on a capillary run, you may experience very strong fluorescence signal (approximately >20,000 RFU) from the labeled fragments therein. High fluorescence signal can prevent the instrument from properly compensating for spectral overlap among the dyes resulting in artifact peaks in one channel derived from the strong signal intensity in another (called "bleedthrough" or "pull-up"). Artifact peaks can corrupt both automated size-calling due to pull-up peaks in the size standard color and the analysis of co-loaded samples when pull-up peaks overlap a bin set for another marker. Certain combinations of fluorophores are more prone to the pull-up effect than others. For example, TAMRA tends to cause pull-up in the ROX channel, but not vice versa (Fig. 3). For this reason, one would choose to use a LIZ-labeled size standard rather than a ROX-labeled standard if you plan to score PCR products labeled with TAMRA.

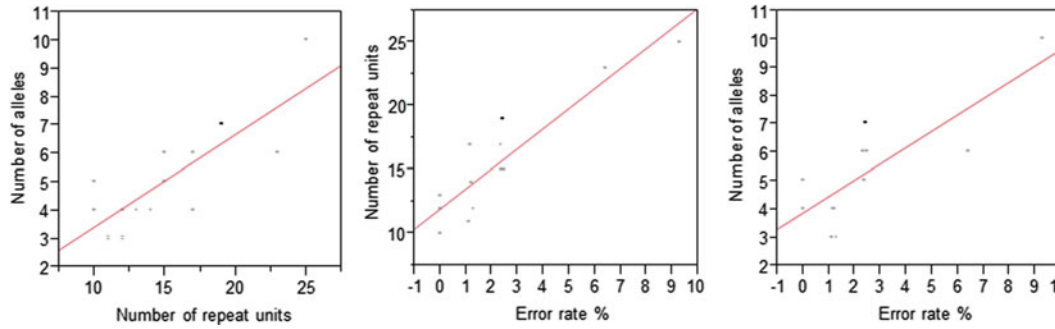


Fig. 4 There is a positive relationship between the repeat size and the number of alleles; however, there is a trade-off associated. Loci exhibiting longer repeat units and high number of alleles will have higher error rates. This is especially true for mononucleotide and dinucleotide repeats. Data generated using 12 mononucleotide SSRs from Flores-Rentería and Whipple (28)

4.4 Stutter in Mononucleotide and Dinucleotide Repeats

Variation in the number of repeats in microsatellite loci is primarily due to polymerase slippage (slipped-strand mispairing) during DNA replication, as well as repair mechanisms during recombination ((62); reviewed in (41)). Slippage can also be generated during PCR reactions making allele designation difficult (40, 43), especially for heterozygotes with adjacent alleles, resulting in high error rates in scoring (18). Mononucleotide repeats are the most common SSRs in the plant chloroplast genome and, due to their high mutation rates, they represent the most variable markers in this organelle (51). According to Guichoux et al. (32) among 100 studies surveyed from 2009 to 2010, none made use of mononucleotide repeat SSRs. This reflects the fact that because mononucleotide repeat SSRs are difficult to accurately assay (58) they are often eliminated at the outset (37). In contrast, dinucleotide SSRs were the most frequently used class of microsatellites (32). Unfortunately, mononucleotide and dinucleotide repeats often show one or more "stutter" peaks arising from multiple PCR products derived from the same reaction template that are typically shorter by one or a few repeats than the full-length product (12). The error rate in allele calling for dinucleotide SSRs is ~5 % with samples amplified by *Taq* polymerase (31), and it could be higher for mononucleotide repeats. Polymerase slippage is positively correlated with the length of the microsatellite ((35, 36); see Fig. 4), making scoring of mononucleotide SSRs >11 bp highly error-prone (18). In contrast, tri-, tetra-, or pentanucleotide repeats appear to be significantly less prone to exhibiting stutter peaks (17, 24, 45). Hence, SSRs with core repeats three to five nucleotides long are sometimes preferred for forensic and parentage applications (17, 38). Note however that stutter bands, when not too strong, can be useful, by helping distinguish true alleles from PCR artifacts (e.g., ref. 54). Note also that a few solutions have been proposed to overcome stuttering problems. The most common solution has been to simply select loci that present the lowest degree of stutter (e.g., refs. 21, 46).

However, mono- and dinucleotide repeats have been used successfully in studies of chloroplast DNA variation in plants (23, 51), SSR-poor fungi (16), or in other circumstances, for assessing microsatellite instability associated with cancer (e.g., ref. 27), where such markers are of special interest.

In addition to the importance of mono- and dinucleotide SSRs mentioned above, there is a methodological relevance in the use of these markers. According to Guichoux et al. (32), focusing on the shortest motifs (such as mono- or dinucleotide repeats) rather than on longer ones (≥trinucleotide repeats) should allow for more dense packing of loci on a given separation system, resulting in larger multiplex designs. This can be important because the capillary electrophoresis-based genetic analyzers used for SSR genotyping make use of no more than four or five fluorophores, thus limiting the number of SSR loci that can be analyzed simultaneously. Given that the allelic range size often reaches up to 50 or 100 bp and that amplicons measuring over 300 bp are rarely used (e.g., refs. 14, 33).

4.5 Reducing Stutter

Stutter bands are typically shorter than the original fragment (56). Thus it has been generally assumed that choosing the largest fragment (bp) will resolve the problem. However, in our experience this is not always right, so improvement during the PCR amplification has to be done in order to reduce stutter. We list the few solutions that have been proposed to overcome stuttering problems:

1. To decrease denaturing temperature to 83S °C (47).
2. Varying the reaction conditions or including additives such as formamide, bovine serum albumin, or dimethyl sulfoxide (9).
3. Adjusting the PCR program by using touchdown or hot start techniques, reducing the number of cycles, or maintaining a stringent annealing temperature (21).
4. To use new-generation polymerases, such as fusion enzymes (26) or PCR kits designed especially for microsatellite analysis (e.g., Multiplex PCR Kit or TypeIT Microsatellite PCR Kit from Qiagen).

Flores-Rentería and Whipple (28) developed a new method to increase the accuracy of scoring mono- and dinucleotide alleles by designing primers that include part of the microsatellite in order to reduce the slippage. This method was tested using primers developed to amplify mononucleotide repeats (≥10 bp) in the chloroplast of *Pinus* spp.

4.6 Null Alleles and Allelic Drop-Out

A microsatellite null allele is any allele at a microsatellite locus that consistently fails to amplify to detectable levels via polymerase chain reaction (PCR) (19). There are at least three potential causes of null alleles or allelic drop-out: (1) poor primer annealing due to mutation on the primer region (e.g., substitutions or indels in one or

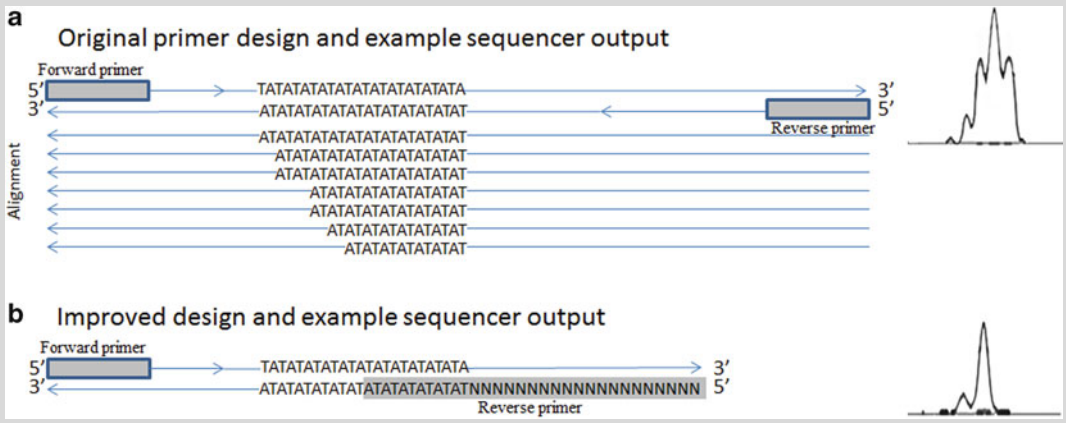
Box 1 Improving Scoring by Reducing Stutter in Mononucleotide and Dinucleotide Repeats

For example, if there is a dinucleotide repeat of $(TA)_{7-12}$ that when amplified, the profile generated on capillary sequencer looks like panel A. There is an easy way to reduce the stutter by designing a new primer that contains part of the microsatellite. In this example we redesign the reverse primer.

1. To amplify and sequence the SSRs of interest using the original primers in at least eight samples under normal conditions (samples from the most diverse source the best).
2. To align your sequences in order to find the range in length of the repeat (panel A). In this case the longest repeat is $(TA)_{12}$ and the shortest is $(TA)_7$.
3. Then a new reverse primer should be designed including the flanking region of the microsatellite and part of the microsatellite. The repeat length in the primer should be equal to the smallest microsatellite detected, minus one or two bases, in our example $(TA)_6$ (panel B).
4. The new reverse primer should be compatible with the original forward primer, e.g., no hetero or homo dimer formation. The unequal length of the forward (let us say 24 bp) and the new reverse (let us say 32 bp) primers does not affect the amplification as long as they have similar melting temperature above 50S °C.
5. You can decide to use the forward region to design your primer containing part of the repeat if the flanking region has better conditions than the reverse flanking region (e.g., G+C content).
6. When using the internal primers the PCR should be performed under standard conditions.
7. This method allows a multiplex assay, if similar melting temperatures are used for all primers.
8. If you are using mononucleotides SSR multiplex primer combinations should not mix A and T repeats to avoid primer-dimer formation.
9. In our experience up to six primer pairs can be multiplexed in a single PCR reaction.
10. You can try also to use higher concentration of the reverse primer.

This method requires a little bit of knowledge about designing primers. However, it is more cost-effective in comparison to the use of fusion polymerases, and may require less troubleshooting than the use of additives. However, the benefit achieved through this method of stutter reduction diminishes with longer repeats as the primer may not be able to be designed with an acceptable annealing temperature due to the necessary length. Further, if the repeat-containing 3′ end of the primer is too long, mispriming may occur at other SSR loci containing the same repeat. Though this effect may not directly interfere with the scoring of a locus, it can consume one of your primers, thus reducing the efficiency of your PCR reaction.

If you want to amplify a mono- or dinucleotide SSR longer than 20 bp using a new reverse primer containing only 10 bp of the repeat you might not have a clear peak. In that case you can design an additional reverse primer containing, for example, 16 bp.



both primer annealing sites). In particular, mutations in the priming site at or near where the 3′ end of the primer anneals are thought to be especially detrimental to PCR amplifications (39) and can contribute to an allele becoming null for a given locus. The allele can be "resurrected" following a redesign of the primers. In most cases internal primers are designed, resulting in a slightly smaller PCR product. Degenerate primers are another alternative, or external primers can be designed when the necessary sequence data is available. (2) Differential amplification of size-variant alleles or "partial nulls" (61). Due to the competitive nature of PCR, alleles of short length often amplify more efficiently than larger ones such that only the smaller of two alleles might be detected from a heterozygous individual. Outcompeted alleles may stochastically amplify more strongly in a second PCR reaction. Alternatively, by loading more sample undetectable peaks become evident. (3) PCR failure due to inconsistent DNA template quality or low template quantity. These problems are insidious because in some cases only one or a few loci (or alleles) fail to amplify, whereas others amplify with relative ease from the same DNA preparation (29, 30). When DNA template at a given locus is poor in some specimens but not others, some samples may appear artificially homozygous rather than heterozygous for the null allele. A potential solution for this is to improve DNA quality by either a further purification step (e.g., ethanol precipitation or column purification) or by re-extracting DNA from the sample in question. Of the above three causes for null alleles, the first one is generally accepted as a legitimate cause of a "true" null allele while causes 2 and 3 are more likely due to technician deficiencies in the amplification process (13).

In addition to these primary causes of null alleles and drop-out, several population genetic phenomena might give the false impression that null alleles are present in a given study. Biological factors such as the Wahlund effect (reduction of heterozygosity in a population caused by subpopulation structure) or inbreeding, for example, can cause significant heterozygote deficits relative to Hardy–Weinberg equilibrium that might be misconstrued as evidence for null alleles (11). Wahlund effect or inbreeding tends to be observed more or less concordantly across loci, whereas the effects of null alleles are locus specific. Therefore the comparison across multiple loci will be useful to discard these possible causes of homozygosity bias.

4.7 Compound Microsatellites

It is often assumed based on a handful of sequence observations that microsatellite loci have a single, discrete repeat sequence (e.g., $(GT)_{14}$). Perhaps in part due to a complex mutational process that leads to variation in microsatellite repeats (22), many microsatellite loci may in fact exhibit variation in the sequence of the repeat structure (e.g., $(GT)_9(GA)_6$; (7, 50)). Such repeats are known as compound microsatellites and are discussed at

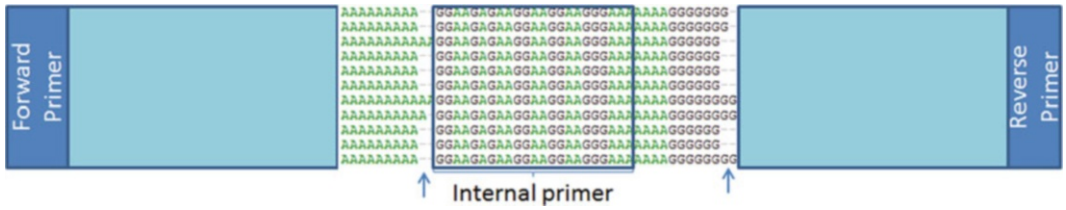


Fig. 5 Compound microsatellite creating homoplasy (*see arrows*). Breaking of the compound microsatellite can be achieved by designing an internal primer; potential region for a redesigned forward or reverse primer is shown in a *black box*

length in Bull et al. (10). Scoring a compound microsatellite may be more challenging than scoring a perfect repeat, as alleles observed within a given population may not be represented in other populations, and since the length of the various repeat motifs may also vary. Unfortunately such complexity can lead to some amount of homoplasy among individuals (e.g., ref. 50), so perfect repeats are desirable. If possible, redesign your primers to only assess one type of repeat (Fig. 5).

4.8 Incomplete Terminal Adenylation

Alternatively, an allele may falsely appear to exhibit such complexity when using *Taq* polymerase and incomplete terminal adenylation results in a peak that is 1 bp shorter than an expected allele (9), regardless of the length of the repeat motif. Most researchers try to ensure complete terminal adenylation when using *Taq* polymerase by using a final extension step (e.g., 60S °C for 15 min) once thermal cycling is complete. Brownstein et al. (9) found that including the "PIGtail" sequence, 5′-GTTTCTT-3′, at the 5′ end of your reverse (non-fluorescently labeled) primer will further facilitate complete terminal adenylation of the fluorescently labeled strand.

4.9 Controls, Confidence, and Error Rate

In the previous paragraphs we described some error sources associated to PCR and the nature of the microsatellites used, and gave potential alternatives or solutions to lower the error rate. Additionally, human error can be introduced directly by DNA contamination, mislabeling samples, or entering wrong data. According to Selkoe and Toonen (55), some amount of error is unavoidable. Regardless of the error source, the error rate within each study should be quantified and reported (reviewed in ref. 49).

Confidence in your scoring procedure can be achieved by including some controls in your data. To ensure that amplification of alleles is consistent throughout the duration of a study, a positive control should be run with every PCR plate, especially any time multiple sequencers are used for genotyping in a single study, or new batches of primers are used (20). According to

Selkoe and Toonen (55) the whole dataset can be genotyped in duplicate or more, as is performed for human parentage or forensics. Conversely, population genetics studies lack the ability to conduct this practical quality check, so accurate reporting of error rates is essential. Fortunately, by keeping track of one's error rate, one can identify and correct the major sources of systematic error in order to bring the overall error rate to an acceptable minimum.

We have established the error rate associated to a marker by repeating marker amplification under same condition in a 96-well plate. Error rate has to be calculated consistently with a simplex or a multiplex design, counting the number of inconsistent genotypes between the first and second attempts. The error rate can then be expressed as either the ratio of incorrect genotypes to the number of repeated reactions (28) or the ratio of incorrect alleles to the total number of alleles (34). Alternatively, the false discovery rate (3) can be employed and has been used to establish and control error rates for a variety of genotyping studies (e.g., refs. 4, 57). By examining the sources of each error, it is possible to determine whether the majority of errors are broadly distributed (such as typographical errors), or biased towards some subset of the data (such as homozygotes in the case of null alleles). For researchers investigating samples of known pedigree as in half-sib/full-sib association mapping studies (e.g., ref. 52), genotyping errors will reveal themselves when one or more alleles segregate inconsistently with Mendelian inheritance patterns. Such an obvious genotyping error will cue the researcher to re-genotype the aberrant samples and can quickly inform whether the trouble is with the PCR chemistry or the initial DNA extraction.

Just a few programs take the error rate into account. One of them is the parentage program CERVUS, which can estimate error rate while also accounting for mutation (42). The effect of error on measures of genetic structure can be estimated using a bootstrapping technique developed by Adams et al. (1). Once the error rate is accounted for, it can be controlled in order to achieve the desired statistical power (3). Due to the potential sources of error incurred during microsatellite allele scoring, we encourage software developers to continue to incorporate error rate into their programs.

Despite the recent advances in DNA sequencing technologies, we expect microsatellites to continue to be utilized as a user-friendly, cost-effective genetic marker system. Such genetic inquiry remains necessary in various research disciplines including ecology and agriculture. With a little experience, it is our sincere hope that a researcher can confidently make use of microsatellites in order to answer their particular research question.

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