

REVIEWS AND
SYNTHESES**Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers**

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Abstract

Recent improvements in genetic analysis and genotyping methods have resulted in a rapid expansion of the power of molecular markers to address ecological questions. Microsatellites have emerged as the most popular and versatile marker type for ecological applications. The rise of commercial services that can isolate microsatellites for new study species and genotype samples at reasonable prices presents ecologists with the unprecedented ability to employ genetic approaches without heavy investment in specialized equipment. Nevertheless, the lack of accessible, synthesized information on the practicalities and pitfalls of using genetic tools impedes ecologists' ability to make informed decisions on using molecular approaches and creates the risk that some will use microsatellites without understanding the steps needed to evaluate the quality of a genetic data set. The first goal of this synthesis is to provide an overview of the strengths and limitations of microsatellite markers and the risks, cost and time requirements of isolating and using microsatellites with the aid of commercial services. The second goal is to encourage the use and consistent reporting of thorough marker screening to ensure high quality data. To that end, we present a multistep screening process to evaluate candidate loci for inclusion in a genetic study that is broadly targeted to both novice and experienced geneticists alike.

Keywords

Homoplasy, linkage, marker isolation, Mendelian inheritance, microsatellites, molecular ecology, neutrality, null alleles, population genetics, simple sequence repeats.

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INTRODUCTION

In the past decade, genetic approaches to answering ecological questions have become more efficient, powerful and flexible, and thus more widespread. Genetic markers such as allozymes, microsatellites and mitochondrial and nuclear DNA sequences can be used to estimate many parameters of interest to ecologists, such as migration rates, population size, bottlenecks, kinship and more (see Table 1). Microsatellites have emerged as one of the most popular choices for these studies in part because they have the potential to provide contemporary estimates of migration, have the resolving power to distinguish relatively high rates of migration from panmixia, and can estimate the relatedness of individuals. Several recent reviews detail the myriad of genetic analysis techniques now available and the ecological questions they can address (Bossart & Prowell

1998; Cruzan 1998; Davies *et al.* 1999; Luikart & England 1999; Shoemaker *et al.* 1999; Sunnucks 2000; Manel *et al.* 2003, 2005; Beaumont & Rannala 2004; Pearse & Crandall 2004). These reviews encourage ecologists to use genetic approaches, but there are still no published texts or manuals to guide a newcomer in the more practical side of adopting and applying these techniques. This synthesis is meant as a companion piece to those reviews to help flatten the learning curve of applied population genetics. Nevertheless, those attempting to use microsatellites for the first time will need to also read many of the more technical papers cited here and seek guidance from an experienced population geneticist.

There are two distinct factors contributing to the recent technical advancements of molecular ecology. First, laboratory techniques have become streamlined and less expensive, enabling the use of large numbers of samples and many loci. Second, improvements in computing technology

Table 1 Brief summary of some ecological questions that can be addressed using neutral genetic markers, sorted by the type of data required

Requires multilocus allele frequency data*
Which population did these individuals originate from?
How many populations are there?
Requires highly polymorphic sequence or microsatellite data†
Did the population expand or contract in the recent past?
Do populations differ in past and present size?
Requires multilocus genotype identification‡
What are the genetic relationships of individuals?
Which individuals have moved? (i.e. mark/recapture natural tags)
Which individuals are clones?
Works with many marker types†
What is the average dispersal distance of offspring (or gametes)?
What are the source–sink relationships among populations?
How do landscape features impact population structure and migration?
What are the extinction/recolonization dynamics of the metapopulation?
Did the population structure or connectivity change in the recent past?

See Pearse & Crandall (2004) and other references in text for more detail.

*These analyses might require >10 microsatellites – the number is inversely correlated with the degree of genetic differentiation across populations. Species with low migration rates and/or small populations will require fewer loci.

†Using >1 locus will substantially dampen interlocus sampling error.

‡Often requires microsatellites, but also possible with AFLP and RAPD fingerprinting techniques – see Sunnucks 2000 for marker comparison. RAPD, random amplified polymorphic DNA; AFLP, amplified fragment length polymorphism.

have inspired the use of intensive statistical approaches such as maximum likelihood, Bayesian probability theory and Monte Carlo Markov chain simulation. These new approaches use more of the information in a data set than the summary statistics of traditional approaches (e.g. F_{ST} , a measure of allele frequency differences across populations), and because the typical data set today contains 10^2 – 10^3 individuals sampled at many loci, there is more power to describe the demography and history of populations and relationships of individuals in a detailed manner. These advances allow many basic ecological questions to be addressed with genetic tools for the first time or in new ways (Table 1). In the past 5–10 years, dozens of software programs using the aforementioned statistical tools have been developed to address these lines of questioning (see Pearse & Crandall 2004).

Many ecologists are not yet aware that in many cases, using genetic tools no longer requires investment in expensive equipment and laboratory bench skills. There are now commercial services that will develop new markers for virtually any species, and many other companies and centralized university facilities that will extract and genotype DNA from a collection of tissue samples and send back a full data set in a matter of weeks for a reasonable fee. These services and their relatively low costs are the direct result of the ubiquitous use of marker isolation and genotyping in the medical sciences for applications such as disease linkage mapping.

In order to facilitate the successful use of microsatellites by newcomers to molecular ecology, our first goal in this synthesis is to provide an overview of the pros and cons of employing microsatellites. Although microsatellite marker isolation is still problematic in certain taxa, new marker isolation and genotyping has become routine in a wide range of taxa (including most vertebrates, many insects and some plants), allowing their use without an in-house laboratory. Our second goal is to outline the process of undertaking new microsatellite marker isolation and its associated costs, in order to provide ecologists and newcomers to population genetics with the information need to decide whether to invest in using genetic tools. Our third goal is to encourage thorough quality testing of genetic data sets by presenting a six-step microsatellite screening protocol. A newcomer to the field is especially vulnerable to omitting one or more of these important steps because no formal protocol has been established in the literature. Importantly, we hope our screening protocol will encourage all eco-geneticists, novice and experienced, to adopt more consistent and thorough reporting of these important steps.

PART I: A REVIEW OF MICROSATELLITES

What are microsatellites?

Microsatellites are tandem repeats of 1–6 nucleotides found at high frequency in the nuclear genomes of most taxa. As

such, they are also known as simple sequence repeats (SSR), variable number tandem repeats (VNTR) and short tandem repeats (STR). As a result of the widespread use of microsatellites, our understanding of their mutational behaviour, function, evolution and distribution in the genome and across taxa is increasing rapidly (Li *et al.* 2002; Ellegren 2004). A microsatellite locus typically varies in length between 5 and 40 repeats, but longer strings of repeats are possible. Dinucleotide, trinucleotide and tetranucleotide repeats are the most common choices for molecular genetic studies. Dinucleotide repeats account for the majority of microsatellites for many species (Li *et al.* 2002). Trinucleotide and hexanucleotide repeats are the most likely repeat classes to appear in coding regions because they do not cause a frameshift (Toth *et al.* 2000). Mononucleotide repeats are less reliable because of problems with amplification; longer repeat types are less common, and fewer data exist to examine their evolution (Li *et al.* 2002).

The DNA surrounding a microsatellite locus is termed the flanking region. Because the sequences of flanking regions are generally conserved (i.e. identical) across individuals of the same species and sometimes of different species, a particular microsatellite locus can often be identified by its flanking sequences. Short stretches of DNA, called oligonucleotides or primers, can be designed to bind to the flanking region and guide the amplification of a microsatellite locus with polymerase chain reaction (PCR). A specific pair of PCR primers is the tangible product of microsatellite marker isolation (elaborated below; see Appendix S1 in Supplementary Material). The widespread availability of oligonucleotides from commercial services makes it simple to order any unlabeled primer sequence and have it delivered to you within days for \leq USD 20 (fluorescently labelled primers needed for use in a DNA sequencer are currently \geq USD 80).

Unlike flanking regions, microsatellite repeat sequences mutate frequently by slippage and proofreading errors during DNA replication that primarily change the number of repeats and thus the length of the repeat string (Eisen 1999). Because alleles differ in length, they can be distinguished by high-resolution gel electrophoresis, which allows rapid genotyping of many individuals at many loci for a fraction of the price of sequencing DNA. Many microsatellites have high-mutation rates (between 10^{-2} and 10^{-6} mutations per locus per generation, and on average 5×10^{-4}) that generate the high levels of allelic diversity necessary for genetic studies of processes acting on ecological time scales (Schlötterer 2000).

Why choose microsatellites?

There are several widely used marker types available for molecular ecology studies, and many questions can be addressed with more than one type of marker (Table 1). A comprehensive review of different marker types is provided

elsewhere (Awise 1994; Sunnucks 2000; Zhang & Hewitt 2003; Schlötterer 2004) and is beyond the scope of this study. Microsatellites are of particular interest to ecologists because they are one of the few molecular markers that allow researchers insight into fine-scale ecological questions. Imagine, for example, a plant ecologist studying flowering time. Using microsatellite markers, our researcher could address a variety of interesting questions such as: Are the individuals or populations that flower early genetically distinct from those that flower late? Do immigrants tend to flower in synchrony with their neighbours or their natal population? Is there a relationship between measures of fitness (flowering time, flower number, seed set, etc.) and genotypic identity? Are the best performers (in terms of those fitness measures) in a particular year relatives?

Regardless of the question, a molecular marker must fundamentally be selectively neutral and follow Mendelian inheritance in order to be used as a tool for detecting demographic patterns, and these traits should always be confirmed for any marker type (see Part III: A Microsatellite Screening Protocol). Here, we outline the desirable traits of microsatellites compared with other marker types such as allozymes, amplified fragment length polymorphisms (AFLP), sequenced loci and single nuclear polymorphisms (SNP), focused on both practicalities and ecological considerations.

Easy sample preparation

An ideal marker allows the use of small tissue samples which are easily preserved for future use. In contrast to allozyme methods, DNA-based techniques, such as microsatellites, use PCR to amplify the marker of interest from a minute tissue sample. The stability of DNA compared with enzymes allows the use of simple tissue preservatives (such as 95% ethanol) for storage. In addition, because microsatellites are usually shorter in length than sequenced loci (100–300 vs. 500–1500 bp) they can still be amplified with PCR despite some DNA degradation (Taberlet *et al.* 1999). As DNA degrades, it breaks into smaller pieces and the chance of successfully amplifying a long segment is proportional to its length (Frantzen *et al.* 1998). This trait allows microsatellites to be used with fast and cheap DNA extraction methods, with ancient DNA, or DNA from hair and faecal samples used in non-invasive sampling (Taberlet *et al.* 1999). Furthermore, because microsatellites are species-specific, cross-contamination by non-target organisms is much less of a problem compared with techniques that employ universal primers (i.e. primers that will amplify DNA from any species), such as AFLP. This feature is of particular importance when working with faecal samples or species, such as scleractinian corals, in which endosymbiont contamination is practically unavoidable.

High information content

Each marker locus can be considered a sample of the genome. Because of recombination, selection and genetic drift, different genes and different regions of the genome have slightly different genealogical histories. Relying on a single locus to estimate ecological traits from genetic data creates a high rate of sampling error. Thus, taking multiple samples of the genome by combining the results from many loci provides a more precise and statistically powerful way of comparing populations and individuals. Furthermore, statistical approaches to the questions of most interest to ecologists often require multiple, comparable loci (see Table 1 and Pearse & Crandall 2004). Although AFLP, allozymes and random amplified polymorphic DNA (RAPD) techniques are also multilocus, none of them have the resolution and power of a multilocus microsatellite study (but for distinct reasons; see Sunnucks 2000). While AFLP markers can be a good alternative choice to microsatellites (Bensch & Akesson 2005). Gerber *et al.* (2000) showed that 159 AFLP loci provided slightly less power to determine paternity than six polymorphic microsatellite markers. Sequencing technology has advanced rapidly, but its cost still prohibits the duplication or triplication of workload by using multiple independent gene sequences in parallel (Zhang & Hewitt 2003). SNP markers hold great promise for future studies but their use in non-model organisms is still nascent (Morin *et al.* 2004). Microsatellites have become so popular because they are single locus, co-dominant markers for which many loci can be efficiently combined in the genotyping process to provide fast and inexpensive replicated sampling of the genome.

Microsatellite markers generally have high-mutation rates resulting in high standing allelic diversity. In species for which populations are small or recently bottlenecked, markers with lower mutation rates, such as allozymes, may be largely invariant and only loci with the highest mutation rates are likely to be informative (Hedrick 1999). A slow mutational process allows the signature of events in the distant past to persist longer. Thus, the selection of loci with high or low allelic diversity will depend on the question of interest. For example, if one is interested in a potential historical barrier to gene flow or tracing the recolonization of territory since the last ice age, markers with lower mutational rates are likely to be the most informative. In contrast, if one is interested in present day demography or connectivity patterns, or detecting changes in the recent past (10–100 generations), microsatellites with higher mutational rates are preferable. Questions of paternity or clonal structure are best addressed using microsatellites with highest allelic diversity, which can provide every individual with a unique genotype 'identification tag' using only a few loci (Queller *et al.* 1993). Similarly, for studies of population structure and migration that employ population allele

frequency estimates, the numerous alleles of high diversity microsatellites act as statistical replicates to lend more power to distinguish populations (Kalinowski 2002; Wilson & Rannala 2003).

What are the drawbacks to microsatellite markers?

Despite many advantages, microsatellite markers also have several challenges and pitfalls that at best complicate the data analysis, and at worst greatly limit their utility and confound their analysis. However, all marker types have some downsides, and the versatility of microsatellites to address many types of ecological questions outweighs their drawbacks for many applications. Fortunately, many of the pitfalls common to microsatellite markers can be avoided by careful selection of loci during the isolation process.

Species-specific marker isolation

PCR-based marker analysis requires primer sequences that target the marker regions for amplification. In order to use the same primer sequence to amplify the same target from many individuals, the region where the primer binds must be identical, with few or no mutations causing interindividual differences. For the gene regions commonly used as sequenced markers, primer regions are highly conserved, such that they are invariant within species and sometimes even across broad taxonomic groups. This sequence conservation necessitates only minor work to optimize a primer set for a new species. In contrast, a given pair of microsatellite primers rarely works across broad taxonomic groups, and so primers are usually developed anew for each species (Glenn & Schable 2005). However, the process of isolating new microsatellite markers has become faster and less expensive, which substantially reduces the failure rate and/or cost of new marker isolation in many cases (Glenn & Schable 2005). Moreover, many commercial and academic laboratories can provide a set of polymorphic microsatellite loci for a new species at reasonable cost in 3–6 months (see Part II: Acquiring microsatellites). Nevertheless, there are some taxa for which new marker isolation is still fraught with considerable failure rate, such as some marine invertebrates (e.g. Cruz *et al.* 2005), lepidopterans (Meglecz *et al.* 2004) and birds (Primmer *et al.* 1997).

Unclear mutational mechanisms

One of the challenges currently being addressed by geneticists is that the mutational processes of microsatellites can be complex (Schlötterer 2000; Beck *et al.* 2003; Ellegren 2004). For the majority of ecological applications, it is not important to know the exact mutational mechanism of each locus, as most relevant analyses are insensitive to mutational mechanism (Neigel 1997). However, several statistics based on estimates of allele frequencies (e.g. F_{ST} and R_{ST}) rely

explicitly on a mutation model. Traditionally, the infinite allele model (IAM), in which every mutation event creates a new allele (whose size is independent from the progenitor allele) has been the model of choice for population genetics analyses, and because it is the simplest and most general model, continues to be widely used as a default.

A model specific to microsatellites, the stepwise mutational model (SMM), adds or subtracts one or more repeat units from the string of repeats at some constant rate to mimic the process of errors during DNA replication that generates mutations, creating a Gaussian-shaped allele frequency distribution (Ellegren 2004). However, non-stepwise mutation processes are also known to occur, including point mutation and recombination events such as unequal crossing over and gene conversion (Richard & Paques 2000). While debate continues about the prevalence of non-stepwise mutation for microsatellites, the current consensus is that the frequency and effects are usually low, and stepwise mutation appears to be the dominant force creating new alleles in the few model organisms studied to date (Eisen 1999; Ellegren 2004). Nevertheless, metrics employing the SMM tend to be highly sensitive to violations of this mutational model (e.g. loci with non-stepwise mutation or constraints on allele size) and thus metrics using the IAM are usually more robust and reliable (Ruzzante 1998; Balloux & Lugon-Moulin 2002; Landry *et al.* 2002). More complex and realistic mutational models that add the probability of non-stepwise mutation to the SMM are beginning to replace the SMM in common genetic analyses, and are already available in several statistical packages (e.g. Piry *et al.* 1999; Van Oosterhout *et al.* 2004).

Hidden allelic diversity

Size-based identification of alleles (i.e. gel electrophoresis – see Appendix S2 in Supplementary Material) greatly reduces the time and expense of microsatellite genotyping compared with sequencing each allele in each individual. However, this shortcut requires the assumption that all distinct alleles differ in length. In fact, alleles of the same size but different lineages can be quite common, a phenomenon termed ‘homoplasy’. Homoplasy dampens the visible allelic diversity of populations and may inflate estimates of gene flow when mutation rate is high (Garza & Freimer 1996; Rousset 1996; Viard *et al.* 1998; Blankenship *et al.* 2002; Epperson 2005). There are two distinct types of homoplasy, ‘detectable’ and ‘undetectable’. Detectable homoplasy can be revealed by sequencing alleles. For instance, point mutations will leave the size of an allele unchanged, and insertions or deletions in the flanking region might create a new allele with the same size as an existing allele. Detectable homoplasy appears to affect only a fraction of genotypes at a fraction of loci, and this bias appears to be marginal in the majority of cases (Viard *et al.* 1998; Adams *et al.* 2004; Curtu *et al.* 2004).

Adams *et al.* (2004) found homoplasy was only common for compound and/or interrupted repeats. Empirical estimates of detectable homoplasy reported only a slight (1–2%) underestimation of genetic differentiation (Adams *et al.* 2004; Curtu *et al.* 2004).

Undetectable homoplasy occurs when two alleles are identical in sequence but not identical by descent (i.e. they have different genealogical histories). Such non-identity occurs from the random-walk behaviour of the stepwise mutation process when there is a ‘back-mutation’ to a previously existing size (e.g. an allele mutates from 5 to 6 repeats and then a copy of this allele mutates from 6 to 5 repeats) or when two unrelated alleles converge in sequence by changing repeat number in two different places in the sequence. As the SMM predicts a 50% chance of back-mutation, undetectable homoplasy may be extensive when mutation rate is high, but can be accounted for in analyses (Slatkin 1995; Estoup & Cornuet 1999).

In general, homoplasy is often a minimal source of bias for population genetic studies limited to populations with a ‘shallow’ history or moderate effective population size, as the chance of homoplasy is proportional to the genetic distance of two individuals or populations (Estoup *et al.* 2002). However, when used for highly divergent groups, such as for phylogenetic reconstruction, high-mutation rate loci may be problematic (Estoup *et al.* 1995). It is important to note that undetected homoplasy plagues all marker types. When appropriate, there are several methods that can be employed to assess detectable homoplasy (see Part III: A Microsatellite Screening Protocol).

Problems with amplification

Finding a useful DNA marker locus requires identifying a region of the genome with a sufficiently high mutation rate that multiple versions (alleles) exist in a given population, and which is also located adjacent to a low mutation rate stretch of DNA that will bind PCR primers in the vast majority (approaching 100%) of individuals of the species. If mutations occur in the primer region, some individuals will have only one allele amplified, or will fail to amplify at all (Paetkau & Strobeck 1995). In addition, primers must bind under repeatable PCR conditions so that genotyping can be performed in serial, by different workers, and by different laboratories. Consistent amplification across all samples can only be assured by trial and error, such that at the middle or end of genotyping all the samples in a study, some loci will have to be discarded because of amplification problems. If this marker attrition is planned for in the initial isolation of microsatellite markers, the chance that amplification problems will ruin a study is minimal. However, several taxa seem more often beset by amplification problems than others, notably, bivalves, corals and some other invertebrate taxa (e.g. Hedgecock *et al.* 2004). A low rate of null alleles

can have a negligible impact on many types of analysis, although for some types of parentage analyses it can be substantial (Dakin & Avise 2004).

PART II: ACQUIRING MICROSATELLITES

Step 1: Searching for existing microsatellite markers

The first step in considering a microsatellite marker study is to search published literature for any existing microsatellite primers for the target species and closely related species. The availability of microsatellite markers for a given species will be a combination of past interest in that species (and related species) and the inherent success rate of microsatellite development for that taxon. There are clear differences in the frequency of microsatellite regions in the genomes of plants, animals, fungi and prokaryotes (Toth *et al.* 2000), and the success rate of isolating microsatellite markers often scales with their frequency in the genome (Zane *et al.* 2002). For example, microsatellites tend to be relatively rare in lepidopterans, birds, bats and prokaryotes, whereas fishes and most mammals tend to have a high frequency of repeat motifs (Neff & Gross 2001). In addition, species with high rates of inbreeding, low population sizes and frequent or severe bottlenecks typically have low average polymorphism and heterozygosity, and on average shorter microsatellites (DeWoody & Avise 2000; Neff & Gross 2001).

Currently, most microsatellite markers are reported in 'primer notes' in *Molecular Ecology Notes*. There is a searchable database online for any microsatellite primers published in this journal (<http://tomato.bio.trinity.edu/>). The sequences themselves are archived in GenBank, and are often submitted long before their use appears in published studies. GenBank can be searched with a web-based engine run by the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) by typing in the species, genus or family name, the term 'microsatellite' and selecting the Nucleotide database.

Sometimes flanking regions are highly conserved across taxa, allowing cross-species amplification of microsatellite loci from primers developed from other species in the same genus or even family, especially for vertebrates such as fishes, reptiles and mammals (Rico *et al.* 1996; Peakall *et al.* 1998). Thus, it is useful to search the databases above for primers developed for congeneric and confamilial relatives of the target species. Success rate of primers may decrease proportionally to the genetic distance between the focal species and the species of origin (Primmer *et al.* 1996; Wright *et al.* 2004). In addition, allelic diversity often decreases when primers are used in non-source species (Primmer *et al.* 1996; Ellegren *et al.* 1997; Neff & Gross 2001; Wright *et al.* 2004), a type of 'ascertainment bias' that can be accounted for if need be (Petit *et al.* 2005). In general,

attempting amplification of existing primers from related species is less expensive and time-consuming than isolating new primers (Squirrell *et al.* 2003), and any successes will save money even if additional markers are needed to augment these appropriated ones.

Step 2: Isolating new markers

In the past decade, the process of isolating new microsatellites has been streamlined with technological advances and protocol optimization to make the process cheaper, more efficient and more successful (Zane *et al.* 2002; Glenn & Schable 2005). See Appendix S1 for a conceptual schematic of a microsatellite locus isolation process. A quick search on the web using appropriate search terms, such as 'microsatellite isolation service', will produce a long list of providers in locations across the globe. Some services specialize in certain taxa, such as plants or mammals, and will generally work with you to tailor the product to your needs. These laboratories typically require 2–6 months to develop markers, and most cost less than USD 1500 per locus, or 10–15 loci for *c.* USD 10 000. Although this expense is not trivial, it is roughly the cost of a PCR machine, and is far less expensive than equipping a full molecular laboratory if you do not already have access to one. The cost and time to delivery also depend on whether the loci are tested for quality and amplification protocols are optimized as part of the isolation service. Some services will even write up a primer note publication with shared authorship. Many of the laboratories that offer microsatellite isolation also offer genotyping services, and can take you from start to finish for your entire study. As an alternative to sending out samples, it is also possible to establish collaboration with an academic laboratory with the necessary technical expertise and equipment, or at many universities, work closely with a central sequencing facility.

PART III: A MICROSATELLITE SCREENING PROTOCOL

Although anyone can send tissue samples to a commercial service and receive a full set of microsatellite markers in a matter of months, it is not trivial to develop an optimal set of loci that will provide reliable results. There are several basic assumptions behind the analyses commonly applied to microsatellite data and each should be explicitly addressed (Table 2).

Loci that are included in analyses despite gross violations of these assumptions or high error rates could lead to inaccurate and biased genetic estimates. In the hopes of motivating a more critical consideration of marker quality control, we present here a detailed guide to evaluating loci for inclusion in a population genetic study. As more details

Table 2 Summary of the quality control screening protocol with checklist of suggested tests

Assumption	Suggested tests for locus quality control
1. Accurately scored genotypes	Re-score a subset of genotypes and calculate error rate
2. Amplification of all alleles	Test for homozygote excess patterns consistent with null alleles (MICRO-CHECKER); calculate frequency of samples that fail to amplify any alleles at just one locus
3. Linkage equilibrium	Use an exact test to search for correlations between alleles at different loci (available in many programs)
4. Selective neutrality	Test for conformity to Ewens sampling distribution (ENUMERATE or PYPOP), test for outlier loci (FDIST2, DETSEL)
5. Mendelian inheritance	Perform defined crosses when possible*; discard loci with cases of >2 alleles per diploid individual
6. Every allele differs in length	Sequence a subset of alleles or employ SSCP – if there is good reason to believe that homoplasy is a significant problem*

*These tests are currently beyond the standards required of most ecological uses of microsatellites and should be viewed as optional (see text for more detail).

about microsatellite mutation behaviour, inheritance mechanisms and distribution across chromosomes are uncovered, the list of suggested tests will likely evolve.

While we cannot know whether or not most studies employ such quality control measures, the majority of publications fail to report the results for most of these tests (Table 3). In our survey of 50 recent microsatellite studies, 28% of studies mention the importance of quality control screening but fail to report any results of statistical tests. Moreover, most testing was carried out *post hoc* and relied heavily on testing for Hardy–Weinberg Equilibrium (HWE). Table 3 shows that roughly twice the failure rate is detected with explicit tests for null alleles, inheritance and neutrality compared with indirect inferences based on testing for HWE. While the continuing trend of shortening manuscripts makes thorough reporting of quality control testing more challenging, the use of web-based data repositories which are commonly linked to printed manuscripts would be an easy solution to this problem, and would facilitate the comparison and meta-analysis of data sets.

Once working primers are developed (see Appendix S1), 20–30 individuals from each of 3–5 broadly distributed populations can be genotyped (see Appendix S2) for the preliminary screening outlined below. When the entire collection of samples in the study is genotyped, the analyses of the tests in the screening process should be repeated and the results reported in any subsequent publication. The recommended steps in the screening process are outlined below (with references that provide more extensive overviews of these topics), and a checklist of necessary tests is presented in Table 2. We note here that some specialized statistical analyses make other critical assumptions, such as conformity to a specific mutational model, constant population size, or migration-drift equilibrium that are considered beyond the scope of this review.

Allele scoring error

There are many steps between extracting DNA and entering a genotype into a database, and at each point a variety of errors can arise. A genotyping error rate of even 1% (i.e. 1% of the alleles in an entire data set are misidentified), which is an uncommonly good value for most studies, can lead to a substantial number of incorrect multilocus genotypes in a large data set (Hoffman & Amos 2005). Sources of error include poor amplification, misprinting (i.e. misinterpreting an artefact peak/band as a true microsatellite allele and including it in the genotype), incorrect interpretation of stutter patterns or artefact peaks (see Appendix S2), contamination, mislabelling or data entry errors (Bonin *et al.* 2004). 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.

A high quality genetic data set starts with good sample preservation. Proper sample preservation can substantially reduce technical difficulties with amplification down the line, so should be planned carefully (Dawson *et al.* 1998). Note that while non-invasive sampling (based on skin, hair or faecal samples) is often useful, it often requires a more intensive protocol for genotyping and leads to a higher error rate than when properly preserved tissue samples are used (reviewed by Taberlet *et al.* 1999; Piggott & Taylor 2003).

To ensure that amplification of alleles is consistent throughout the duration of a study, a positive control should be run with every PCR batch – especially any time multiple sequencers are used for genotyping in a single study, or new batches of primers are used (Delmotte *et al.* 2001). Red flags should be heeded by re-extracting and re-amplifying questionable genotypes (e.g. heterozygotes with closely sized alleles, faint alleles – see Appendix S2 for examples of hard-to-call genotypes). If necessary, the whole data set

Quality control screening step	Frequency of reporting (%)	Survey result (%)
Genotype scoring error rate	10	2.1 ± 2.4
Indirect evidence for null alleles*	84	13.6 ± 25.2
Explicit tests for null alleles	36	34.6 ± 33.5
Evidence for linkage equilibrium	78	10.8 ± 24.2
Evidence for sex linkage	12	5.3 ± 7.7
Indirect evidence for deviation from neutral expectations*	26	1.6 ± 5.8
Explicit tests for conformity with neutral expectations	8	5.3 ± 10.5
Found signs in data set consistent with 'non-Mendelian' inheritance*	34	1.8 ± 7.8
Explicit tests for 'non-Mendelian' inheritance with defined crosses or pedigrees	16	4.7 ± 11.2
Incidence of homoplasious alleles per locus	4	1.4 ± 1.9

Frequency of Reporting indicates the percentage of the 50 studies that performed each test. The Survey Result values are the mean percentage of loci that failed the test ± 1 SD. We present both the values for those studies that tested explicitly for each quality control step, and those that inferred a violation *post hoc* after detecting an unexpected deviation from Hardy–Weinberg Equilibrium (HWE; noted by asterisk). We excluded studies that used microsatellite markers taken from previously published research studies to minimize the chance that tests of assumptions were carried out previously and therefore not reported in the current study. A list of the references for the 50 studies is included in Appendix S3 in Supplementary Material.

*Includes tests of deviation from HWE.

can be genotyped in duplicate (or more), as is performed for human parentage or forensics.

Error rate can be calculated by repeating marker amplification in a random subset of 10–15% of the total number of samples, and counting the number of inconsistent genotypes between the first and second attempt. Error rate is then expressed as either the number of incorrect genotypes divided by the number of repeated reactions, or the number of incorrect alleles divided by the total number of alleles (Hoffman & Amos 2005). 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). Information on error type and frequency allows estimation of the effects of the error on the results (e.g. inflated homozygosity, reduced kinship estimates, etc.; Bonin *et al.* 2004). The effect of error on measures of genetic structure can be estimated using a bootstrapping technique developed by Adams *et al.* (2004), and the parentage program CERVUS can estimate error rate while also accounting for mutation (Marshall *et al.* 1998). Analyses based on individual multi-locus genotypes are more sensitive to error than those based on average allele frequencies. For example, a per-locus error rate of 5% in a three-locus data set results in 95% accuracy in allele frequency estimation, but means that only 85% of individuals were genotyped correctly at all three loci. Some

Table 3 Survey of quality control screening of microsatellite loci reported in 25 recently published microsatellite studies in each of the journals *Evolution* and *Molecular Ecology*

amount of error is unavoidable, but we argue that the error rate within each study should be quantified and reported. In some cases, removing loci with the highest error rates from analyses may improve statistical power.

Hardy–Weinberg Equilibrium and null alleles

The most commonly reported test of loci is conformity to HWE, in which observed genotype frequencies are compared with the frequencies expected for an ideal population (random mating, no mutation, no drift, no migration). A 'heterozygote excess' (also known as 'homozygote deficit') occurs when the data set contains fewer homozygotes than expected under HWE, and a 'heterozygote deficit' (also known as 'homozygote excess') occurs when there are more homozygotes than expected under HWE. Currently, tests used to determine statistically significant deviation from HWE have low power when allelic diversity is high and sample sizes are moderate (Guo & Thompson 1992). However, failure to meet HWE is not typically grounds for discarding a locus.

Heterozygote deficit, the more common direction of HWE deviation, can be due to biological realities of violating the criteria of an ideal population, such as strong inbreeding or selection for or against a certain allele. Alternatively, when two genetically distinct groups are inadvertently lumped into a single sampling unit, either

because they co-occur but rarely interbreed (unbeknownst to the sampler), or because the spatial scale chosen for sampling a site is larger than the true scale of a population, there will be more homozygotes than expected under HWE. This phenomenon is called a Wahlund effect and may be a common cause of heterozygote deficit in population genetic studies (Johnson & Black 1984; Nielsen *et al.* 2003). Both of these causes of heterozygote deficit should affect all loci, instead of just one or a few.

Another common cause of heterozygote deficit is amplification failure of certain alleles at a single locus. 'Null alleles' are those that fail to amplify in a PCR, either because the PCR conditions are not ideal or the primer-binding region contains mutations that inhibit binding. As a result, some heterozygotes are genotyped as homozygotes and a few individuals may fail to amplify any alleles. Often the mutations that cause null alleles will only occur in one or a few populations, so a heterozygote deficit might not be apparent across all populations.

A simple way to identify a null allele problem is to determine if any individuals repeatedly fail to amplify any alleles at just one locus while all other loci amplify normally (suggesting the problem is not simply poor quality DNA). If re-extraction and amplification still fail to produce any alleles at that locus, it is likely that the individual is homozygous for a null allele. In addition, a statistical approach to identifying null alleles can match the pattern of homozygote excess (large alleles, random distribution of alleles, etc.) to the expected signatures of several different causes of homozygote excess and estimate the frequency of null alleles for each locus. The software program MICRO-CHECKER is designed for this goal (Van Oosterhout *et al.* 2004). A more technical way to detect null alleles is to examine patterns of inheritance in a pedigree (e.g. Paetkau & Strobeck 1995).

Redesigning primers to bind to a different region of the flanking sequence, or adjusting PCR conditions can often ameliorate null allele problems (Callen *et al.* 1993; Pemberton *et al.* 1995). Many researchers are quick to use highly stringent PCR conditions without considering the downside that it inflates the chances for null alleles. A low incidence of null alleles is usually only a minor source of error for most types of analyses. Nevertheless, the effect of null alleles on estimates of genetic differentiation remains unassessed to date. In addition, for certain analyses that require high accuracy in genotyping, such as parentage analysis, even rare null alleles can confound results and any loci with strong evidence of null alleles should be excluded.

'Large allele dropout' is another way that alleles can be missed – the longer allele in a heterozygote does not amplify as well as the shorter one and appears too faint to be detected in the genotype scoring process (Wattier *et al.* 1998). Large allele dropout occurs because the replication

process in PCR is more efficient for shorter than longer sequences, and so it will be most pronounced when alleles in a heterozygote are very different in size. Re-amplifying individuals homozygous for small alleles and increasing their sample concentration in the DNA sequencer run is one way to combat this source of genotyping error.

Gametic disequilibrium

When two loci are very close together on a chromosome, they may not assort independently and will be transmitted to offspring as a pair. Even if loci are not linked physically on a chromosome, they can be functionally related or under selection to be transmitted as a pair (hence the more accurate term gametic disequilibrium is starting to replace the term 'linkage disequilibrium'). While functional linkage would be unusual for microsatellite loci, microsatellites can be clustered in the genome (Bachtrog 1999) and gametic disequilibrium should always be tested.

Gametic disequilibrium creates pseudo-replication for analyses in which loci are assumed to be independent samples of the genome. To avoid increased Type I error, one locus in the pair should be discarded if significant disequilibrium is found consistently between loci. Like tests of HWE, gametic disequilibrium testing has low power for highly polymorphic loci, so examining confidence intervals on estimates is recommended. Several user-friendly software programs, such as ARLEQUIN (Schneider *et al.* 2000), FSTAT (Goudet 1995), GENEPOP (Raymond & Rousset 1995), GENETIX (Belkhir *et al.* 1998) and MICROSATELLITE ANALYZER (Dieringer & Schlötterer 2003), include tests for gametic disequilibrium by searching for correlations between alleles at different loci. One type of linkage that this test will not catch is sex linkage; however, sex linkage will produce an apparent heterozygote deficit that resembles a null allele problem. Testing for sex linkage is reasonably straightforward when samples of individuals of known sex are available: where one sex is consistently homozygous at a locus, sex linkage is indicated (Wilson *et al.* 1997). Lastly, there are many ecological questions that can benefit from the study of linked loci (Gupta *et al.* 2005). For instance, interpopulation variation in linkage can correlate with the history of bottlenecks (Tishkoff *et al.* 1996).

Selective neutrality

Reviews by Kashi & Soller (1999) and Li *et al.* (2002) detail a suite of putative functional roles of microsatellite DNA (such as chromatin organization, and regulation of gene activity and recombination), demonstrating that microsatellites themselves can be under selection. Furthermore, several heritable human diseases, such as Huntington's disease, are directly caused by mutations in microsatellite loci (Ranum &

Day 2002). Alternatively, a microsatellite may sit adjacent to a gene under selection and appear non-neutral because of hitchhiking. The use of microsatellites to construct disease linkage maps suggests many may be closely linked to genes under selection. These examples indicate that neutrality of microsatellite markers should not be taken for granted, and instead should be tested and reported in published studies.

Existing neutrality tests take several different approaches, but all lack the power to detect anything but the strongest signatures of selection (Ford 2002). In many cases, this low power is not a serious problem, because most common methods for estimating the average level of gene flow among populations (e.g. F_{ST} , rare alleles and maximum likelihood) are relatively robust to weak selection (Slatkin & Barton 1989). Including multiple loci helps to average out selection, because selection is not expected to fix mutational similarities across many independent genes in different populations (Lewontin & Krakauer 1973). Jackknifing multilocus estimates of genetic structure can reveal the influence of each locus on the pattern; the program `FSTAT` provides this test.

Explicit tests of neutrality can be applied to each locus individually. The Ewens–Watterson test is based on the premise that a locus free from forces of selection should have a distribution of allele frequencies that matches the Ewens statistical sampling distribution, but only strong deviation is detectable (Slatkin 1994). In addition to selection, past change in population size, population subdivision and deviation from an IAM (likely for many microsatellites – see Schlötterer *et al.* 2004) can cause deviation from the Ewens distribution, so a locus may also appear to be under selection if it grossly violates the assumptions of the model.

A different approach to detecting selection is based on the premise that selection should not affect many independent genes in a similar manner; thus, one way to test for neutrality is to assess the variance in allele frequencies (estimated with F_{ST}) among many loci. Outlier loci are considered suspect and can be removed from data sets if warranted (Lewontin & Krakauer 1973). Two software packages use this approach to evaluate neutrality but make an effort to reduce unrealistic assumptions for which the Lewontin & Krakauer (1973) method was originally criticized. `FDIST2` considers the total number of subpopulations in its test for outliers in the relationship between F_{ST} and heterozygosity (Beaumont & Nichols 1996). `DETSEL` (Vitalis *et al.* 2001) modifies this approach by considering pairs of populations individually, eliminating the need to know the exact number of subpopulations. Any locus that fails a test for selection should be excluded from analyses based on neutral assumptions, such as inferences of connectivity, migration rate, F_{ST} , R_{ST} , etc. However, loci under selection can prove extremely interesting in their own right when examining biological patterns.

Mendelian inheritance

Mendelian inheritance of alleles is a requirement for almost all population genetic analyses and the first major review of microsatellite inheritance studies found Mendelian inheritance was almost never rejected for diploid vertebrate species (Jarne & Lagoda 1996; Dakin & Avise 2004). However, there are increasing reports of what appears to be ‘non-Mendelian’ patterns of inheritance of microsatellites (Smith *et al.* 2000; Dobrowolski *et al.* 2002). Whenever possible, inheritance should be evaluated and reported. Performing defined crosses is the only way to test explicitly for Mendelian inheritance. Because relatively few studies report tests for Mendelian inheritance, it is still unclear how common non-Mendelian inheritance is across taxa. However, our survey of 50 recent studies found that on average more than one locus in 15 appeared to violate Mendelian inheritance when tested for explicitly (Table 3). A large fraction of ‘non-Mendelian’ ratios of alleles in offspring of defined crosses is apparently caused by null alleles. In this case, the ‘non-Mendelian’ pattern of inheritance is simply a technical artefact; the locus does follow Mendel’s laws but the invisible alleles mask this fact. Potential causes of true non-Mendelian behaviour are sex linkage, physical association with genes under strong selection, centres of recombination, transposable elements, or processes during meiosis such as non-disjunction or meiotic drive (segregation distortion). These processes can have severe effects, such as only one parental allele being passed on to all offspring.

Performing defined crosses and genotyping a large number of offspring can be quite challenging or impractical in some species, and straightforward in others, such as those that brood their young. Microsatellite loci in any polyploid species have a high likelihood of occurring multiple times throughout the genome and this will confound analysis, so in particular inheritance should always be examined for polyploids (Ardren *et al.* 1999). Even in diploid or haploid species, duplication of loci can be common and potentially problematic. Any case of a locus displaying more than two alleles per individual (that is not traceable to cross-contamination of samples) should be discarded from most analyses. It is important to note that automated sequencers are set by default to call only two alleles per locus, and will return apparently valid allele calls regardless of the actual number of amplification products produced; for this reason, automated sequencer allele calling should always be double-checked by an experienced operator.

Homoplasmy

The simple assumption that each allele can be identified unambiguously by its size is probably not met by many loci

(Estoup *et al.* 2002). Homoplasmy becomes most pronounced when comparing very genealogically distant (and often geographically distant) individuals or groups, as one or both lineages must have experienced two mutation events following their divergence to create a homoplasious pair of alleles (Angers *et al.* 2000). Thus, homoplasmy is expected to be most problematic for applications in which populations are distantly related, but may also be problematic for species with very large population sizes or for loci with strong allele size constraints and high-mutation rate (Estoup *et al.* 2002). In particular, phylogenetic applications in which populations are actually distinct species are particularly likely to suffer biases from marker homoplasmy. Because the bias introduced by homoplasmy is expected to be slight, quantitative estimation of the rate of homoplasmy by the techniques described below is only warranted in special cases.

'Detectable' homoplasmy can be evaluated by sequencing a certain sized allele from several individuals and looking for differences in sequence. Choosing individuals from different populations may increase the chance of detecting homoplasious alleles, as it is less likely for a homoplasious mutation event to occur in the time since a single population was formed (Estoup *et al.* 2002). A more efficient method than sequencing is to employ single-strand conformational polymorphism (SSCP; Angers *et al.* 2000), which uses gel electrophoresis to separate alleles of the same length but different sequence (Sunnucks *et al.* 2000).

Choosing a final set of loci

The general consensus in the field of molecular ecology is that in most cases, the more loci included in a study, the more reliable the resultant data set will be. However, including loci that do not pass the screening process above can lower both the precision and accuracy of genetic estimates. Therefore, using only the top performers from this screening process should minimize errors that arise from the sources discussed above. At the same time, reducing the number of loci also reduces statistical power and genome-wide sampling declines. Clearly there is a trade-off between these sources of bias, and a newcomer is advised to consult an experienced statistician or geneticist in undertaking this process.

Simulating data sets with similar levels of allele frequency differentiation among populations as observed in preliminary data can help in determining necessary sample sizes and number of loci for the desired statistical tests. EASYPOP is a free program that allows such data set simulation for power analyses (Balloux 2001). In many cases, power can be boosted equally by (1) adding individuals from the sampled population, (2) adding loci, or (3) selecting loci with more alleles over loci with few alleles. However, the effect of

adding individuals saturates more quickly than the other two options for estimates of F_{ST} (Kalinowski 2005). The latter two options, adding alleles by adding more loci and using loci with higher polymorphism, produce a similar effect. Adding loci will reduce the variance in genetic estimates caused by locus-specific phenomena, such as genetic drift or weak selection, because each locus is an independent sample of the genome (Kalinowski 2002). Moreover, using loci with higher polymorphism can inflate the error in allele frequency estimates unless sample sizes also increase concurrently (Ruzzante 1998; Gomez-Uchida & Banks 2005; Kalinowski 2005).

Highly variable microsatellites (e.g. loci with >25 alleles or 85% heterozygosity) have a distinct set of pros and cons. Genotype scoring error may rise due to increased large allele dropout (Buchanan *et al.* 2005) and increased stutter (Hoffman & Amos 2005). The high rates of homoplasmy associated with high-mutation rates (the typical cause of high allelic diversity) can introduce bias into allele frequency estimates, dampening estimates of F_{ST} and leading to substantial inflation of gene flow estimates (Jin & Chakraborty 1995; Slatkin 1995; Gaggiotti *et al.* 1999; Epperson 2005). A negative correlation between heterozygosity and F_{ST} can apparently occur at high-mutation rate loci (O'Reilly *et al.* 2004; Olsen *et al.* 2004) but can be accounted for by breaking loci into subgroups for analysis, or using modifiers that make loci more comparable (Buonaccorsi *et al.* 2002; Olsen *et al.* 2004; Hedrick 2005). On the other hand, highly variable loci have increased power to estimate genetic structure (Epperson 2004), distinguish close relatives for parentage (Queller *et al.* 1993) and assign individuals to the correct source population (Wilson & Rannala 2003).

CONCLUSION AND NEXT STEPS

Much of the hesitation researchers have with using microsatellite markers in ecology stems from the fact that detailed studies or meta-analyses of microsatellites and their mutational and amplification behaviours are still largely the purview of model organisms and human genetics. While microsatellites always require careful evaluation, problems such as unclear mutational mechanism, null alleles and homoplasmy are often inconsequential for ecological measures. Nevertheless, it is always important to explicitly examine the assumptions behind the data even when they are difficult to verify, and whenever possible to address sources of error and bias in molecular studies. Although it is still not currently the norm in the field to perform and report all these tests (Table 3), we argue that any published study should strive to test for and present this information. Increased reporting on the characteristics of new loci will hasten our understanding of the behaviour of this marker

type and improve existing approaches to handling their pitfalls. Similarly, the increased availability of these powerful molecular tools to a wider group will hasten the novel application of microsatellite markers, conceptual approaches to problem solving and data analysis, and availability of markers, samples and data sets.

Although a microsatellite marker study today can be completed in less time, for less money and with less technical expertise than before, the proper use of microsatellite data still takes appropriate training – and a thorough grounding in the principles of population genetics and molecular evolution. Even when loci are carefully screened and selected, interpreting ecological meaning from genetic analyses – even the most simple parentage analyses or gene flow estimates – can be tricky. Those attempting to use microsatellites for the first time will require in-depth reading on microsatellite evolution and statistical genetic analysis, many of which are cited throughout this study. Several more specialized reviews on microsatellites that we recommend as a start are Estoup & Angers (1998), Chambers & MacAvoy (2000), Schlötterer (2000), Sunnucks (2000) and many of the chapters in Goldstein & Schlötterer (1999). In addition, several volumes on statistical analysis of genetic data present the underpinning of the statistical approaches to evaluating genetic marker data, including Nei (1987), Weir (1996) and Balding *et al.* (2003). However, many important technical details about the proper use of genetic markers are still only poorly described in the literature. While the appendices included here are meant to provide a conceptual overview of some of the practicalities of using microsatellites, they are by no means exhaustive. Seeking out the collaboration of a molecular ecologist who holds a proven track record with the techniques and analyses of interest (not necessarily the taxa of interest) early in the design of an experiment is an imperative for newcomers and will undoubtedly make the learning process more efficient and successful.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available online for this article from <http://www.Blackwell-Synergy.com>:

- Appendix S1** Flowchart of microsatellite development.
Appendix S2 Guide to scoring microsatellite genotypes.
Appendix S3 References for studies used to generate Table 3.

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