Microsatellite marker identification using genome screening and restriction-ligation

Helena Korpelainen, Kirsi Kostamo, and Viivi Virtanen
University of Helsinki, Helsinki, Finland

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We have identified a fast and easy method for finding microsatellite markers that utilizes genome screening with inter-simple sequence repeat (ISSR) primers to detect microsatellite regions and to obtain sequence information flanking one side of the microsatellites and a restriction-ligation technique with a specific adaptor to allow sequence walking to obtain sequence information flanking the other side of the microsatellites. Two main alternatives of the method (with or without cloning) are presented. We successfully utilized the method when identifying microsatellite markers for 21 bryophyte species, three algal species, and for the raccoon dog. The proportion of polymorphic markers equaled 95%. We observed that microsatellites are commonly found within the sequenced ISSR amplification products (54% in the present study), in which case specific primers can be identified for the microsatellite without a further restriction-ligation step. It is evident that the DNA regions amplified by ISSR markers commonly represent microsatellite hotspots. We propose that the identified method and the knowledge of the common presence of additional microsatellite repeats within ISSR amplification products are especially attractive to researchers who conduct small-scale microsatellite identification, such as researchers in population genetics and conservation biology.

INTRODUCTION

Genetic fingerprinting has been utilized in the identification of individuals, breeds, cultivars and species, in genetic mapping in connection with animal and plant breeding or medical applications, and in a range of population genetic and ecological applications (1–6). Among DNA fingerprinting methods, species-specific microsatellite analyses are of major importance. Microsatellites are highly abundant within eukaryotic genomes (6,7). The markers consist of repeated 1- to 6-bp-long DNA motifs. Different forms (alleles) vary in the number of units of the repeat motifs. The genomic areas containing microsatellites are known to be highly variable, which makes their analyses useful for many applications (3,6). Even in species in which the level of genetic variation detected by other methods may be low, a considerable level of variation is often detected using microsatellites (8–10). A disadvantage is that the present methodology used to identify microsatellite markers is quite time-consuming and expensive (11), although a number of attempts to identify improved techniques have been carried out, and a number of different protocols are available (3,12,13).

The aim of the present study was to develop a new methodology for a faster and easier way to identify microsatellite markers when compared with traditional methods. The approach includes genome screening techniques with inter-simple sequence repeats (ISSR) primers (14) in order to find microsatellite regions and to obtain sequence information flanking one side of the microsatellites, as well as a restriction-ligation technique with a specific adaptor to allow sequence walking in order to obtain sequence information flanking the other side of the microsatellites. Each ISSR primer contains a repeat sequence flanked by one or a few different nucleotides acting as an anchor. To allow wide testing of the applicability of the identified method, we used the method on a range of plant material and also one animal species.

MATERIALS AND METHODS

The process for microsatellite identification was initiated with the screening of genomic DNA, extracted using DNeasy® Plant Mini kit (Qiagen, Valencia, CA, USA) for the plant species in question, and using standard procedures for animal tissues (15) for the raccoon dog material, with a set of 12 ISSR primers. Different primers or primer combinations were used until a desired number of microsatellite regions (i.e., well-amplified ISSR products) were detected. The PCRs involved the use of two different primers (the possible 6 × 6 combinations shown in Table 1) when following procedure A and the use of single primers when following procedure B (any primer of the 12 primers listed in Table 1). There are many other possible primer sequences besides those listed in Table 1 and used in the present study. The selection of primers was based on knowledge of the occurrence of different repeat sequences in genomes (3). Microsatellite primer identification was tested on plant material, representing 21 species of bryophytes and three algal species collected from different parts of the Northern Hemisphere, mainly from

Table 1. ISSR Primer Sequences Used in the Genome Screening

AG/GA Group		AC/CA Group	
Primer Name	**Primer Sequence**	**Primer Name**	**Primer Sequence**
AG-T	$[AG]_8T$	AC-T	$[AC]_8T$
AG-C	$[AG]_8C$	AC-C	$[AC]_8C$
AG-G	$[AG]_8G$	AC-G	$[AC]_8G$
GA-T	$[GA]_8T$	CA-T	$[CA]_8T$
GA-C	$[GA]_8C$	CA-A	$[CA]_8A$
GA-A	$[GA]_8A$	CA-G	$[CA]_8G$

The primers were used either one at a time (procedure B) or as pairs in cross-amplifications (procedure A), including one primer from both primer groups (all combinations possible). Annealing temperatures were 50°–52°C. Only some of the primers or primer pairs were used when screening the genome of each taxon. ISSR, inter-simple sequence repeat.

Northern Europe, and on one animal species, the raccoon dog.

Procedure A

Cross-amplification including the three following types of PCRs was conducted: (*i*) amplification with only one ISSR primer; (*ii*) amplification with another ISSR primer; and (*iii*) amplification with both ISSR primers at the same time. Amplification reactions were conducted in a volume of 20 µL. The reaction mixture contained about 10–20 ng genomic DNA, 1.2 U DyNAzyme™ II DNA polymerase (Finnzymes, Espoo, Finland), 1× PCR buffer, 0.4 µL 10 mM dNTP mixture, and 1 µL of a single 5 µM primer or 1 µL each of two 5 µM primers. The PTC-200 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) was programmed for 4 min denaturation at 94°C, followed by 45 cycles of denaturation at 94°C for 45 s, annealing at 50°–52°C for 45 s, and elongation at 72°C for 90 s. An additional 8-min elongation followed the last cycle. All amplification products were electrophoretically separated on 1.4% agarose gels. Only clear fragments, mostly in size range of 300–900 bp, resulting from two-primer amplifications and therefore containing different microsatellite repeats at the ends of the sequence were excised and purified with QIAquick® Gel Extraction kit (Qiagen) or a comparable product and sequenced with one of the two primers. The use of two different primers and the presence of different microsatellite repeats at the ends of the sequence are necessary for a satisfactory sequencing result. Single-primer control PCRs are conducted to distinguish the two-primer amplification products from the single-primer amplification products. Based on the sequencing result, the first specific microsatellite primer was identified for the microsatellite at one end or both ends of the sequenced fragment.

To obtain sequence information for the design of the second specific microsatellite primer, 12 µL genomic DNA (about 15–20 ng/µL) were treated with two four-cutter restriction enzymes leaving blunt ends following the protocol: restriction for 4 h at 37°C utilizing *Hae*III and *Rsa*I restriction enzymes in separate reactions, each in a volume of 8 µL containing 6 µL genomic DNA, 4 U of either restriction enzyme, 1× restriction buffer, and 8 µg bovine serum albumin (BSA). Then a two-stranded adaptor, composed of a 44-bp sequence (5′-GAACTAGTCT CGACTCCAGTCAGAGATTCCAC CGCCGTGACCGC-3′) and an 8-bp sequence with 5′ phosphorylation and an amino modifier at the 3′ end (5′-pGCGGTCAC-NH_2-3′) was ligated to the restricted DNA.

The use of an amino residue to block the polymerase catalyzed extension of one strand and the generation of amplification products in the absence of the specific primer has been described by Siebert et al. (16) and Lian et al. (17). The adaptor was constructed from two single-stranded oligonucleotides, which were mixed in equal molarities with the final concentration of 50 µM each, kept at 99°C for 2 min, and allowed to cool to room temperature before use. The ligation reaction was conducted for 2 h at 22°C in a volume of 20 µL containing the previous 8-µL mixture of restricted DNA, 0.5 µL double-stranded 50 µM adaptor, 5 U T4 ligase, 1× ligation buffer, 2 µL 50% polyethylene glycol (PEG) 4000, and followed by inactivation at 65°C for 15 min. After ligation, PCR amplifications were conducted with the first specific primer and the adaptor primer (5′-GAACTAGTCTCGACTCCA GTCAG-3′) in 20-µL reaction volumes containing 2 µL restriction-ligation DNA, 0.5 U DyNAzyme II Hot Start DNA polymerase (Finnzymes), 1× PCR buffer, 0.4 µL 10 mM dNTP mixture, and 1 µL of both 5 µM primers. The thermal cycler was programmed for 10 min denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 25 s, and elongation at 72°C for 40 s. An additional 8-min elongation followed the last cycle. The PCRs were followed by electrophoretic separation of the amplification products, fragment excision and purification, and sequencing with the adaptor primer. Based on the sequencing result, the second specific primer was identified. The first specific primer and the second primer, including a fluorescent label at the 5′ end, were used together when conducting microsatellite genotyping for the species in question.

Some microsatellites may be found within the sequenced ISSR amplification products, in which case the specific primers can be immediately identified for that microsatellite, and no further restriction-ligation step is needed. In the present study, the sequence walking involving the restriction and adaptor ligation procedure was conducted only for those ISSR fragments that did not contain a microsatellite within the sequenced amplification product (i.e., the area between the two ISSR primer sites).

When designing the first specific primer, it is important to consider that there are no recognition sites for the used restriction enzymes between the specific primer and the microsatellite and that the annealing temperature should match closely enough with the annealing temperature of the adaptor primer. Restriction enzymes resulting in blunt ends were used to allow a

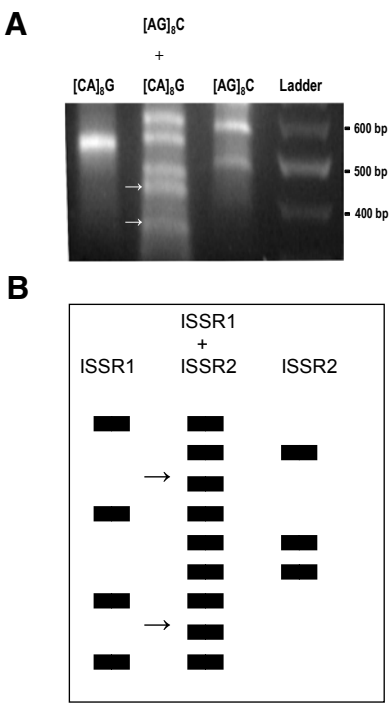

Figure 1. Genome screening. (A) An agarose gel showing the inter-simple sequence repeat (ISSR) amplification of *Polytrichum juniperinum* with primers $[CA]_8G$ (left), $[CA]_8G$ and $[AG]_8C$ (middle), and $[AG]_8C$ (right). (B) Diagram of a comparable cross-amplification including three types of PCRs: amplification with ISSR primer 1, amplification with ISSR primers 1 and 2 at the same time, and amplification with ISSR primer 2. In both panels A and B, PCR products that clearly result only from amplifications with both primers are marked with an arrow.

technique involving only one universal adapter. The aim of the restriction-ligation procedure was to generate DNA fragments that include the first specific primer, the microsatellite region, and enough DNA flanking the microsatellite on the other side of the microsatellite to allow the identification of the second specific primer. The expected length of the resulting fragments is 256 bp plus the adaptor. The use of a modified 3′ end in the short strand of the adaptor prevents amplifications between adaptors ligated to both ends of the restricted DNA fragment and promotes amplification between the adaptor and the microsatellite-specific primer. The microsatellite area of interest is located within the amplified region.

Procedure B

Amplification with single ISSR primers (similar to those used in procedure A) in 20-µL reactions was conducted, followed by electrophoretic separation of the amplified fragments, and excision and purification of clear fragments, mostly in size range of 300–900 bp . The purified DNA was concentrated to 3–5 µL and cloned using the TOPO® TA Cloning kit (Invitrogen, Carlsbad, CA, USA). The amounts of reagents used were reduced by 50% in proportion to the amounts recommended by the manufacturer. Positive clones were selected from the plates and transferred into 50 µL double-distilled water. DNA amplification was conducted in 20-µL reaction volumes containing 4 µL clone solution, 0.5 U Phusion™ High-Fidelity DNA polymerase (Finnzymes), 1× PCR buffer, 0.4 µL 10 mM dNTP mixture, and 1 µL each of primers M13-f and M13-r (at the concentration of 5 µM) included in the cloning kit. The thermal cycler was programmed for 30 s denaturation at 98°C, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 20 s, and elongation at 72°C for 50 s. An additional 8-min elongation followed the last cycle. The amplification products were separated electrophoretically, excised from the gel, and purified. Following the cloning step, sequencing was carried out using either the M13-f or M13-r primer. The following primer identification and the use of the restriction-ligation technique took place as in procedure A. Some microsatellites may be found within the cloned and sequenced ISSR amplification products, in which case the specific primers can be immediately identified for that microsatellite and no further restriction-ligation step is needed.

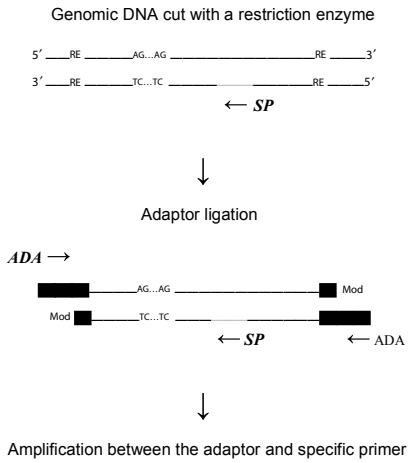

Figure 2. General outline of the restriction-ligation technique used to discover the DNA sequence flanking the microsatellite site (marked as AG…AG/TC…TC). The template-specific primer (SP) has been developed based on the sequencing of an inter-simple sequence repeat (ISSR)-screening generated DNA fragment. The two-stranded adaptor contains an amino modification (Mod) at the 3′ end of the short strand to prevent amplification between two adaptors. RE, cutting site for a restriction enzyme; ADA, adaptor sequence specific primer.

RESULTS AND DISCUSSION

Genome screening with a set of ISSR primers (Table 1) was conducted in each target species using either cross-amplification with two primers at a time (Figure 1) and followed by sequencing without cloning (procedure A) or single-primer amplification combined with the sequencing of cloned products (procedure B). In all, 54% of the sequenced ISSR products contained perfect, imperfect, or composite microsatellite repeats between the two ISSR primer binding sites (Table 2). For those microsatellites, specific primer pairs were identified for further genetic fingerprinting. Most of the remaining ISSR regions were subjected to the restriction and adaptor ligation procedure (Figure 2) to allow sequence walking and the characterization of the sequences flanking both sides of the ISSR primer binding site to allow the further identification of a specific primer pair for the microsatellite (i.e., the ISSR primer site). The sequence walking was conducted either for one or both ends of the ISSR product. The success rate of the microsatellite identification based on

Table 2. Microsatellite Markers Identified Using this Approach

Taxon	No. Species	No. Markers Identified	Polymorphic Markers No. (%)	Markers Identified No. (%)		Success Rates of Sequenced ISSR Products Resulting in a Scorable Marker	
				Directly from Sequence	With Sequence Walking	Directly from Sequence	With Sequence Walking
Rhodophyta	2	10	7 (70%)	0 (0%)	10 (100%)	0%	40%
Chlorophyta	1	4	4 (100%)	0 (0%)	4 (100%)	0%	31%
Bryophyta	21	175	169 (97%)	142 (81%)	33 (19%)	63%	42%
Mammalia	1	2	not tested	2 (100%)	0 (0%)	67%	not tested
Total	25	191	180 (95%)	144 (75%)	47 (25%)	54%	41%

Inter-simple sequence repeat (ISSR) products resulting in a scorable marker after direct sequencing of ISSR products (representing DNA regions between two ISSR primer sites; either procedure A or B) were not exposed to further sequence walking. Polymorphism was defined as any variation at the marker locus detected among about 20 or more individuals genotyped per species. The species for which markers were developed are listed in Supplementary Table S1.

Table 3. Microsatellite Repeat Types Detected as a Result of Direct Sequencing of ISSR Products[a] or Following Sequence Walking[b]

Repeat Motif	Motif Type No. (%)	Repeat Motif	Motif Type No. (%)
Sequence Walking			
AC or CA	12 (26%)		
AG or GA	35 (74%)		
Direct Sequencing		Direct Sequencing	
AC or CA	15 (10.4%)	CAR	1 (0.7%)
AG or GA	42 (29.2%)	AGGA	1 (0.7%)
AT or TA	4 (2.8%)	ACCG	1 (0.7%)
GC or CG	3 (2.1%)	TAGA	1 (0.7%)
GAC or CAG	2 (1.4%)	TATN	1 (0.7%)
AGC or CGA	6 (4.2%)	ACAG	1 (0.7%)
GAA	2 (1.4%)	AATC	1 (0.7%)
ACA	1 (0.7%)	ACTC	1 (0.7%)
AGG	1 (0.7%)	CTCA	1 (0.7%)
GCA	4 (2.8%)	CATM	1 (0.7%)
GAG	3 (2.1%)	AAGTG	2 (1.4%)
CAA or AAC	3 (2.1%)	ACTGG	1 (0.7%)
CAT	1 (0.7%)	CAACG	1 (0.7%)
CCA	2 (1.4%)	GCACTCT	1 (0.7%)
TAA	1 (0.7%)	TCTTCC	1 (0.7%)
R	1 (0.7%)	TTCATTCACTA	1 (0.7%)
M	1 (0.7%)	TAKAKA	1 (0.7%)
SA	2 (1.4%)	$[TG]_8T_{10}$	1 (0.7%)
GN	2 (1.4%)	$[TCT]_4[TC]_8$	1 (0.7%)
KK	3 (2.1%)	$[TC]_6GA[TC]_4$	1 (0.7%)
RR	6 (4.2%)	$[AG]_4C[AG]_2T[RR]_8$	1 (0.7%)
CY	1 (0.7%)	$[TC]_6T[TC]_3Y_8$	1 (0.7%)
CK or KC	3 (2.1%)	$[RCCA]_3TTTCA[RCCA]_6$	1 (0.7%)
GM	1 (0.7%)	$[CA]_6T[ACC]_3$	1 (0.7%)
RRR	1 (0.7%)	$[GCCG]_2[CGC]_3[TC]_6$	1 (0.7%)
ARR	1 (0.7%)	$[GGCT]_4GG[GGCTT]_5$	1 (0.7%)
ASR	1 (0.7%)	$[GCA]_3GGC[GCA]_3GGG[GCA]_4$	1 (0.7%)
CCR	1 (0.7%)	$[RRA]_{11}N_6[RRA]_{25}N_6[RRA]_{13}N6[RRA]$	1 (0.7%)
GAN	1 (0.7%)		

Sequence walking was always targeted to obtain an inter-simple sequence repeat (ISSR) repeat motif (AC, CA, AG or GA; see Table 1).
[a]Representing DNA regions between two ISSR primer sites.
[b]The repeat motif being the same as in the used ISSR primer.

sequence walking conducted for already sequenced products equaled 41% (Table 2). Most amplification products following the restriction-ligation procedure and resulting in successful sequence walking and the identification of specific primer pairs were in the size range of 170–400 bp. The total number of microsatellites identified while testing the methodology equaled 191 markers (identified either directly from the sequence or via sequence walking), of which 95% were found polymorphic (any variation detected among about 20 or more individuals genotyped per species).

Among the sequence walking-based microsatellites, the proportions of AC (or CA) and AG (or GA) repeats (the only possible repeats due to the selection of ISSR primers used in the genome screening) equaled 26% and 74%, respectively (Table 3). Also, among microsatellites resulting from the sequencing of the ISSR amplification products, the AC (or CA) and AG (or GA) repeats were most frequent, 10% and 29%, respectively. Among the 144 microsatellites resulting from direct sequencing, 57% consisted of perfect or imperfect dinucleotide repeats, while the proportions of mono-, tri-, tetra- and pentanucleotide repeats were 1%, 22%, 6% and 3%, respectively. In all, 10% of the repeat motifs contained longer or more complex repeat elements. The number of repeats in the sequenced microsatellites ranged from 5 to 66 among simple repeats. Taxon-specific information on the identified microsatellite markers are given in Supplementary Table S1, available online at www.BioTechniques.com.

Microsatellites are widely used as genetic markers because they are co-dominantly inherited, are commonly highly polymorphic, and can be scored reliably. A major drawback is the time and cost required to identify adequate marker sets. For humans and for many model organisms or economically important organisms, extensive sets of microsatellite markers are available. However, such markers are presently not available for most less well-known organisms, which may still be important targets for genetic research due to causes, such as conservation activities or basic research. We have now identified a new alternative for present methods used in microsatellite identification (3,11–13). The technique eliminates the need for library screening for the identification of clones containing short repeat sequences. Especially, the fact that the procedure does not necessarily require cloning makes it a feasible and attractive alternative for researchers who do not have facilities for cloning or who conduct small-scale microsatellite identification, such as researchers in population and ecological genetics and in conservation biology interested in the benefits of using highly informative microsatellite markers. The present method is also beneficial when a large set of organisms are to be examined for a limited set of microsatellite markers within a relatively short time.

Microsatellite-type elements tend to accumulate in certain genomic regions

instead of being evenly distributed, as observed in the present study (i.e., the common presence of other repeat elements near the ISSR primer binding sites, which also represent repeat elements) and also by Estoup et al. (18) and Altenheim et al. (19). This feature indicates that microsatellites can be commonly found when sequencing amplification products resulting from PCR with ISSR primers, without further sequence walking and the identification of specific primers for the ISSR-repeat site. We discovered that perfect or near-perfect tandem repeats were found in 54% of the sequenced ISSR amplification products. Previously, Geldenhuis et al. (20) have reported a study in which microsatellite-like markers were identified for a pathogenic fungus following ISSR screening and the cloning and sequencing of the PCR products, and a comparable investigation has been conducted for a peat moss by Provan and Wilson (21). Our methods allow this kind of microsatellite screening either with or without cloning, but also with or without the characterization and primer identification for the ISSR repeat itself as a result of sequence walking based on the use of the restriction-ligation method with a specific adaptor system.

Among the ISSR primers, the dinucleotide repeats combined with one-nucleotide anchors work best. The repeat sequences AG and AC used in the present study are suitable for both procedures A and B, while the repeat sequences AT and GC have differing temperature requirements and may cause problems in the amplification and sequencing of cross-amplification products (procedure A; H. Korpelainen, personal observation). The microsatellite repeat motifs AG and AC are common in many organisms (3). Also ISSR primers containing three-nucleotide repeats (e.g., $[TGT]_6$ and $[CTC]_6$) can be used, but the number of resulting microsatellite areas is usually lower. In general, when the repeat length increases, the probability of getting amplification products generally decreases as a consequence of a smaller number of target microsatellites present in the genome and the greater distance between the microsatellites, which hampers the amplification. If the goal is to find specific microsatellites with trinucleotide or longer repeat motifs, the ISSR screening procedure is likely to require the use of a greater number of primers than when searching for dinucleotide repeats. When conducting direct sequencing of ISSR amplification products, we observed that 40% of the repeat sequences detected near the ISSR primer (all based on AC or AG repeats) area were AC (or CA) or AG (or GA) repeats. Such an excessive proportion of similar repeats indicates the presence of a local accumulation of related repeat sequences. Dinucleotide repeats including regular, irregular, and complex motifs formed 57% of all microsatellites detected as a result of direct sequencing of ISSR amplification products.

When the procedures conducted with (procedure B) or without cloning (procedure A) are compared, it is clear that the procedure without cloning is easier, cheaper, and faster. Disadvantages concern the cross-amplification products, which are not always reliably distinguishable from single-primer products, and the sometimes inconsistent quality of sequencing when using the ISSR primer as a sequencing primer. The procedure involving cloning is technically more demanding and slightly more expensive than the procedure without cloning. However, the quality of sequencing is consistently high. Long and high-quality sequences also increase the possibility of directly finding usable microsatellite markers for which both specific primers can be identified without the otherwise following restriction-ligation step.

The restriction-ligation technique worked adequately, however nonspecific, multiple, or poor amplifications took place, apparently resulting from sequence variation in the region of the specific primer or from the presence of closely similar sequences elsewhere. The use of one specific primer only (i.e., the combination of a specific primer and the universal adaptor primer) lowers the specificity of the PCR when compared with amplifications with two specific primers.

Despite some shortcomings concerning either the sequence quality in the procedure conducted without cloning or the specificity of the amplification following the restriction-ligation steps, the new methodology possessed a quite high rate of success. Microsatellites were detected in 54% of the products following the original ISSR amplification, and microsatellite search involving sequence walking had a success rate of 41%. The methodology is applicable to any organism possessing short repeat sequences. Additionally, the new methodology makes it possible to estimate the frequency of microsatellites present in the genome of the target species already at an early stage of the marker identification. The short procedure also leads to a lowered risk of mistakes occurring during the identification process. Since the accumulation of repeat sequences is evident, the original amplification with an ISSR primer indicates the likely presence of sequence variation in that general genomic area. Therefore, the following sequencing will result in information that has potential beyond that related to microsatellite markers, for example concerning single nucleotide polymorphism (SNP) polymorphism applications. Such SNP (and microsatellite) applications may have potential in many identification scenarios, such as when identifying plant cultivars or endangered species.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Address correspondence to Helena Korpelainen, Department of Applied Biology, P.O. Box 27, FI-00014 University of Helsinki, Finland. e-mail: helena.korpelainen@helsinki.fi