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TECHNICAL ADVANCES

Multiplex SNP-SCALE: a cost-effective medium-throughput single nucleotide polymorphism genotyping method

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Abstract

We describe a convenient, cost-effective and flexible medium-throughput single nucleotide polymorphism (SNP) genotyping method, Multiplex SNP-SCALE, which enables the simultaneous amplification by polymerase chain reaction (PCR) of up to 25 (or potentially more) loci followed by electrophoresis in an automated DNA sequencer. We extended the original SNP-SCALE method to include (i) use of a commercial multiplex PCR kit, (ii) a four-dye system, (iii) much-reduced (2-μL) reaction volumes, (iv) drying down of template DNA before PCR, (v) use of pig-tailed primers, (vi) a PCR product weighting system, (vii) a standard optimized touchdown PCR thermocycling programme, and (viii) software (SNP-SCALE PRIMER DESIGNER) that automatically designs suitable SNP-SCALE primers for a batch of loci. This new protocol was validated for different types of SNPs. The method is cost- and time effective for medium-scale evolutionary and ecological projects involving 10s to 100s of loci.

Keywords: genotyping, locked nucleic acid (LNA), medium throughput, nonmodel organism, single nucleotide polymorphism (SNP), small reaction scale, universal fluorescent-labelled oligonucleotide (UFO) primer

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Introduction

Single nucleotide polymorphisms (SNPs) are the most abundant form of genetic variation, and are widely distributed in both coding and noncoding regions of most genomes. SNPs have been widely exploited in human clinical biology, such as in the HapMap project (The International HapMap Consortium 2003), while several useful properties of SNPs make them advantageous in molecular ecology, evolutionary genetics and conservation biology (reviewed in Brumfield *et al*. 2003; Morin *et al*. 2004). These useful properties include their codominant inheritance, low likelihood of homoplasy, the ability to target markers in specific regions of the genome (Aitken *et al*. 2004), and relatively easy ascertainment in almost any species (e.g. Primmer *et al*. 2002). SNPs are likely to become particularly useful where it is necessary to type a large number of markers dispersed throughout the genome, such as in population genomics (Luikart *et al*. 2003) and gene mapping (Slate 2005). Many commercial SNP genotyping protocols have been developed, using various assay methods based on hybridization, ligation, primer extension or enzymatic cleavage (reviewed by Syvanen 2001 and Chen & Sullivan 2003). However, the majority of these methods are designed for high-throughput human or model-organism studies, and 100s or 1000s of loci must be analysed before the protocols become cost-effective, although some commercially available SNP typing protocols, including Sequenom's MassARRAY, might be suitable for smaller projects. Ecological or evolutionary studies of nonmodel organisms, which typically involve 10s–100s of loci for 100s–1000s of samples, would therefore benefit from a cost-effective medium-throughput generic genotyping method.

One of the common limitations in molecular studies of wild species is the quantity and quality of DNA. This can be overcome by polymerase chain reaction (PCR)-based analyses. Allele-specific PCR (AS-PCR) is one of several techniques that make use of the PCR procedure for SNP genotyping (Newton *et al*. 1989; Okayama *et al*. 1989). AS-PCR relies on variation in primer–template binding efficiency to discriminate among target alleles. The typical reaction contains two allele-specific primers labelled with different

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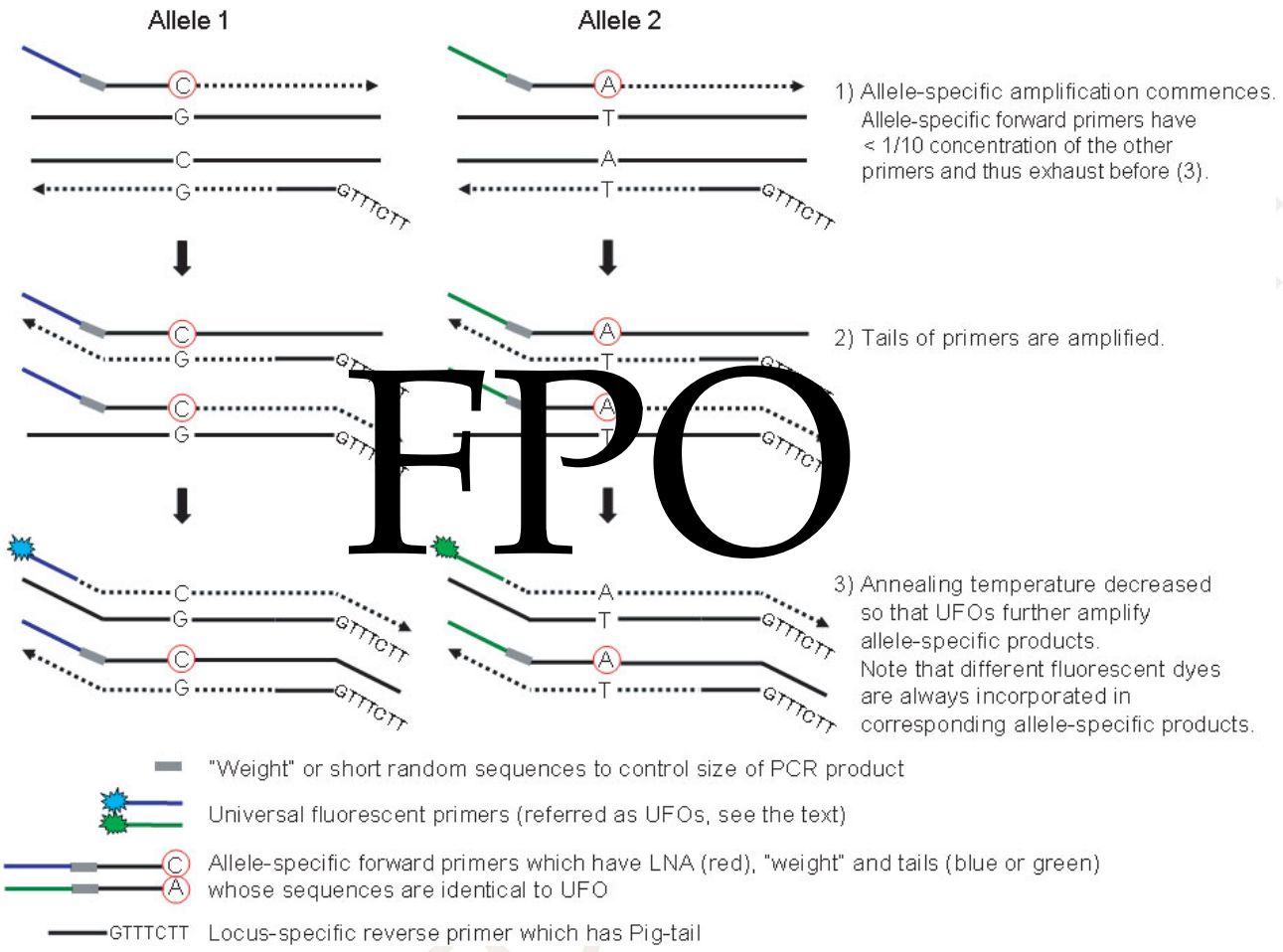

Fig. 1 Overview of Multiplex SNP-SCALE procedure.

dyes, each identical in sequence except for the 3′ base that complements its corresponding SNP base. There are two drawbacks of this method: the high cost of fluorescent dyes and the low allele specificity of the assay. SNP-SCALE (Hinten *et al*. 2007) solved these problems by the use of universal fluorescent-labelled primers to reduce costs, and locked nucleic acids (LNA) at the 3′ end of each allele-specific primer; LNAs have a higher binding affinity and specificity to their complementary base than standard nucleic acids (Koshkin *et al*. 1998; Singh *et al*. 1998; Latorra *et al*. 2003). However, the original SNP-SCALE protocol is based on singleplex PCR and is not sufficiently time efficient to enable the analysis of 10s–100s of loci.

Here, we present a new SNP-SCALE protocol based on multiplex PCR in a much-reduced reaction volume. This cost-effective and medium-throughput protocol, consisting of only a single PCR and electrophoresis in a DNA sequencer with a fluorescent dye detection system, allows us to genotype 25, or potentially more, SNP loci simultaneously. We also compared the success rates between alternative protocols and between different types of SNPs to assist potential users to choose the best protocol.

Methods

Protocol overview

The protocol is outlined in Fig. 1. The fundamental reaction in the new protocol is the same as in the original SNP-SCALE formulation: for each locus, two allele-specific forward primers and a locus-specific reverse primer amplify the targeted SNP region. The allele-specific forward primers, which are at relatively low concentration, include distinct 5′-tails that are identical in sequence to two universal fluorescently labelled forward primers (hereafter UFO, universal fluorescent oligonucleotides, following Hinten *et al*. 2007) that differ in size and colour. The initial PCR products that incorporate the UFO sequences are subsequently amplified by using the UFOs and the locus-specific reverse primer in the same PCR. The PCR products are then separated by electrophoresis and each allele at each locus is discriminated by both size and colour.

In this new protocol, we implement multiplex PCR by making several adaptations to the original SNP-SCALE protocol. These are (i) using the QIAGEN Multiplex PCR

Table 1 Universal fluorescent primers (referred to as UFOs, following Hinten *et al*. 2007). Relative migration is the observed size difference of PCR products including each UFO relative to UFO3-FAM, the UFO that gave the smallest observed product size. Both the length and dye of a UFO determine its relative migration

Name with dye	5′–3′	mer	Relative migration (base pair)
UFO1-HEX	CAGGGTTTTCCCAGTCACGAC	21	0.7–1.5
UFO1-VIC	Same	Same	1.1–2.4
UFO2-FAM	AGCGGATAACAATTTCACACAGGA	24	3.5–4.2
UFO3-FAM	AGCCGTTGCTACCCTCGTTC	20	0
UFO3-PET	Same	Same	2.4–4.0
UFO4-FAM	GTTCTGAGGGTGGCGGTTCT	20	0.2–0.6
UFO4-NED	Same	Same	0.4–0.9
UFO5-HEX	CATGGGTTCCTATTGGGCTTG	21	1.7–2.1
UFO5-NED	Same	Same	1.6–2.1
UFO6-HEX	GCAAAACCCCGCTAATCCTAATC	23	2.6–3.3
UFO7-HEX	AATCAGTGAGGCCACCGAGTAAA	23	2.7–3.3
UFO7-NED	Same	Same	2.9–3.9
UFO7-PET	Same	Same	5.4–6.3

Kit (QIAGEN); (ii) the use of a four-dye system rather than two dyes; (iii) the use of a pigtail (an oligonucleotide tail of GTTTCTT; Brownstein *et al*. 1996) at the 5′ end of each locus-specific reverse primer to reduce noise due to the inconsistent addition of adenine by *Taq* DNA polymerase; (iv) reaction volumes as small as 2 μL, (v) drying down of template DNA before PCR; (vi) the addition of random sequence 'weights' to allele-specific forward primers to make otherwise similar-sized PCR products distinct, allowing greater multiplexing; (vii) the use of an optimized standard thermocycling programme so that further optimization is not needed for every individual locus; and (viii) the use of software that automatically designs suitable SNP-SCALE primers for a batch of loci. The weight is designed so as not to bind to either the template DNA or UFO, so annealing temperatures are not affected.

Design and preliminary testing of new UFOs

We designed new UFOs in order to implement a four-dye system, as only two UFOs were used in the original SNP-SCALE protocol (Hinten *et al*. 2007). As UFOs bind to the artificial tail of another primer, in theory, any sequence would work as a UFO as long as (i) it meets the general requirements for primers, and (ii) it does not bind to the target genomic DNA. For the sake of convenience, we designed UFOs by choosing primers that bind to *Enterobacteria* phage M13 sequence (GenBank Accession no. 56718463). We chose initial candidate primers whose annealing temperatures (T_m) were around 64 °C, as for the existing UFOs (Hinten *et al*. 2007), using PRIMER 3 (Rozen & Skaletsky 2000). We confirmed the absence of sequences similar to these primer sequences in the whole genome of *Arabidopsis thaliana*, which is a close relative of one species that we studied (see below), using BLAST. From the initial candidate primers, two 20-mer primers, one 21-mer primer and two 23-mer primers that had relatively low matches to any genomic sequence (e > 2.5) were chosen as the base sequence for the new UFOs. They were labelled with the fluorescent dyes 6-FAM, HEX, NED or PET. One to three dyes were tested for each of five base sequences, making 13 UFOs in total (Table 1).

To test the amplification and size separation of SNPs using the new UFOs in comparison with the existing UFOs, we used new and existing UFOs in the SNP-SCALE protocol detailed in the following section for two SNP loci, ALS075 and ALS149 (T. Kenta *et al*. unpublished) in northern rockcress (*Arabidopsis lyrata* spp. *petraea*). We used DNAs of four *A. lyrata* plants as templates. The genotypes of those plants were known by sequencing; one plant was homozygous at both loci, another was the alternative homozygote at both loci and the others were heterozygous at both loci. We tried six pairs of UFOs in singleplex reactions and three combinations of UFOs in multiplex reactions to test compatibility among all possible pairs of UFOs. The relative observed size of PCR products obtained using each UFO is summarized in Table 1, together with details of each UFO. The estimated sizes of PCR products of the same allele at the same locus varied even when the same size of UFO was used. This variation was due to migration differences both among dyes and among the UFO base sequences. The former variation was 0–3.8 bp while the latter was 0–1.0 bp. Among all dyes, PET-labelled products showed the biggest difference between expected and observed size. Their observed sizes were 2.0–3.8 bp larger than for FAM labels, while the differences among the other dyes were within 1.0 bp. The pattern of relative migration speed among dyes was consistent with a technical report published by Applied Biosystems (Hauser *et al*. 2007). Amplification by UFO6-HEX was relatively poor when used in a multiplex set comprising UFO3-FAM,

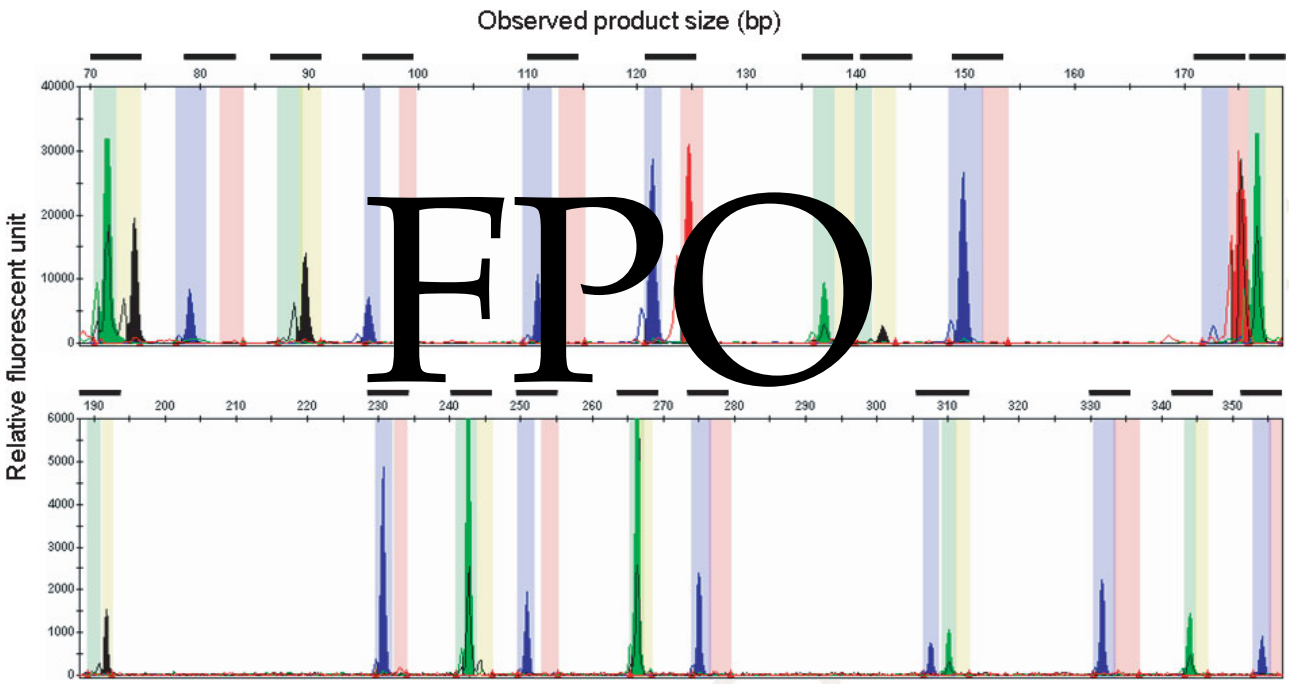

Fig. 2 An electropherogram of 21-plex SNP-SCALE using multiplex set 10 in *Arabidopsis lyrata*. GENEMAPPER 3.7 (Applied Biosystems) output was edited. Bars above the size axis indicate each locus. The locus ALS076 at *c.* 310 bp is tri-allelic. Note that the scales of the vertical axes are different between the upper and lower figures, indicating that larger products tend to have smaller peaks. At each locus, the two alleles are discriminated by both size and colour. Green (HEX) peaks are accompanied by smaller black (NED) peaks at the same positions, indicating that there has been some colour bleed-through due to usage of a nonstandard dye set (see the text). However, this degree of bleed-through does not compromise the scoring of the adjacent yellow allele peak because of the amplified allele size difference.

UFO4-FAM, UFO6-HEX and UFO7-HEX. Although filter set G5 on the ABI sequencers is designed for the set of FAM, VIC, NED and PET labels, we found that HEX, which is available at relatively low cost (e.g. 20-mer HEX-labelled oligonucleotides are currently available in the UK at less than one-quarter of the cost of similar VIC-labelled primers), can satisfactorily substitute for VIC. There was sometimes a considerable colour leak from HEX (green in Fig. 2) to NED (black); the different alleles labelled by those dyes, as for all loci, were discriminated by size in SNP-SCALE.

Validating Multiplex SNP-SCALE

We tried 11 sets of multiplex reactions, each of which consisted of a 5–30-plex, for a total of 45 SNP loci in *A. lyrata* and 21 loci in Soay sheep (*Ovis aries*).

Each sample was PCR amplified in a 2-μL reaction volume containing 0.1–20 ng genomic DNA, 1× master mix (including hotstart *Taq* DNA polymerase, PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and unknown additives) and 0.5× Q-Solution (containing unknown additives), as supplied in the QIAGEN Multiplex PCR Kit (QIAGEN), 0.02 μM of each allele-specific forward primer, 0.2 μM of each locus-specific primer and 0.50 μM of each UFO (with the exceptions of UFO1-FAM, UFO4-FAM and UFO5-HEX that were used at 0.17, 0.71 and 0.59 μM concentration, respectively, as the amplification efficiencies of these UFOs were different from the others). DNA was dried down either by incubation at room temperature overnight or at 70 °C for 30 min before PCR. The PCR mixes were overlaid with 6 μL or 3 μL of mineral oil in 96-well or 384-well plates, respectively, in *A. lyrata*, whereas 384-well plates were used without mineral oil in *O. aries*. Reactions that excluded Q-Solution were also tried in some multiplex sets and the results were compared to those including it. Allele-specific forward primers included LNAs at their 3′ end (Proligo). All allele-specific forward primers and locus-specific reverse primers were designed using PRIMER 3 (Rozen & Skaletsky 2000) to have an annealing temperature (T_m) of around 60 (range 58–62) °C and 65 (63–67) °C, respectively, and a low self-complementarity value (annealing temperatures were calculated excluding tails and weights). We did not check the annealing temperature and self-complementarity of those primers including tails because a preliminary test suggested that, as expected, this did not improve the PCR results. The inclusion of LNAs in allele-specific forward primers raises annealing temperature by approximately 5 °C. Because empirically, the actual annealing temperature (T_a) is lower than T_m by > 5 °C, the T_a for both classes of primers became < 60 °C. The primer design procedure

described above can now be carried out automatically using our new SNP-SCALE PRIMER DESIGNER software (www.sheffield.ac.uk/molecol/software~/snp.html). SNP-SCALE PRIMER DESIGNER uses PRIMER 3 (Rozen & Skaletsky 2000) for calculating properties of allele-specific forward primers and for finding suitable matching locus-specific reverse primers.

The PCR thermocycling conditions were identical for all multiplex sets and loci: an initial denaturation step at 95 °C for 15 min to activate the hotstart *Taq* polymerase, followed by 10 touchdown cycles of denaturation at 94 °C for 30 s, annealing at 60–51 °C (decreasing by 1 °C per cycle) for 90 s, followed by 40 subsequent similar cycles with annealing at 50 °C for 90 s, finally followed by an extension at 60 °C for 30 min to allow complete adenine addition. PCR was carried out in a Tetrad or Dyad DNA engine thermal cycler (MJ Research).

After dilution of 20–50 times with water, 0.5 μL of diluted PCR products could be taken out easily, without interference by mineral oil, and mixed with 9.5 μL Hi-Di formamide (Applied Biosystems) including GeneScan-500 ROX or 500 LIZ size standards (Applied Biosystems). Samples were run on an ABI 3730 capillary electrophoresis machine (Applied Biosystems) with the POP7 matrix using filter set D or G5, respectively. Fragment sizes and peak heights (relative fluorescent units) of PCR products were scored using the SNaPshot analysis method in GENEMAPPER 3.7 software (Applied Biosystems).

The observed sizes of PCR products were compared to the expected sizes, to determine the threshold difference in expected size above which any two given loci can be safely multiplexed without their sizes overlapping. We used the theoretical size based on genomic sequence as the expected size, except that 3.0 bp was added to PET-labelled products to reflect their slow migration speed (Table 1). The difference (D) between observed size (O) and expected size (E) is expressed by $D = O - E$. We are interested in $(D_1 - D_2) = (O_1 - O_2) - (E_1 - E_2)$, that is the difference between expected and observed 'distance between any two loci', where subscripts 1 and 2 indicate any two loci. The distribution of $(D_1 - D_2)$ was obtained by bootstrapping with 10000 permutations using R 2.4.0 (R Development Core Team 2006).

We evaluated the success of our assay for each locus at each of two steps: PCR amplification and genotype scoring. All 66 loci were used for the former while 16 loci in *A. lyrata* and all 21 loci in *O. aries* were used for the latter. If the largest peak height of alleles at a locus was less than 100 relative fluorescent units, PCR amplification was considered to have failed. At least eight individuals were used to check the amplification of each locus, and amplification was regarded as successful if > 95% of individuals were amplified. In theory, some loci might fail to amplify due to primer incompatibility with another locus. We used AUTODIMER 1.0 (Vallone & Butler 2004) to try to predict such incompatibilities in multiplex PCR and evaluated whether the program successfully predicted which loci would fail.

To score genotypes at each SNP, absolute total (T) and relative (R) peak heights of the two alleles at a locus (x, y) were calculated by $T = \mathrm{Log}_{10}(x + y)$, $R = x/(x + y)$, and T was plotted against R for each sample to generate a peak intensity cluster plot, with the help of a Microsoft Excel macro (www.sheffield.ac.uk/molecol/software~/snp.html, Hinten *et al*. 2007). In this macro, if the peak of an allele was not detected, then peak height was set at a default value of 10 relative fluorescent units to reflect the background noise level. At least 48 individuals were used in a genotyping quality check for each locus. Genotyping of each SNP was regarded as successful if the heterozygous and two homozygous genotypes formed distinctly separate clusters on the peak-intensity cluster plot.

In those SNPs where one allele is a C/G while the other is an A/T (defined here as allotype SNPs), the C/G alleles could possibly have a slightly higher annealing temperature than the A/T alleles and thus start being amplified earlier during the SNP-SCALE touchdown cycle, leading to a biased relative peak height. If this were to happen, then genotyping quality would be lower in allotype SNPs than in those where the alternative alleles are C and G, or A and T (defined here as autotype SNPs). Two tests were carried out to test for this possible artefact of SNP-SCALE. First, the silhouette score, which provides a numerical estimate of cluster quality, was calculated using CLUSTERA (Lovmar *et al*. 2005) for the peak-intensity cluster plot for every locus. These scores were compared between allo- and autotype SNPs, using *t*-tests, after normalizing using the mean and SD of each species to remove any bias in distribution between the species. Second, the peak height of alleles with dye I (y) was regressed on the peak height of the other allele with dye II (x) in the equation $y = \alpha x$ for all successfully genotyped allotype loci. Then the slope values, α, were normalized using the mean and SD of each species and compared using *t*-tests between two groups of loci: those with the C/G allele labelled with dye I, and those with the C/G allele labelled with dye II.

Results and discussion

Multiplex amplification was highly successful (Table 2) using the QIAGEN Multiplex PCR Kit (QIAGEN). We obtained 100% success for eight multiplex sets and 67–86% success for the other three multiplex sets when using the best conditions in terms of the presence of Q-Solution, an additive included in the multiplex PCR kit. The Q-Solution had favourable or adverse effects depending on the specific multiplex set and changed the number of successful loci by one to two in each of three multiplex sets. This result is consistent with the user's manual, which reports that there are cases where Q-Solution may work adversely. The

Table 2 Summary of loci used and amplification success for each multiplex set. Different alleles of a locus are labelled by a pair of different UFOs and discriminated by size and colour. In a multiplex set, the same pair of UFOs is used for many loci and those products are discriminated by size. APS loci and ALS loci will be published elsewhere (J. Gratten *et al.* unpublished and T. Kenta *et al.* unpublished, respectively)

	Number of loci				
Multiplex set	Total	Amplified with Q-Solution	Amplified without Q-Solution	UFOs	Loci used
Ovis aries					
1	6	Not tried	6	1-VIC, 2-FAM	APS005, APS009, APS010, APS027, APS040 and APS065
2	5	Not tried	5	1-VIC, 2-FAM	APS011, APS030, APS031, APS037 and APS071
3	5	Not tried	5	1-VIC, 2-FAM	APS003, APS006, APS008, APS012 and APS035
4	5	Not tried	5	1-VIC, 2-FAM	APS013, APS016, APS028, APS033 and APS063
Arabidopsis lyrata spp. *petraea*					
5	20	17	15	1-HEX, 2-FAM	ALS004, ALS007, ALS009, ALS011, ALS014, ALS019, ALS026, ALS049, ALS057, ALS059, ALS075, ALS092, ALS104, ALS137, ALS140, ALS145, ALS149, ALS152 and ALS155
6	7	7	Not tried	3-PET, 4-FAM, 5-HEX, 7-NED	ALS021, ALS027, ALS052, ALS087, ALS092.2, ALS120, ALS130
7	6	4	Not tried	3-PET, 4-FAM, 5-HEX, 7-NED	ALS002, ALS022, ALS037, ALS071, ALS076 and ALS079
8	6	6	Not tried	3-PET, 4-FAM, 5-HEX, 7-NED	ALS015, ALS025, ALS069, ALS081, ALS099, ALS102
9	6	6	Not tried	3-PET, 4-FAM, 5-HEX, 7-NED	ALS011.2, ALS035.2, ALS048, ALS065, ALS074 and ALS101
10	21	19	21	3-PET, 4-FAM, 5-HEX, 7-NED	Multiplex set 6, 7, 8 and 9 excluding ALS015, ALS022, ALS079 and ALS099
11	30	25*	24*	1-HEX, 2-FAM, 3-PET, 4-FAM, 5-HEX, 7-NED	Multiplex set 6, ALS009, ALS011, ALS014, ALS026, ALS049, ALS075, ALS104, ALS145 and ALS149

*These numbers include different loci. Five loci were successful only in either one of these reactions.

21-plex trial worked at 100% success for all individuals in multiplex set 10 (Fig. 2). In multiplex set 11, 25 of 30 loci were successfully amplified for all eight individuals, while 95.8% of genotypes [230/(8 individuals × 30 loci)] were obtained, including those loci classified as unsuccessful. Variation in peak height among loci tended to increase with the number of loci in the multiplex, although this did not cause a problem in scoring individual loci using the SNaPshot analysis method in GENEMAPPER 3.7 (Applied Biosystems). Noise bands were relatively abundant in the lower size range (< 80 bp). There was a general tendency for relatively low-concentration DNA samples to be more prone to amplification failure.

All five loci that used the PCR product weighting system were successfully amplified, confirming its usefulness. Although we only tried weighting by up to 3 bp, there is no reason why bigger weights should not be successful. Noise due to 1-bp differences was less obvious or negligible when pigtails were used (Fig. 2), compared to the original SNP-SCALE method (Hinten *et al*. 2007). Noise bands of the same colour sometimes appeared in the vicinity of target bands. These noise peaks created much less of a problem in peak scoring when loci of adjacent sizes used a different pair of UFOs. AUTODIMER did not predict any of the loci whose amplification failed, while some pairs of loci that were predicted to form primer dimers by AUTODIMER amplified well.

The observed size of PCR products was biased by up to 6 bp from the expected size (Fig. 3a), making ($D_1 - D_2$) biased by up to 12 bp (Fig. 3b). The 1% and 0.1% quantiles of ($D_1 - D_2$) were –7.0 and –9.7, respectively. This means that any two loci 7.0 and 9.7 bp apart from each other in expected size have only a 1% and 0.1% risk of overlapping. Given that the size range of a SNP locus is limited to 5 bp and that a 7-bp interval is necessary, electrophoresis from 80 to 500 bp could accommodate a 35-plex set without prior size checking.

Genotype scoring was successfully performed in 16/21 (76%) and 16/16 (100%) loci in *Ovis aries* and *Arabidopsis lyrata*, respectively. The best and the worst examples of peak-intensity cluster plots are shown in Fig. 4. We attribute lower genotyping success in *O. aries*, relative to *A. lyrata*, to

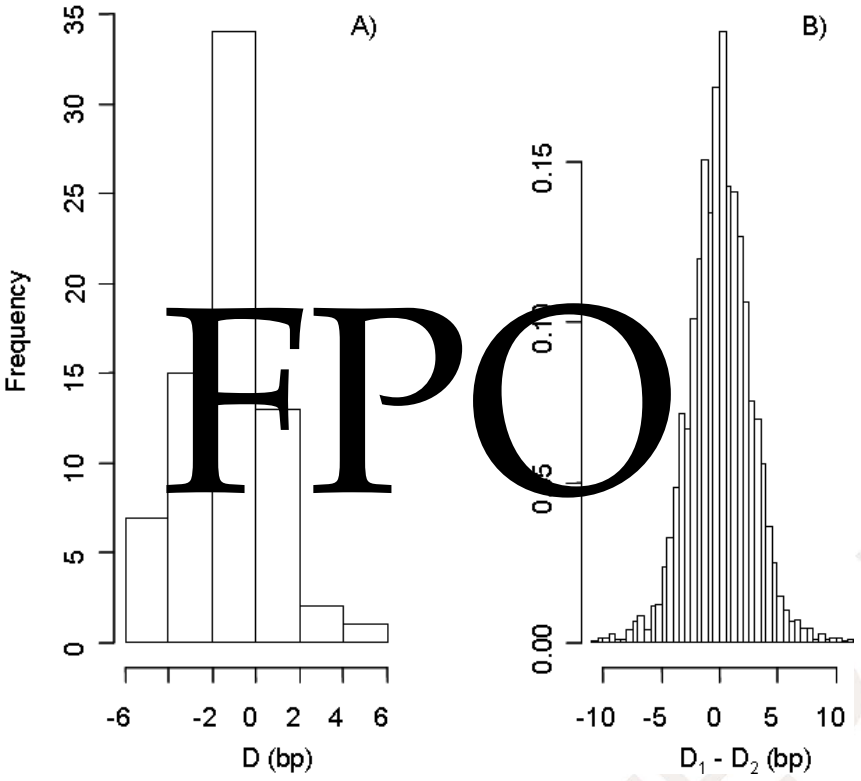

Fig. 3 Histogram of (a) difference (D) between observed and expected size for each locus and (b) difference between expected and observed 'distance between any two loci' ($D_1 - D_2$) generated by bootstrapping with 10 000 permutations.

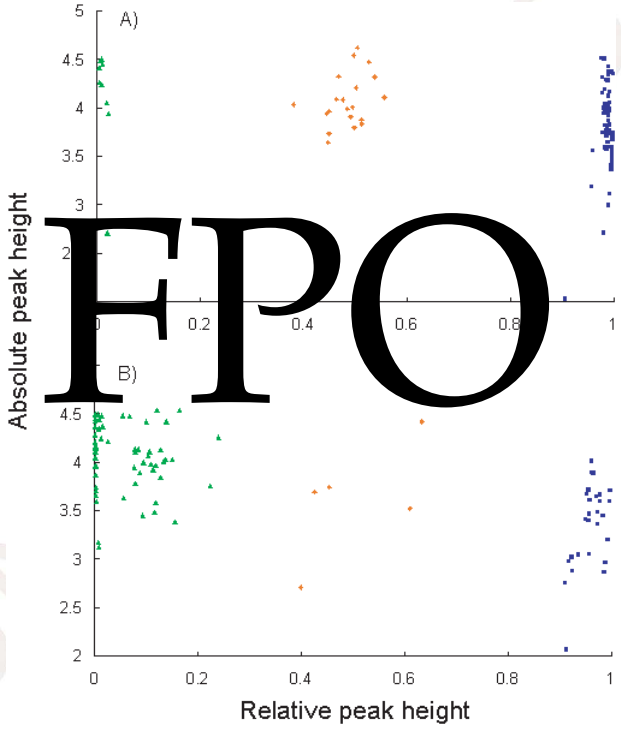

Fig. 4 Examples of peak-height cluster plots of loci displaying the best (A, ALS140) and the worst (B, ALS059) genotyping quality (based on silhouette score) in *Arabidopsis lyrata*.

more variable DNA quality and/or the failure to use mineral oil in *O. aries* PCRs. First, *O. aries* DNA was extracted from samples collected over a 20-year period, whereas DNA from *A. lyrata* was extracted from samples collected in the past two years. Second, low-level evaporation due to the absence of mineral oil might have led to changes in reagent concentrations, resulting in a decrease in the allele-specificity of the reaction, particularly in the later PCR cycles.

The silhouette score was not significantly different between the allo- and autotype SNPs ($P = 0.19$, 2-tailed Welch's t-test, $t = -1.48$, d.f. = 6.18). For allotype SNPs, the slope, α, was not significantly biased towards the C/G allele vs. the A/T allele ($P = 0.39$, 2-tailed Welch's t-test, $t = -0.88$, d.f. = 23.06). These results indicate that Multiplex SNP-SCALE does not have a systematic bias with respect to the category of allelic variation.

Multiplex PCR Kits (QIAGEN) cost four to eight times more per reaction than alternative *Taq* polymerase-based PCRs. The extra reagent cost is therefore compensated for by using a multiplex of four to eight loci, which we found to be easily achievable. Moreover, multiplexing also increases the efficient use of equipment and, most importantly, labour. Multiplex SNP-SCALE is a quick and simple procedure, consisting of only a single PCR and electrophoresis, and is flexible in terms of the number of loci analysed: it is easy to make a new multiplex set by adding or removing loci.

Our usage of small-volume (2-μL) reactions further reduces costs. Although QIAGEN recommends 50-μL reaction

Box 1 Recommended procedure for Multiplex SNP-SCALE.

1 Choose two pairs of UFOs whose sizes differ by 2–3 bp within a pair (see Table 1).
2 Run a PCR with the same thermocycling condition as SNP-SCALE using only UFOs as primers and confirm that they do not amplify significant noise bands. Otherwise, design your own UFOs.
3 For each locus, design a locus-specific reverse primer and a pair of allele-specific primers with tails whose sequences are the same as each UFO. The most important steps in the primer design are checking the annealing temperature and self-complementarity of primers excluding tails. It is not necessary to check between-locus primer compatibility. Any type of SNP can be analysed using Multiplex SNP-SCALE. Make the sizes of PCR products as different as possible, although PCR products < 80 bp may be subject to overlap with noise bands. Use a weight system to separate the sizes of otherwise overlapping loci.
4 Choose a multiplex set by putting each locus at a minimum 7-bp interval. Pre-mix primers of a multiplex set and try a multiplex reaction. There is no need for any preliminary single-plex checking. To minimize the time-consuming primer-mixing step, we recommend trying SNP sets in a 6–8-plex first, and then combining different sets once the sizes and positions of noise bands have been identified. Primer-mix stocks for each multiplex should be prepared at a high enough concentration to allow subsequent dilution (e.g. mixing two sets is equivalent to diluting them to half concentration). Reactions both with and without Q-Solution should be attempted, particularly when the plex number is high.

volumes, our small-volume reactions have proven to be consistently stable. We attribute this to the use of mineral oil to block evaporation and the initial drying down of the DNA. By drying down the DNA, any variation in the pipetted volumes of the DNA or the premixed PCR solution does not affect the concentration of any component of the reaction (other than possibly the DNA, which appears to be less critical). Indeed, this technique is generally useful in reducing costs, while maintaining consistency, in a wide range of applications. For example, we have also found multiplexed microsatellite amplification to be highly successful using these small reaction volumes (T. Kenta, unpublished data).

The cost of Multiplex SNP-SCALE, including all the consumables and labour required throughout the steps from primer design to genotyping analysis, is currently in the range of £0.04–£0.15 per genotype for 384–1920 samples using 15–25-plex reactions. The reduced labour cost of primer design achieved by using SNP-SCALE PRIMER DESIGNER software contributes to this low cost. There is a relatively high initial cost for primers, currently *c.* £43 per locus, and so the final per-genotype cost largely depends on the number of samples analysed. The per-genotype cost is similar for any total number of loci when the number of the plex exceeds 15. This cost is considerably less than for the original SNP-SCALE method (£0.12–£0.22 per genotype) and is comparable to commercially available medium-throughput SNP-typing systems, although these may require a high initial investment in new equipment or be less flexible in terms of the ease with which loci can be added or removed from the multiplex sets.

Finally, the Multiplex SNP-SCALE procedure is summarized in Box 1, incorporating the recommendations described in detail above. We conclude that Multiplex SNP-SCALE is cost- and time-effective for medium-scale evolutionary and ecological projects involving 10s to 100s of loci.

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