Molecular Ecology (2000) **9**, 1699–1710

COMMENTARY

SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology

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Abstract

All genetic markers are estimators of DNA nucleotide sequence variation. Rather than obtaining DNA sequence data, it is cheaper and faster to use techniques that estimate sequence variation, although this usually results in the loss of some information. SSCP (single-stranded conformation polymorphism) offers a sensitive but inexpensive, rapid, and convenient method for determining which DNA samples in a set differ in sequence, so that only an informative subset need be sequenced. In short, most DNA sequence variation can be detected with relatively little sequencing. SSCP has been widely applied in medical diagnosis, yet few studies have been published in population genetics. The utility and convenience of SSCP is far from fully appreciated by molecular population biologists. We hope to help redress this by illustrating the application of a single simple SSCP protocol to mitochondrial genes, nuclear introns, microsatellites, and anonymous nuclear sequences, in a range of vertebrates and invertebrates.

Keywords: allele genealogies, DNA sequence, PCR, population genetics, SSCP

Received 25 March 2000; revision received 25 June 2000; accepted 25 June 2000

Introduction

Acquisition of DNA sequences is now a fundamental component of most phylogenetic, phylogeographic, and molecular ecological studies (Avise 1994; Templeton 1994; Moritz & Lavery 1996; Smith & Wayne 1996). The importance of DNA sequence data is increasing and many new analyses require them (review in Sunnucks 2000). While obtaining DNA sequence has become less expensive and time-consuming, it is still a limiting step in most projects. Often in population biology, many samples must be sequenced, yet many individuals may carry the same sequence. SSCP (single-stranded conformation polymorphism) offers a simple, inexpensive and sensitive method for detecting whether or not DNA fragments are identical in sequence, and so can greatly reduce the amount of sequencing necessary (Kanazawa *et al*. 1986; Orita *et al*. 1989a,b; reviews in Hayashi 1991, 1992; Hayashi & Yandell 1993).

SSCP has been extensively applied in biomedical research and there are scores of published adaptations and refinements. Despite some successful applications in population biology (e.g. Antolin *et al*. 1996; Burt *et al*. 1996; Girman 1996; Bagley *et al*. 1997; Friesen *et al*. 1997; Ortí *et al*. 1997a; Bagley & Gall 1998; Jaeckel *et al*. 1998; Gasser *et al*. 1999; Hedrick *et al*. 1999; Sommer & Tichy 1999; Steel *et al*. 2000; Congdon *et al*., 2000; Trewick *et al*., 2000), molecular ecologists have yet fully to embrace SSCP technology.

Many researchers are discouraged from using SSCP by reports of the extensive optimization required in clinical applications. However, the requirements of clinical work and molecular ecology differ in an important manner. Clinical work is often concerned with detecting rare alleles that are very similar to common ones. For example, many diseases are associated with rare point mutations. So in clinical screening, failure to detect a single base change can be literally a matter of life or death. In contrast,

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population studies generally seek to estimate genetic differences among individuals or groups of individuals, so small amounts of undetected variation will usually have negligible impact on the inferences drawn. Indeed, most commonly used screening techniques in molecular ecology detect only a proportion of the underlying genetic variation. For example, 14 randomly chosen restriction enzymes applied in a polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) survey of population samples of a skink *ALD-1* region detected only one of 20 variable sites (Slade *et al*. 1993). In contrast, SSCP will detect the great majority (typically >90%) of the sequence variation in most DNA regions in the size range commonly assayed in molecular ecology (review in Girman 1996; some details below).

Using illustrations from our work applying a generic SSCP protocol, we hope to dispel the myth that the technique requires extensive optimization when it is applied to most questions in molecular ecology. With little or no optimization, our protocol can reveal high levels of polymorphism in genetic markers investigated in molecular ecology. We suggest some applications of SSCP, and present some accumulated information on procedures and interpretation.

Materials and methods

PCR–SSCP: general method

SSCP entails electrophoresis of single-stranded (ss)DNA fragments of suitable size through a nondenaturing polyacrylamide gel, followed by visualization. We focus here on SSCP of PCR fragments, although the technique is applicable to other kinds of nucleic acid fragments (references above). Under appropriate conditions (notably low temperature and nondenaturing conditions), DNA strands fold into structures that migrate according to their shape. DNA strands of different sequence generally do not assume the same shape, and so have distinct gel mobilities. Recent evidence suggests that these mobility differences are based primarily on tertiary rather than secondary structure of the DNA molecules (Liu *et al*. 1999). The sensitivity of the technique is generally inversely proportional to the size of fragment (e.g. single base pair differences resolved 99% of the time for 100–300 bp fragments, >80% for 400 bp ones; references in Girman 1996). Fragments at least as large as 775 bp may be analysed successfully (Ortí *et al*. 1997a).

Sensitivity of detecting sequence variation and the appearance of the banding patterns associated with a given sequence may alter with experimental details. When it is essential that a given variant or all variants must be revealed, one can attempt to achieve this by altering temperature, gel and buffer compositions and running conditions (Hayashi 1991, 1992; Hongyo *et al*. 1993; Ortí *et al*. 1997a; Highsmith *et al*. 1999; Liu *et al*. 1999; Nataraj *et al*. 1999; Yip *et al*. 1999). This degree of optimization will not be necessary for most applications involving screening genetic variation in molecular ecology (see Introduction). We now give details of our generic protocol. For many conditions we give bounds within which the technique usually works well. We have found little optimization to be necessary. Usually acceptable protocol details (gel running, etc.) are found within one or two attempts, after which, conditions for a given marker assay should be held constant.

PCR–SSCP protocol

The protocol uses basic materials found in molecular population biology laboratories. Few adaptations should be necessary for most applications, although laboratories without access to radioisotopes will need an alternative method of visualization. Common alternatives include silver-staining (commercial kits or standard silver-staining protocols can be applied, e.g. Atkinson & Adams 1997; Sommer & Tichy 1999), and fluorescent dyes (e.g. Turenne *et al*. 2000; Zumstein *et al*. 2000). While ethidium bromide can detect SSCP variation (Ortí *et al*. 1997a), it does not stain single-stranded DNA well, and SYBR gold (Molecular Probes) can be used (see Small & Gosling 2000). Standard reagents such as buffers and dyes follow Sambrook *et al*. (1989).

1 Optimize PCR conditions to obtain strong, clear products as visualized on agarose gels stained with ethidium bromide, in 10 μL reaction volumes with 10–50 ng DNA, *Taq* polymerase (Promega, 0.5 units), 1× *Taq* reaction buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100), 2 mM $MgCl_2$, 200 μM of each dNTP, and 5–10 pm primers.
2 Carry out radio-incorporation PCRs as in (1) above but in the presence of 0.05 μL [α^{32}P or α^{33}P] dATP (10 mCi/mL) (or other radio-labelled dNTP).

Equal (200 μM) concentrations of the four unlabelled dNTPs may be optimal for most templates in SSCP, while for microsatellite analysis on denaturing gels we use only 20 μM unlabelled dATP. Nonetheless, for most templates we find that our standard microsatellite PCR products also give acceptable SSCP patterns when run on nondenaturing (SSCP) gels. Conversely, our standard SSCP products can be successfully run on denaturing gels to screen for length variation (in addition to their primary function of screening for sequence variation on nondenaturing gels). Obtaining both length and sequence information can be very useful for detecting microsatellite allele homoplasy (example below).

We find ^{33}P gives clearer results than ^{32}P, as is generally the case with radioassays provided that ^{33}P gives sufficient signal. While we use radio-incorporation for practical reasons (notably utilization of the radioisotope already available in the laboratory, and fewer processing steps), end-labelling primers generates only one band per allele, which can aid clarity and interpretation (see Friesen *et al*. 1997). It is possible that incorporation PCR and end-labelling will have different effects on the conformers of a given DNA sequence, because of the presence of more labelled nucleotides in the former case.

3 At the end of the PCR cycling, add 10 μL standard formamide loading dye to PCR products, heat to 90 °C for 3 min, then place on ice. The exact temperature and time of denaturation seem to make little difference, but they should be standardized. Running some reactions as double-stranded DNA on each gel can aid interpretation. To achieve this, the samples are loaded in nondenaturing gel loading buffer, and are not heated.

4 Prepare nondenaturing polyacrylamide gels. Our stock gel-mix contains 8% acrylamide (37.5:1 acrylamide: bis-acrylamide, e.g. BIORAD product 161 0148), 5% glycerol, 0.5× standard TBE buffer, and is stored refrigerated. Immediately before pouring a gel, 2 μL TEMED (e.g. Sigma T8133) and 2 μL 25% w/v ammonium persulphate per mL of gel mix are added to catalyse the polymerization reaction. In our experience, gel mix that has been stored for more than one or two weeks gives DNA bands that are sharp but wavy rather than sharp and straight.

5 Electrophorese 2–4 μL of each PCR product through a nondenaturing acrylamide gels in 0.5× TBE running buffer at 4 °C and 5–15 W for 2–12 h. Typically on a 29-cm gel, we might run a 220-bp product for 2 h at 15 W, and a 450-bp one for 8 h.

We find that standard sequencing equipment performs well, and have tried gels 58.5 cm long × 21.5 cm wide, 29.0 × 21.5 and 42.5 × 38.0. Within the bounds above, the precise running conditions do not seem to be critical for most variation to be resolved, and application of moderate conditions based on a little experience or one or two trials usually yields usable results. Three main variables are gel length (shorter gels generally give sharper, straighter bands, and migration is faster), size of PCR product (the relationship of molecular weight with migration distance is not precise, but there is a strong trend that smaller products run faster), and power (which seems to predict migration in a linear fashion). Bromophenol blue and xylene cyanol dyes are helpful indicators: with all PCR products we have tested so far, all useful SSCP bands have migrated more slowly than xylene cyanol, and this can be used to help choose the running time in a new system.

Note that incomplete denaturation is a possible cause of unclear patterns: reduction of the amount of PCR product denatured and loaded is a simple remedy, if the PCR product is sufficiently concentrated (Hongyo *et al*. 1993).

It is generally best to keep the apparatus cool, ideally using cooling and recirculating buffer, but it is sufficient to place the apparatus in a cold room, refrigerator, or using a fan blowing over an ice bucket. Satisfactory results can be achieved at ambient temperatures, and this makes possible the application of fluorescent visualization in automated sequencers. It is most important to maximize constancy and repeatability of the temperature (Hayashi 1992; Hongyo *et al*. 1993).

6 Dry and autoradiograph gel.

7 Develop and examine autoradiograph after 4–72 h exposure.

For most PCR products we see two distinct systems of bands on the gel. One system is multibanded, slower migrating, weaker and finer than the other, which is usually single-banded. Both systems should be examined, as variation may be more apparent in one system than the other (see also Ortí *et al*. 1997a).

Multiple bands seen in SSCP analysed PCR products resulting from radioactive incorporation may be attributable to: (i) differing conformation of the two strands of renatured single-stranded DNA; (ii) multiple stable single-stranded conformations per strand; (iii) unfolded single-stranded DNA due to lack of renaturation under certain conditions; (iv) double-stranded homoduplex DNA due to lack of denaturation or to reannealing of the single strands (see Atkinson & Adams 1997) and/or; (v) double-stranded heteroduplex DNA created during PCR or reannealing (e.g. in heterozygous individuals; see Murray *et al*. 1999).

These different DNA systems can be distinguished by combinations of four sorts of comparison: end-labelling one primer vs. incorporation PCR, homozygotes vs. heterozygotes, denatured vs. nondenatured samples, and denaturing gels with absolute size markers vs. nondenaturing gels. However, SSCP protocols can be used to great effect without knowing which sort of DNA is being examined — it is simply necessary to confirm that one can deduce with appropriate confidence whether two DNA fragments share the same sequence or not (see also Murray *et al*. 1999).

8 Ascribe gel phenotypes.

On each gel, all templates are placed in categories (gel phenotypes) that are provisionally regarded as sharing

DNA sequence within phenotype, and not sharing sequence among phenotypes. It may be necessary to re-run samples from the same gel next to each other, because sometimes, similar ones are not sufficiently close for easy comparison, and occasional gel irregularities may disrupt certain comparisons. When this process is completed with confidence, comparisons can be made among gels, wherein representatives of each phenotype from each gel are re-run on the same gel.

The logistics of within and among gel comparisons depend on the number of populations and haplotypes. When there are few haplotypes the process is quite trivial. When there are many haplotypes and populations, a number of re-runs may be necessary. Measurements of band migrations, and loading the same sample at the edges and centre of the gel may be helpful in particularly complex situations. These investigations are not peculiar to SSCP analysis; for example, the underlying logic is the same as that accepted for decades in allozyme electrophoresis.

9 Sequence multiple representatives of each gel phenotype.

When gel phenotypes have been ascribed with confidence, replicate individuals are sequenced. The degree of replication is decided upon as in other scientific procedures, that is, it will reflect the degree of confidence desired, given the consequences of making an incorrect inference and the resources available.

In the examples we give here, unless specified, we sequenced double-stranded PCR products by automated or manual sequencing (manual technique in Sunnucks & Hales 1996) using the PCR primers referenced in the text.

10 Incorporate SSCP into an efficient and pragmatic screening programme.

SSCP is only one of a suite of techniques for detecting sequence differences. If certain haplotypes/alleles are difficult to distinguish and it is important that discrimination be achieved, rather than optimizing SSCP, it may be more efficient to use other approaches (e.g. sequence-directed restriction enzyme assays) in conjunction with SSCP.

Cutting bands from dried acrylamide gels and reamplifying

In a range of applications, it is often difficult but necessary to separate alleles, homologues or paralogues of identical length. SSCP offers a solution to this problem by separating PCR products on the basis of sequence — they can then be visualized and sequenced or reamplified if necessary (see also Ortí *et al*. 1997a). While we present a protocol for radioassay gels, E. Lessa (personal communication) has pointed out that DNA bands cut from silver-stained gels can be re-amplified by PCR, contrary to an earlier report (Lessa & Applebaum 1993).

We use a standard technique that works well for denaturing and nondenaturing gels: (i) Staple an X-ray film to the dried gel and expose by autoradiography. (ii) Remove the film and process it. (iii) Use the staple holes to align the film and gel. Mark the positions of bands of interest on the gel by pushing a needle though the film and gel. Excise the gel fragment. (iv) Soak gel fragment in 40 µL TE for 10–15 min, then ease the gel off the paper with clean forceps or a needle, and discard the paper. (v) Heat the gel fragment in the TE to 90 °C for 3 min. Use 1 µL of the solution as PCR template.

Applications and examples in population biology

The following examples from our recent projects encompass a wide range of questions and organisms. Minor or no modifications were made to the standard protocol given above.

1. Ensuring that intraspecific variation is adequately represented in phylogenetic sequencing projects

Phylogenetic approaches are used for a plethora of questions from biodiversity prioritization to phylogeography (e.g. Crozier 1997; Moritz & Faith 1998). For these applications intraspecific as well as interspecific variation should be quantified. However, intraspecific variation is often under-sampled, owing to time and expense. Particularly at lower taxonomic levels, this carries the risk that intrataxonomic variation swamps intertaxonomic variation. Application of SSCP presents a rapid and inexpensive method to ensure adequate representation of intrataxonomic sequence variation.

Cytochrome b *variation in* Macropus *wallabies.* We carried out a phylogenetic analysis of the distinctiveness of two marsupial species introduced to Kawau Island, New Zealand into sympatry: tammar and parma wallabies (*Macropus eugenii* and *M. parma*) (Taylor *et al*. 1999). Eight *M. eugenii* and seven *M. parma* from Kawau Island, representatives of both extant *M. eugenii* subspecies, and reference individuals of other taxa were subjected to PCR–SSCP for a ≈ 370 bp region of cyt *b* (Fig. 1, see legend for details). The patchy and thin distribution of Australian *M. parma*, and its threatened conservation status, make samples extremely difficult to obtain, and none could be screened. New PCR products and sequence were obtained using the widely applied 'conserved' primers L14724, Pääbo (1990) and H15149, Kocher *et al*. (1989) that produce a 490-bp fragment. The SSCP/sequence analysis

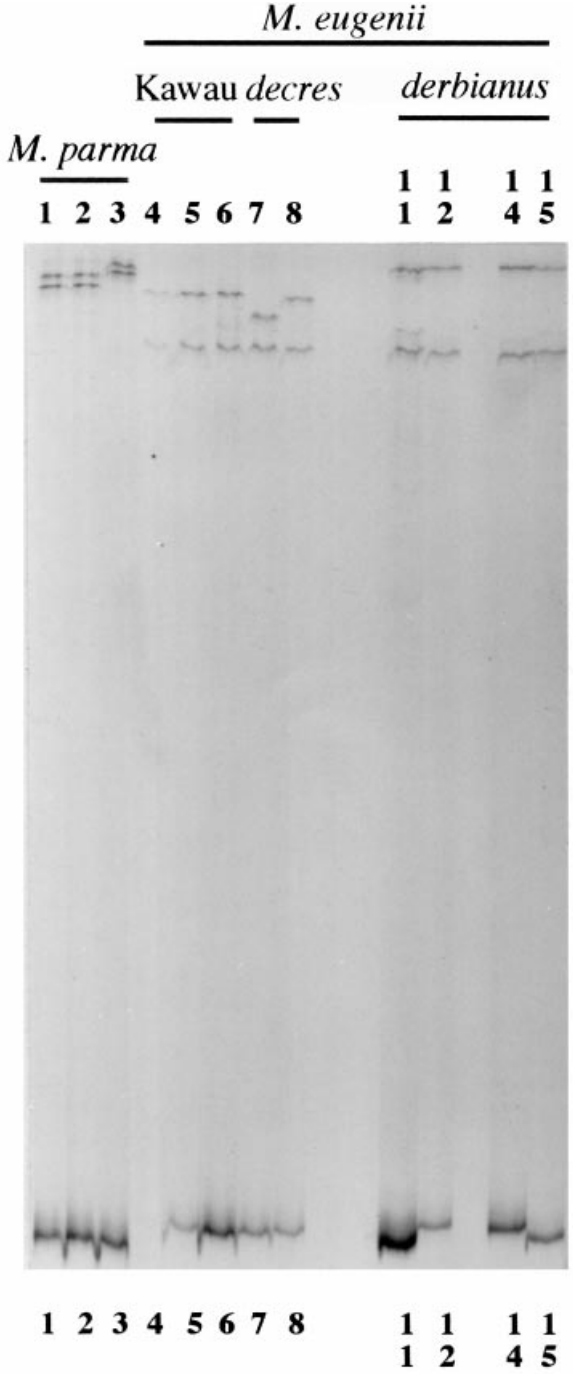

Fig. 1 PCR–SSCP autoradiograph showing a 370-bp fragment of mitochondrial cytochrome *b* (primers L14841 and H15149, Kocher *et al*. 1989 which gave similar SSCP results to the primers mentioned in the text), in various *Macropus* wallabies as indicated. Lanes 12 and 14 *M. eugenii derbianus* are from Garden Is. 'Kawau' and '*decres*' refer to the Kawau Is. (New Zealand) population and Kangaroo Is. subspecies, respectively, of *M. eugenii*. Intrapopulation variation is 1–2 bp, but easily detected. Running conditions, 15 W for 4 h, 29 cm gel.

revealed only slight intrapopulation variation. Two or three representatives of each SSCP phenotype were sequenced. SSCP easily detected small sequence differences; for example, the two *M. parma* haplotypes differ by two substitutions, and *M. eugenii* haplotypes from Kangaroo Is. at a single site (Fig. 1).

2. Screening large population samples for mitochondrial DNA (mtDNA) sequence variation

Uncovering and exploring unexpected patterns of sequence variation: mtDNA cytochrome oxidase I (COI) in the onychophoran Euperipatoides rowelli. Samples of *Euperipatoides rowelli* Reid (1996) were obtained from a continuous forest (details in Sunnucks & Wilson 1999; Sunnucks *et al*. 2000). A total of 285 *E. rowelli* were analysed for sequence variation in a 456-bp product of mitochondrial COI by SSCP using the commonly applied conserved invertebrate primers C1-J-1718 and C1-N-2191 (Simon *et al*. 1994). Twenty-seven haplotypes were distinguished and representatives sequenced. Seven of these were unique and were sequenced only once, nine were sequenced twice from the same individual, and eight were sequenced from between two and six individuals. Additional variants were never found by this repeated sequencing.

Mitochondrial DNA (mtDNA) diversity and divergence was remarkably high: up to eight haplotypes differing at 12.7% of sites were found in the same log, while microsatellite data indicate interbreeding on this spatial scale (Curach & Sunnucks 1999; Sunnucks & Wilson 1999). On the other hand, samples from some relatively large geographical areas had low haplotypic diversity. These complex patterns of diversity, divergence and heterogeneity would probably have been overlooked by a more traditional approach. Large-scale sampling of intraspecific variation may be particularly important for small, sessile organisms, which may show extremely high genetic divergence (see also Thomaz *et al*. 1996; Burton *et al*. 1999). SSCP greatly reduced the amount of sequencing necessary (40 samples sequenced compared to 285 if sequencing alone had been used), even in the face of high diversity.

Technical notes. Haplotypes with similar SSCP patterns were usually very similar in sequence. For example, haplotypes 1, 8, 10, 12, 13 and 17 differ by 2–3 bp (<0.7%), and are similar on the gel (Fig. 2). Some pairs look different but are similar in sequence (e.g. 18 and 25 only differ by 3 bp), but there were no outstanding instances of haplotype pairs that looked very similar while showing large sequence divergence.

As with most of our applications of SSCP, the two major banding systems on the gels were synergistically informative. For example, haplotypes 1 and 8 have similar

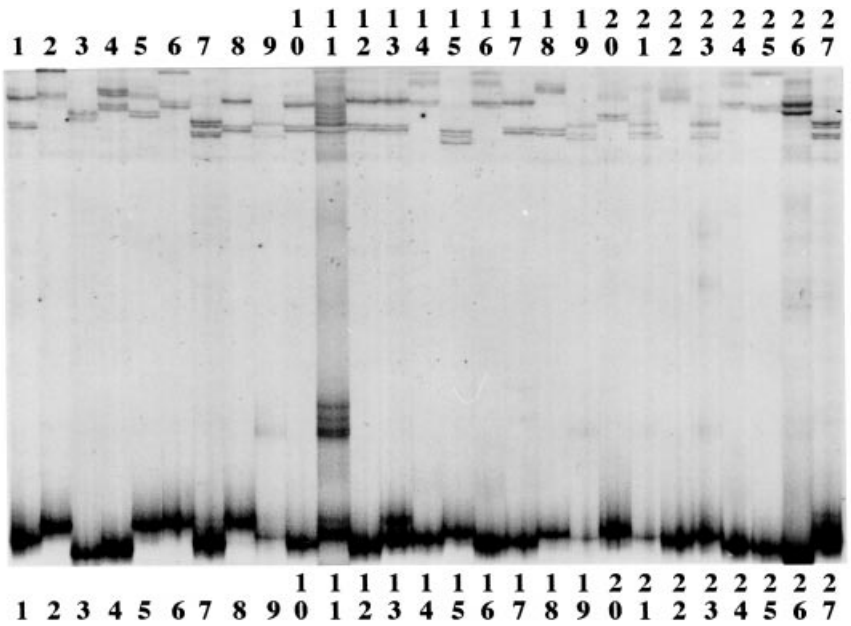

Fig. 2 PCR–SSCP autoradiograph showing a 456-bp fragment of mitochrondrial COI in *Euperipatoides rowelli*, showing the 27 haplotypes found to be different during population screening and confirmed by sequencing. Running conditions: 15 W for 4 h, 29 cm gel.

upper bands, but a large difference in migration of the lower ones: they differ by 3 bp. Conversely, haplotypes 6 and 8 have similar lower bands but different upper ones, and differ by 27 bp. The lane for haplotype 11 is overloaded, and it has a extra banding system of intermediate mobility. This may be double-stranded DNA that has not been denatured, but we have not confirmed this.

A small group of related sequences showed consistently pale phenotypes, even though they amplified strongly as visualized on agarose/ethidium bromide gels (e.g. # 9 on Fig. 2). Possibly incorporation of radionuclide into these sequences is disproportionately low, or high-mobility SSCP conformations are produced and lost under standard running conditions. In any case one should not assume that weak lanes indicate PCR failure.

mtDNA haplotypes that consistently show multi-banded lower systems of bands (e.g. haplotype 13 on Fig. 2) might be examined closely for the presence of multiple templates, as more than one band of this banding system per PCR template is unusual (see Discussion).

Finding a mtDNA marker with appropriate variation for a given taxonomic level: mtDNA control region and cytochrome b *in silverside fish* Odontesthes *complexes*. *Odontesthes* silverside fish provide an exciting model for studying biogeographic processes in South America (Beheregaray & Sunnucks 2000). For phylogeographic reconstruction, 180 *O. argentinensis* and 250 *O. perugiae* plus outgroups were screened by SSCP for variation in a 416-bp section of mtDNA control region, using primers 'D' and 'E' from Lee *et al*. (1995). On sequencing one or more exemplars of each SSCP pattern, extremely high diversity was revealed within these two taxa: 139 *O. argentinensis* sequenced revealed 63 haplotypes, and 120 *O. perugiae* yielded 89 unique sequences. Diversity was also high within populations, e.g. 20 haplotypes in 22 individuals from one population. Single substitutions were detected by SSCP in 75 pairwise haplotype comparisons, and different SSCP phenotypes always had different sequences (0.2–4.8%).

Phylogenetic analyses could not separate the most recently diverged groups, but it was possible to resolve relationships between the two species complexes and nearby *Odontesthes* species by sequencing the commonest SSCP phenotypes in each species. The pattern of marker utility could be determined in advance from the SSCP gels, so that, had the intermediate level of resolution not been of interest, the marker could have been abandoned and the sequencing effort saved.

A slower-evolving mitochondrial marker was needed for investigating deeper evolutionary relationships. A total of 130 *O. argentinensis* and 80 *O. perugiae* was screened for SSCP/sequence variation in 438 bp of the cytochrome *b* gene (primers L14724 and H15149, as for *Macropus* wallabies, above). In stark contrast to control region, cyt *b* showed only 18 gel phenotypes, and a small number of sequences (32) confirmed that the region was too invariant, yielding only 10 different *O. argentinensis* and eight *O. perugiae* sequences.

SSCP can be at least an order of magnitude more efficient than sequencing all templates: mtDNA control region of the European wild rabbit Oryctolagus cuniculus *in Australia*. As part of a study of the invasion biology and evolution of the European wild rabbit O. cuniculus in Australia (Zenger 1996), a 565-bp fragment of mtDNA control region was sequenced manually for 252 individuals. Seven different

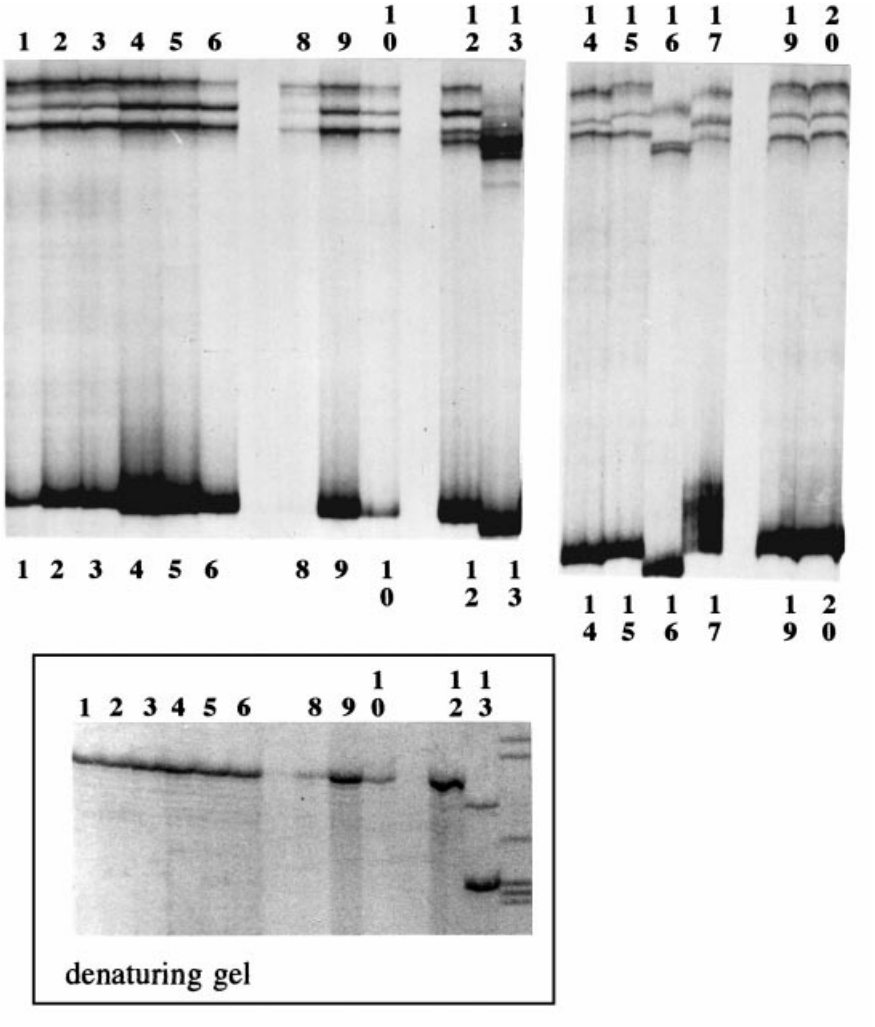

Fig. 3 PCR–SSCP autoradiographs (lanes 1–13 from one gel and lanes 14–20 from another) of a ≈ 220 bp fragment of intron EF1 α in aphids. Lanes 1–10 are *Sitobion miscanthi* group; 12, *S.* near *fragariae*; 13, unidentified aphid from Western Australia collected along with *Sitobion*; 14 *S. ibarae*; 15, *S. rubiphila*; 16, *Metopolophium dirhodum*; 17, *Macrosiphum euphorbiae*; 19–20, *S. miscanthi* group. Running conditions: 15 W for 2.5 h, 29 cm gel.Inset: Aliquots of the PCR products seen in PCR–SSCP lanes 1–13, re-run on standard denaturing gel (as used for microsatellites), with M13 sequencing ladder in the rightmost lane.

haplotypes were detected differing by one to 13 substitutions (primers dLl-588 5′-AGGCTCCTGCCCCACCAGC-3′, dLr-1254 ACATCCACAGTTATGTGTGAGC, designed from published rabbit sequence).

Subsequently, the same samples were screened using SSCP. All 252 individuals were allocated correctly to their haplotype. SSCP categorization required four SSCP gels and was completed in three days including re-running of samples for quality control. It would have been necessary to sequence only two representatives of the seven haplotypes (14 templates; one or two days' work using manual sequencing) rather than all 252. The complete sequencing approach using manual sequencing had occupied much of a one year MSc project. Thus, SSCP matched the resolution of complete sequencing, but was over an order of magnitude more efficient in time, labour, and resources.

3. *Developing and screening sequence-variable markers*

Nuclear intron variation: EF1a intron variation in Sitobion *aphids. Sitobion* aphids are useful models for studying the evolution of sex and parthenogenesis (e.g. Sunnucks *et al*. 1996; Simon *et al*. 1999; Wilson *et al*. 1999). Sequence variable markers are desirable for these projects. Conserved primers amplifying variable single copy nuclear (scn) DNA provide a source of potential nuclear marker regions (e.g. Friesen *et al*. 1997; Villablanca *et al*. 1998). However, each candidate must be assessed for sequence variation with high sensitivity, complications owing to paralogous copies must be excluded, and techniques for rapid screening are highly desirable. SSCP offers much in both marker development and screening.

SSCP has been effective in revealing variation in a ≈ 220 bp PCR product amplified by a pair of primers (EF1 and EF2, Palumbi 1996) for an intron in the elongation factor gene 1α (EF1α) of aphids (Fig. 3). For example, members of a functionally parthenogenetic set of *Sitobion* aphid genotypes can be ascribed to one of two species (Wilson *et al*. 1999). One species differs at only one site from the other in this EF1α region (e.g. lanes 1–10 vs. lane 12, Fig. 3). This difference cannot be assayed by any known restriction enzyme. Three representatives of one

SSCP phenotype and seven of the other have been sequenced, revealing no additional variation. Different phenotypes are seen in other *Sitobion* aphids (upper right panel, Fig. 3) and these always have different sequences.

SSCP analysis indicated that some species have multiple, nonallelic, but length-identical copies of the region (e.g. *Macrosiphum euphorbiae*, lane 17 on Fig. 3); this was subsequently confirmed by sequencing. This facility is likely to prove particularly useful in rapid development of scnDNA markers, as functional genes frequently arise by duplication and modification (Tomarev & Piatigorsky 1996). Alleles of identical length but different sequence can be separated by re-amplifying from dried SSCP gels. The most ready alternative, cloning, is more error-prone and expensive (Ortí *et al*. 1997a).

Technical note. PCR–SSCP product (obtained with equal concentrations of the four unlabelled nucleotides) run on denaturing gels (rather than nondenaturing, as required for SSCP) usually gives adequate resolution for screening length variation (Fig. 3, inset panel). In this example, an aphid with a distinct SSCP phenotype has an EF1α allele some 12 bp shorter than those of all known *Sitobion* aphids. It also has another PCR product in the same size range.

4. *Getting the most out of microsatellites: length homoplasy and sequence variation*

Allele length homoplasy (alleles with different evolutionary histories but having the same length, e.g. Ortí *et al*. 1997b) is a challenge to application of microsatellites in molecular phylogeography and systematics. SSCP offers an inexpensive and rapid approach to this issue, in that it reveals same-length alleles with different sequences. We have obtained informative SSCP patterns from most microsatellite loci we have tested (two examples follow).

Microsatellite length homoplasy in grey kangaroos. Two populations of grey kangaroos (*Macropus giganteus*) from southern New South Wales (NSW) and northern Queensland (QLD), Australia were tested for genetic differentiation using microsatellite loci. Most loci showed fixed differences between populations and a clear pattern of genetic distinction, but some loci had alleles that were apparently common in both geographical regions. SSCP screening of one such locus (Me15, Taylor & Cooper 1998) indicated sequence variation among same-size alleles from different populations (Fig. 4). Sequencing of an allele of the same length from NSW and QLD revealed nine substitutions in 233 bp, three of these within the repeat motif. Thus substantial sequence divergence in microsatellites was not reflected in length variation. Importantly, because one third of the substitutions created impurities within the repeat motif, the locus at the two geographical locations may evolve differently. Such sequence variation could have important implications for the application of mutation models and other assumptions about microsatellites.

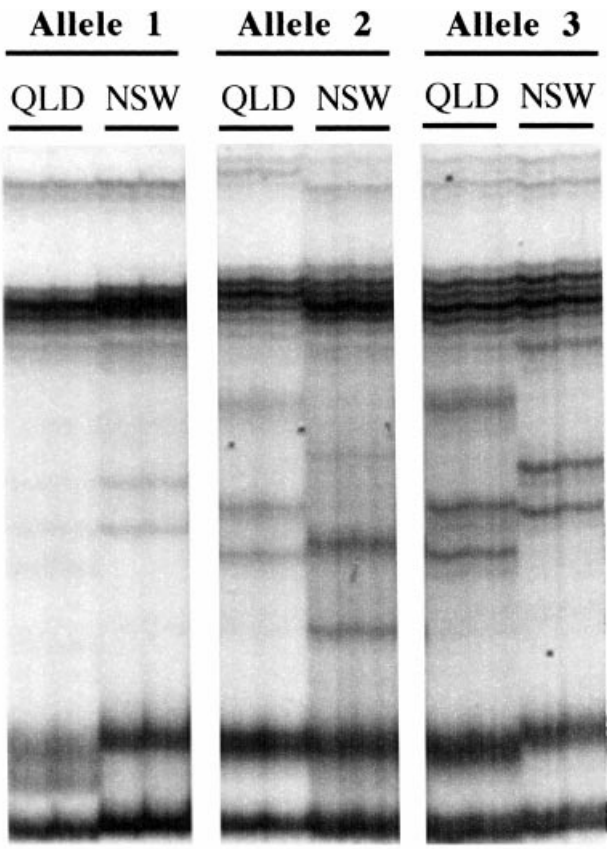

Fig. 4 PCR–SSCP autoradiograph indicating microsatellite length homoplasy (at microsatellite locus Me15). Three different allele lengths are shown: for each, two homozygous individuals from Queensland (QLD) and two from New South Wales (NSW) were compared. All three alleles appear to have different sequences at the two locations. This was confirmed by sequencing for Allele 1, at which the QLD version differs from the NSW one at nine nucleotide positions, three of which reduce the length of pure repeat.

We reiterate that in most cases, standard microsatellite PCR products give adequate SSCP patterns when run on nondenaturing gels: there is usually no need to repeat amplifications for SSCP. Thus, SSCP screening for microsatellite length homoplasy could be a routine part of microsatellite analysis (also suggested by Estoup & Cornuet 1999). Uncovering the proportion of alleles and loci harbouring undetected sequence variation will help to understand the risk of false inferences about population processes, and sequencing effort can be focused.

Sequence variation in length-invariant microsatellites: Sitobion *aphids.* For species with low microsatellite variation, a

major cost of developing loci is synthesis of primers that subsequently reveal little or no length variation. However, a proportion of these loci will contain useful sequence variation that can be detected by SSCP. We obtained variable SSCP patterns for some length invariant loci in *Sitobion* aphids. For example, locus S12 (A.C.C. Wilson & P. Sunnucks unpublished) showed three different SSCP patterns, confirmed by sequencing to differ at single sites. The relationships among aphids based on these data were consistent with earlier data on up to 14 microsatellite loci (Sunnucks *et al*. 1996; Wilson *et al*. 1999).

5. *Separating complex mixtures of same-length PCR templates: cloned pseudogenes or multigene families*

Nuclear sequence markers: unravelling length-identical 'alleles' in Sitobion *aphids*. Sequencing with arbitrary primer pairs (SWAPP) is an approach to generate scnDNA markers (Burt *et al*. 1994). SSCP can facilitate this by identifying regions that are sequence-but not length variable (Bagley *et al*. 1997), and by separating same-length PCR products.

We attempted to generate a SWAPP marker in *Sitobion* aphids, starting with a pair of randomly chosen primers that give complex variable banding patterns in *Sitobion* aphids (Gillings & Holley 1997). We targeted a band of ≈ 300 bp that amplified in the presence of both primers, which were used to sequence the region. The sequences revealed apparently useful intra- and interspecific variation, so we designed *Sitobion*-specific primers within the PCR product (sERICf 5'-AGAGAGAGCGAAGGTTTGCG-3' and sERICr GTTTGTAAACACCACCACACG). These produced a very clear single band of 275 bp product (called 'sERIC') on denaturing gels. However, SSCP and sequencing revealed that the primers amplify a series of length-identical but sequence-variable fragments (at least six different ones in some individuals). The sequences were consistent with a moderately variable open reading frame, plus a noncoding section.

SSCP was applied in two ways to try and identify true alleles from the mixtures of sequences within individuals. First, a random sample of cloned PCR molecules per individual were analysed by SSCP, under the assumption that heterozygous individuals would show two common alleles. The second approach assumed that the 'real' alleles would have higher melting temperatures than spurious products (this may not always be valid, but allelism can subsequently be tested in inheritance studies). Genomic DNA was subjected to PCR–SSCP at increasing annealing temperature (T_a). Only one or two alleles per individual were apparent at the highest successful T_a. PCR at this T_a was then used to try and match the SSCP patterns of alleles in genomic DNA to those from the different cloned fragments from the same individual (Fig. 5). This procedure seems to have worked.

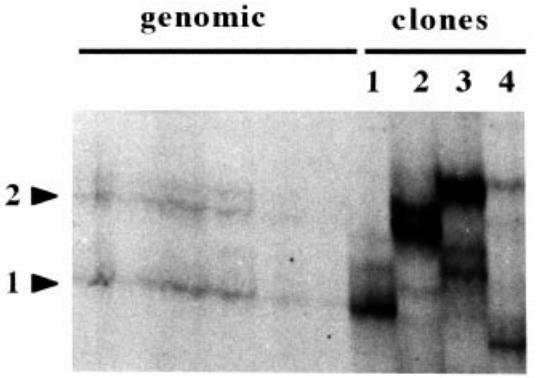

Fig. 5 PCR–SSCP autoradiograph comparing cloned and genomic 275 bp 'sERIC' fragments in aphids. The six lanes labelled 'genomic' were produced in high-stringency PCR from different individuals — all presented the same phenotype: band '1', and paired bands '2'. These bands putatively match clones 1 and 2, respectively. Running conditions 15 W for 11 h, 42.5 cm gel.

Under its assumptions, clones 1 and 2 are indicated as true alleles. Consistent with this, clone 1 is identical in sequence to products cloned from other closely related genotypes, and clone 2 is only 2.2% divergent from clone 1. The two sequences differ by six transitions and a deletion. Sites 42, 63 and 135 lie at third positions in codons within a putative open reading frame with stop codon. After the deletion (pos. 233) variant sites are not in-frame, consistent with a noncoding region. These two sequences lie in a parsimony cluster with strong bootstrap support (data not shown). The sequences were obtained in replicate by standard techniques from minipreparations, using primers to the plasmid into which the DNA regions were cloned.

Despite the apparent success of these novel applications of SSCP, we suspended work on sERIC as a genetic marker. In part this was because SSCP patterns of clones from the same aphid were more similar than those from different aphids, as confirmed by sequencing (data not shown), suggesting that clonal lineages were subject to concerted evolution.

mtDNA nuclear transposed copies. Multiple integrations of mtDNA into the nuclear genomes of organisms occur widely (review in Zhang & Hewitt 1996). In such cases, SSCP can be used to screen large numbers of individual cloned PCR fragments, and determine how many sequences are present. This may help to identify functional mtDNA; for example, in *Sitobion* aphids the most common sequence is the true mtDNA (Sunnucks & Hales 1996). More directly, individuals in a new project can be screened in advance of sequencing to gain an indication of whether nuclear integrations are present. If they are, gel phenotypes may be multibanded or smeary

compared to known single-copy material, but it should be noted that false negatives are common (S. Trewick personal communication). Recently SSCP has been used to detect and separate heteroplasmic copies from the same individual (Steel *et al*. 2000).

Technical note. We have found that boiling a small piece of bacterial colony (or cells from overnight culture) in 40 μL TE and using 1 μL of this as PCR template works well in SSCP–PCR.

Summary and conclusions

We have illustrated the application of SSCP under the following categories:

1 Ensuring that intraspecific variation is adequately represented in phylogenetic sequencing projects.
2 Screening large population samples for mtDNA sequence variation.
3 Developing and screening sequence-variable markers.
4 Getting the most out of microsatellites: length homoplasy and sequence variation.
5 Investigating complex mixtures of similar length sequences: pseudogenes and multigene families.

While this list is far from exhaustive, it demonstrates the wide range of uses to which SSCP can be put in population biology. We have presented results from a broad array of PCR primers for which SSCP has given very useful and clear information with little or no adaptation from a basic protocol using standard equipment. Many of the primers reported here are conserved ones in common use, and encompass vertebrate and invertebrate examples, mtDNA, nuclear introns and microsatellites.

SSCP is only one of a suite of sensitive techniques for assaying sequence variation (reviews in Lessa & Applebaum 1993; Girman 1996; Gasser 1997). Other common techniques include targeted restriction fragment length polymorphism (RFLP) analysis (e.g. Slade *et al*. 1993), heteroduplex analysis and its derivatives (e.g. Campbell *et al*. 1995), denaturing gradient gel electrophoresis (DGGE, e.g. Fodde & Losekoot 1994) and temperature gradient gel electrophoresis (TGGE, see Girman 1996). All these techniques have their advantages and disadvantages in terms of sensitivity, optimization, cost and requirements for equipment (e.g. some comparisons in Nataraj *et al*. 1999; Langefors *et al*. 2000). Overall, we find that SSCP presents a very favourable combination of characteristics that is not found in any other technique listed above (see also Girman 1996). Perhaps most importantly, sensitivity is high but no specialized equipment or reagent is needed, minimal optimization is required for most applications (although virtually complete sensitivity can be achieved if necessary, Liu *et al*. 1999), and a PCR product can be analysed for length and sequence variation, reamplified or isolated for sequencing (Girman 1996; Ortí *et al*. 1997a).

Acknowledgements

We are indebted to Jamie Foster for provision of the generic SSCP protocol. Enrique Lessa, Nick Campbell, Loren Reiseberg, several anonymous reviewers, Steve Trewick and Mary Morgan-Richards provided useful comments on earlier versions of the manuscript. We thank Valma Neville for bringing the dried band excision protocol to our attention, and Vianney Brown and Keith Herbert for photographic expertise. This is Key Centre for Biodiversity & Bioresources publication 307.

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This paper arose from cooperative research in a laboratory shared by several research groups at Macquarie University. Paul Sunnucks researches population biology primarily of invertebrates, and works with PhD students Alex Wilson (sex and parthenogenesis in aphids) and Luciano Beheregaray (molecular evolution of silverside fish). Many of the data from onychophora were obtained by Jordan French in a single semester Honours project. Andrea Taylor and PhD student Kyall Zenger have diverse interests involving the application of microsatellites and other markers to marsupial populations.
