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Mitochondrial D-Loop "Signatures" Produced by Low-Stringency Single Specific Primer PCR Constitute a Simple Comparative Human Identity Test

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Summary

We have developed a technique called "LSSP-PCR" (low-stringency single specific primer PCR) that detects single or multiple mutations in DNA. A purified DNA fragment is submitted to PCR by using a single primer specific for one of the extremities of the fragment, under conditions of very low stringency. The primer hybridizes specifically to its complementary extremity and nonspecifically to multiple sites within the fragment, in a sequence-dependent manner. A complex set of reaction products is thus created that, when separated by electrophoresis, constitutes a unique "gene signature." We here report the application of LSSP-PCR to the detection of sequence variation in the control (D-loop) region of human mtDNA, which is known to differ significantly between unrelated individuals. We prepared human DNA samples from blood and amplified a 1,024-bp portion of the mtDNA control region, using primers L15996 and H408. The amplified mtDNA fragments were then reamplified under LSSP-PCR conditions by using L15996 or H408 as drivers to produce complex signatures that always differed between unrelated individuals and yet were highly reproducible. In contrast, all mother-child pairs tested were identical, as expected from the matrilineal inheritance of mtDNA. Thus, the use of LSSP-PCR to produce D-loop signatures constitutes a powerful new technique for mtDNA-based comparative identity testing.

Introduction

Human mtDNA exhibits several characteristics that render it useful for human identification and establishing family relationships (Stoneking et al. 1991; Ginther et al. 1992). First, it is haploid, does not undergo recombination, and exhibits matrilineal inheritance (Giles et al. 1990). Second, it is generally homoplasmic in humans (Monnat and Loeb 1985). Third, it is highly variable, mostly in the 1,024-bp control (D-loop) region, which evolves fivefold faster than does the remainder of the molecule (Vigilant et al. 1989; Ward et al. 1991; Lundstrom et al. 1992). In this context, apparently unrelated Caucasian individuals are very rarely identical, differing on average in 7 nt, making mtDNA virtually matrilineage specific (DiRienzo and Wilson 1991; Ginther et al. 1992). On the other hand, within a matrilineage, the sequence is very stable (Ward et al. 1991; Lundstrom et al. 1992). The identity of an individual can thus be tested by comparison with any matrilineal relative. Fourth, mtDNA is very abundant, being present in thousands of copies per cell. This natural amplification is convenient when dealing with small or degraded samples (Handt et al. 1994). The main disadvantages of the study of mtDNA, as compared to nuclear genome markers, are that paternity cannot be tested and that it cannot be used to distinguish between members of the same matrilineage. Thus, although with limited application in the identification of criminals, it is extremely useful in the identification of victims of crimes or accidents.

We have recently developed a simple and rapid PCR-based technique for detecting DNA sequence variation that is both highly sensitive and informative (Pena et al. 1994*a*; Pena and Simpson, in press). It consists of submitting a purified DNA fragment to multiple cycles of PCR amplification in the presence of a single oligonucleotide primer ("driver"), specific for one of the extremities of the fragment, under conditions of very low stringency. The driver hybridizes with high specificity to its complementary extremity and with low specificity to multiple sites within the fragment, in a sequence-dependent manner. The reaction thus yields a large number of products that can be resolved by polyacrylamide gel electrophoresis to give rise to a multiband DNA fragment "signature" that reflects the underlying sequence.

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Changes as small as single base mutations can drastically alter the multiband pattern, producing new signatures. We have called this technique "LSSP-PCR" (low-stringency single specific primer PCR). LSSP-PCR is generally applicable to the detection of single or multiple mutations in any gene-sized DNA fragment and has been used for detection single base changes in human genetic diseases (Pena et al. 1994*a*, 1994*b*) and for the genetic typing of papilloma virus (Villa et al. 1995), *Leptospira,* and *Trypanosoma cruzi* (A. R. Vago, A. M. Macedo, R. P. Oliveira, E. Chiari, L. M. C. Galvão, D. A. Reis, A. J. G. Simpson, and S. D. J. Pena, unpublished results). We here describe the application of LSSP-PCR for the detection of sequence variation in the D-loop of human mtDNA. We demonstrate that the technique is simple, very sensitive, and reproducible and that different template sizes can be used efficiently. Thus, the study of mtDNA signatures with LSSP-PCR represents a valuable new comparative identity test for humans.

Material and Methods

PCR Amplification of D-Loop Fragments

Total genomic DNA was isolated from peripheral blood by digestion with SDS-proteinase K followed by phenol and chloroform extractions and isopropanol precipitation (Pena et al. 1991). The final DNA concentration was determined by agarose gel electrophoresis against known standards. DNA from hair follicles was prepared by the Chelex (BioRad) procedure (Walsh et al. 1991) and subjected to PCR without quantification. For most experiments, a 1,024-bp fragment, encompassing the entire mtDNA control region, was amplified using the primers L15996 (5′-CTC CAC CAT TAG CAC CCA AAG C-3′) and H408 (5′-CTG TTA AAA GTG CAT ACC GCC A-3′) as described by Vigilant et al. 1989. Alternatively, primer L15996 or primer L15926 (5′-TCA AAG CTT ACA CCA GTC TTG TAA ACC-3′) was used with primers H16197 (5′-TGA TTG CTG TAC TTG CTT GTA AG-3′), H16277 (5′-CAC CCC TCA CCC ACT AGG ATA-3′), H16347 (5′-ATG GGG ACG AGA AGG GAT TTG A-3′), H16401 (5′-TGA TTT CAC GGA GGA TGG TG-3′), H16498 (5′-CCT GAA GTA GGA ACC AGA TG-3′), or H580 (5′-TTG AGG AGG TAA GCT ACA TA-3′), to obtain different-sized fragments, as described in the Effect of Template Size section. The reaction mix consisted of 0.25 U of *Taq* DNA polymerase, 5 pmol of each primer, 50 μM dNTPs, 1.5 mM MgCl, 50 mM KCl, 0.1% Triton X-100, and 10 mM Tris HCl pH 9.0, in a final volume of 25 μl. The reaction program involved an initial 5 min denaturation followed by 30 cycles of annealing at 56°C for 1 min, extension at 74°C for 1 min, and denaturation at 94°C for 45 s.

LSSP-PCR

For the production of LSSP-PCR signatures, previously amplified D-loop fragments were run on 0.8% agarose gels (14 × 10 × 0.7 cm) by using TBE (89 mM Tris-borate, 2 mM EDTA, pH 8.0) at 90 V/cm for 1 h and stained with ethidium bromide. In the gel, standards with known concentrations of DNA were run simultaneously, so that the amounts of DNA in the bands of interest could be estimated. The band was then excised by punching out with a plastic transfer pipette. All procedures were performed with an ultraviolet transilluminator with emission at high wavelength (320 nm) to avoid DNA breaks. The volume of band aspirated was 100–150 μl. Since we had an estimate of the total amount of DNA in the gel band and knew its geometry, it was possible to calculate the concentration in the agarose plug. Throughout this procedure, strict chemical and bacteriological sterile conditions were maintained. All pipette tips were plugged (A.R.T. Tips, Promega) to avoid contamination. Samples of ~15 ng, still in the agarose plugs, were melted and mixed with the following reagents in a final volume of 10 μl: 1.6 U of *Taq* DNA polymerase (Promega), 48 pmol of primer L15996 (in the large majority of experiments) or H408, 50 μM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, and 10 mM Tris-HCl pH 9.0. Amplification was achieved by using 40 cycles involving denaturation at 94°C for 1 min, annealing at 30°C for 1 min, and extension at 72°C for 1 min. Ten microliters of each reaction were analyzed on acrylamide/bisacrylamide gels containing 6.% monomer and 3.3% cross-linking. The gels had dimensions of 17 × 12 × 0.3 cm and were run in TBE for 2 h at 110 V, being afterwards silver stained as described by Santos et al. (1993). Alternatively, fluorescent mtDNA signatures were achieved using fluorescein-labeled L15996 primer in a 25-μl reaction volume, of which 10 μl were analyzed by gel electrophoresis and 5 μl were studied in an automatic laser fluorescence (A.L.F.-Pharmacia), using a 6-h run on a denaturing 6% urea-polyacrylamide gel and default run conditions. The data from the run were then displayed with the Fragment Manager software (Pharmacia).

Three different thermocyclers were used in the LSSP-PCR experiments, all from MJ Research. Two were of the MiniCycler model, while the third was of the PTC-100 model, which has a slower ramp speed.

DNA Sequencing

DNA sequencing of region I of the D-loop of human mtDNA was performed exactly as described by Ginther et al. (1992).

Results

Optimization and Reproducibility

With the objective of establishing reproducible conditions for LSSP-PCR, we initially optimized five basic

parameters: amount of template, number of cycles, annealing temperature, magnesium concentration, and period of extension. The standard amount of template band for LSSP-PCR was 15 ng. However this amount could be varied at least five times either way without significantly affecting the resulting signatures. The number of cycles has a marked effect on the signatures up to 35 cycles, the pattern subsequently becoming highly stable (fig. 1*a*). On this basis, we elected 40 cycles as our basic procedure. Next, we verified the outcome of changes in the annealing temperature. Variations in the range 25°C–35°C had no marked effect on the signatures, but at >40°C there is a deterioration of the signatures (data not shown). Thus, we elected to maintain the annealing temperature of 30°C used in our initial report (Pena et al. 1994*a*). The concentration of Mg^{++} was varied from 0.5 to 6 mM and found to be optimal in the range 1.5mM–3 mM. In the original LSSP-PCR strategy we had used a simple cycle that had only an annealing temperature of 30°C and a denaturing temperature of 94°C—extension occurred in the ramp from 30°C and 94°C. However, we observed variation in the LSSP-PCR patterns obtained in different thermocyclers, for which we suspected that varying ramping speeds might be responsible. Thus, we introduced a 1-min extension period at 74°C, which altered the LSSP-PCR signatures (fig. 1*b*) but rendered them reproducible between thermocyclers (data not shown). With this optimization of parameters, mtDNA signatures were found to be highly reproducible with DNA obtained from different individuals of the same matrilineage and on different days, even when experiments were spaced over a period of 4 wk (fig. 2).

Variability

To evaluate the applicability of LSSP-PCR for individual identification, we used DNA from 30 unrelated individuals. We amplified the whole D-loop in a 1,024-bp fragment with the primers L15996 and H408, purified the product, and subjected it to LSSP-PCR using the primer L15996 in order to obtain a "signature" pattern. In all 30 cases, the signatures were different (fig. 3). D-loop signatures could likewise be obtained with primer H408, but L15996 gave a consistently more complex and more polymorphic banding pattern. Because of its position, L15996 is more likely to detect sequence variation in region I of the D-loop, which is more variable than region II (Ginther et al. 1992).

LSSP-PCR in D-Loops with Known DNA Sequence

The control region of human mtDNA is flanked by the proline and phenylalanine tRNA genes and contains two hypervariable segments named "region I" and "region II" (Vigilant et al. 1989). To ascertain more precisely the effect of sequence variation on the mtDNA

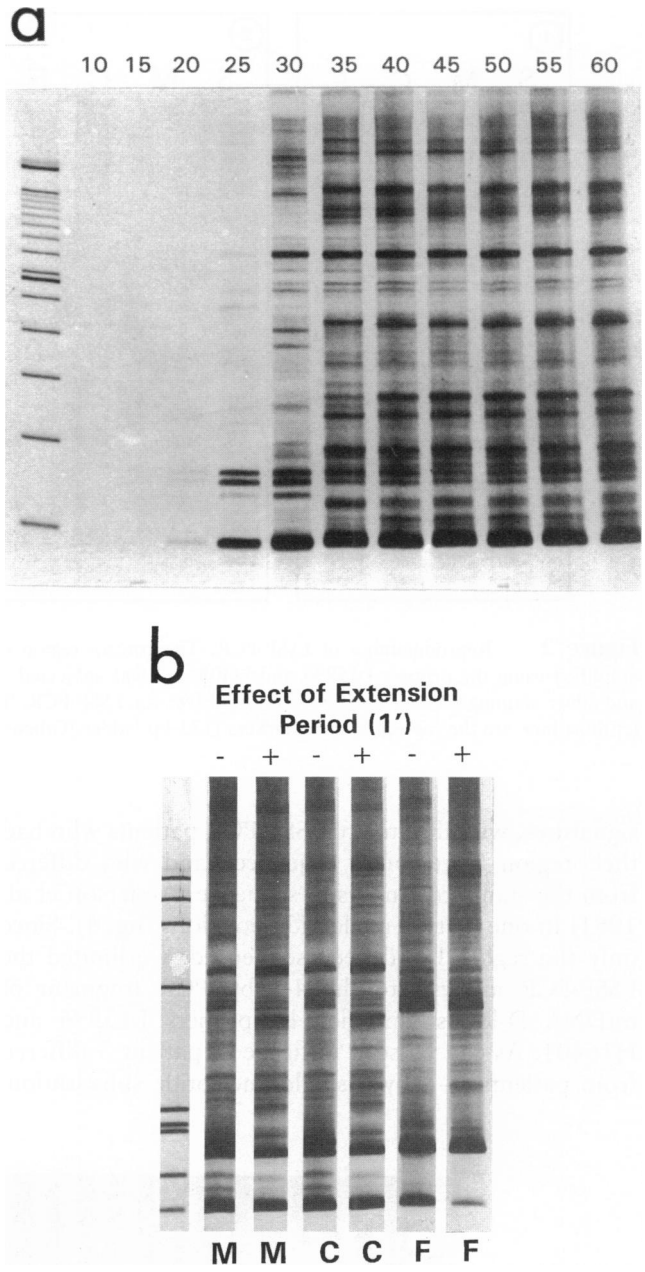

Figure 1 Optimization of LSSP-PCR. *a*, Number of cycles. The template was a fragment of 1,024 bp from the control region of human mtDNA amplified using the primers L15996 and H408. A single premix was prepared for the LSSP-PCR procedure and divided between 11 different microtubes. Primer L15996 was the driver for LSSP-PCR. After the indicated number of cycles, one tube was removed and analyzed on a silver-stained 6% polyacrylamide gel. In the leftmost lane are the molecular size markers (100-bp ladder, starting at 200 bp). *b*, Effect of extension period. The control region of the mtDNA of a mother (M)–child (C)–father (F) trio was amplified using the primers L15996 and H408 and was subjected to LSSP-PCR with (+) and without (−) an extension period of 1 min at 74°C. Afterwards, the reaction products were visualized on silver-stained 6% polyacrylamide gels. The left lane contains the molecular size markers (fragments of ϕX174 phage DNA digested with *Hae*III) with sizes of, from the bottom, 194, 234, 271, 281, 310, 603, 872, 1,078, and 1,353 bp.

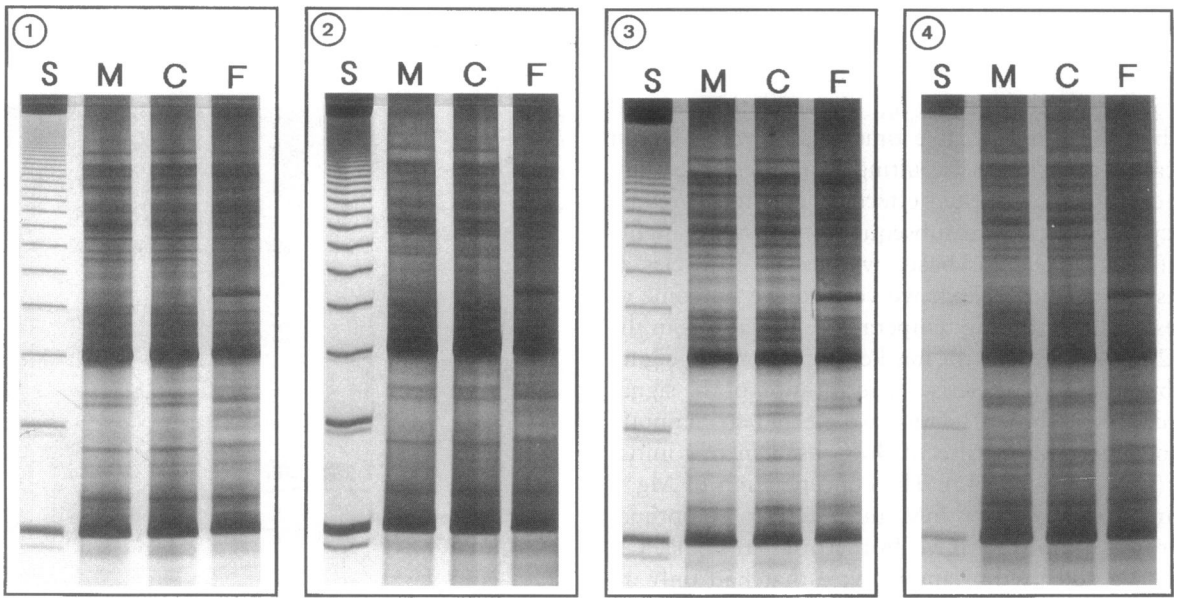

Figure 2 Reproducibility of LSSP-PCR. The control region of the mtDNA of a different mother (M)–child (C)–father (F) trio was amplified using the primers L15996 and H408 and was subjected to LSSP-PCR, followed by polyacrylamide gel electrophoresis in 6% gels and silver staining. Primer L15996 was the driver for LSSP-PCR. The whole procedure was repeated once a week for 4 wk (1–4). On the leftmost lane are the molecular size markers (123-bp ladder [Gibco-BRL], starting at 246 bp).

signatures, we subjected to LSSP-PCR patients who had their region I previously sequenced and who differed from the standard Anderson sequence (Anderson et al. 1981) in one or two nucleotide positions (fig. 4). Since only the region I had been sequenced, we limited the LSSP-PCR analysis to the 447-bp PCR fragment of mtDNA D-loops amplified by primers L15996 and H16401. As can be seen in figure 4, patient 3 differed from patients 4–6 by a single nucleotide substitution, patients within groups 1–3 and 4–6 differed by two mutations, and patients 1 and 2 differed from 4–6 by three changes. All LSSP-PCR signatures are unlike each other, and there does not appear to exist a clear correlation between the degree of sequence divergence and the differences between signatures. Likewise, there does not emerge any obvious relation between the position of the single mutation and the signature alterations. At any rate, this experiment demonstrates the high sensibility

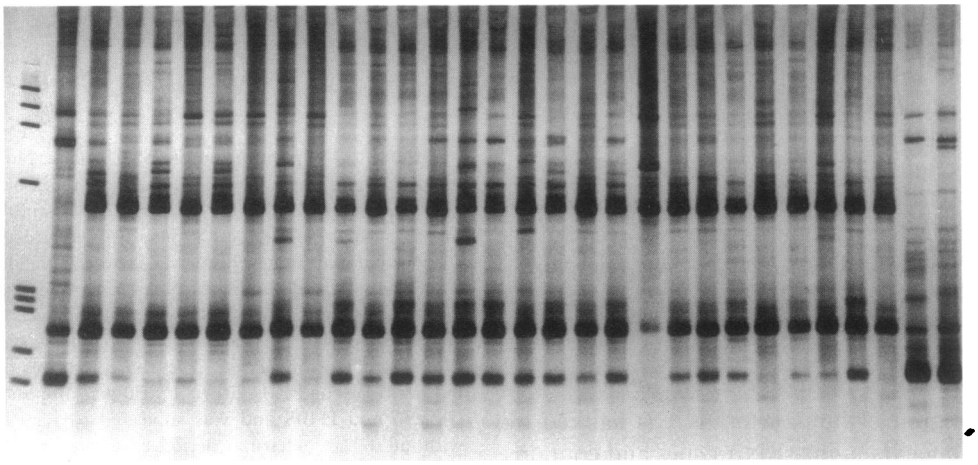

Figure 3 Silver-stained 6% polyacrylamide gel, showing the D-loop signatures obtained by LSSP-PCR in the control region of mtDNA of 30 unrelated individuals. Primer L15996 was the driver for LSSP-PCR. No two signatures are identical. In the first lane (left to right) are the molecular size markers (ϕX174 DNA digested with *Hae*III), from the bottom: 194, 234, 271, 281, 310, 603, 872, 1,078, and 1,353 bp.

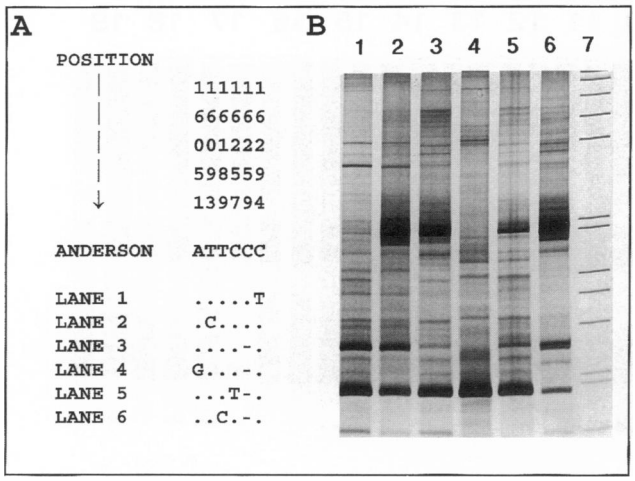

Figure 4 LSSP-PCR profiles of six unrelated individuals known to have different nucleotide sequences in their mtDNA. Since only region I had been sequenced, we limited the LSSP-PCR analysis to the 447-bp PCR fragment amplified by primers L15996 and H16401. On the left (*A*) are shown the nucleotide sequences of the individuals (numbered 1–6) in comparison to the standard Anderson sequence (Anderson et al. 1981). The electrophoretic profiles of individuals 1–6 are displayed on the right (*B*). Lane 7 corresponds to molecular size markers (1-kb ladder [Gibco-BRL]).

of LSSP-PCR for the detection of single base changes, as had been shown in previous publications (Pena et al. 1994*a*, 1994*b*).

Identity Testing

With the purpose of studying the applicability of LSSP-PCR as an identity test, we performed two stringent experiments. In the first trial, 38 blood samples constituting 19 mother-child pairs were tested blindly by LSSP-PCR with the objective of pairing them correctly. The results are shown in figure 5, where it can indeed be seen that in 18 instances the LSSP-PCR signatures of the mother-child pairs were identical. In one single instance (pair 6, fig. 5) there was the presence of an extra band in the mother (lane 12, arrow) that was, however, superimposed on a background of absolute mother-child identity. This is the first mother-child difference that we have observed in >50 pairs analyzed, and it was apparently an artifact, since it could not be reproduced in other experiments. In spite of this artifact, the pairing of the samples was absolutely correct. On the other hand, the fingerprints of all 19 pairs differ among each other, emphasizing the matrilineage specificity of the LSSP-PCR D-loop signatures.

In real-life situations, generally only one or a few evidence specimens are compared to several reference DNA samples, often of different tissue of origin. To mimic this situation in an experimental environment, DNA from 10 different individuals was prepared from hair samples. DNA was also isolated from blood drawn from two of these subjects and D-loop signatures produced for all 12 specimens. The test was to identify blindly the origin of the two blood samples. As shown in figure 6, all 10 individuals had different signatures, and the assignment of blood samples X and Y to individuals 1 and 4, respectively, is straightforward.

Effect of Template Size

In forensic case work one regularly has to work with degraded samples, and it may be convenient to work with fragments of the D-loop instead of with all 1,024 bp. To evaluate whether LSSP-PCR would be applicable to smaller templates, seven fragments of the mtDNA control region from the same individual, varying from 246 to 1,194 bp, were used to produce signatures. In all cases, the L15996 was used both as the upper primer in the primary amplification and as the driver in LSSP-PCR. As demonstrated in figure 7, signatures of considerable complexity could be obtained with fragments as small as 246 bp. An eighth fragment of 1,091 bp was used for a different test. It had L15926 as the upper primer in the first amplification and L15996 as the driver for the LSSP-PCR step. The signature obtained not only showed that internal primers can, as expected, be used for LSSP-PCR but that the signatures obtained were identical to those of the fragment initiated with L15996 (fig. 7).

Discussion

Our initial experiments with LSSP-PCR of the D-loop region of human mtDNA (Pena et al. 1994*a*) suggested that it had the necessary sensitivity to be used as an identity test. However, controlled experiments had to be performed to ascertain whether it had the required reproducibility. Throughout this work, all experiments were carried out with the utmost care to avoid any bacteriological or chemical contamination. Because of the very low stringency conditions, any extraneous DNA that contaminates the LSSP-PCR reaction will be amplified and confuse the results. Our data demonstrate clearly that, after optimization of the procedure, the reproducibility of LSSP-PCR was excellent, using identical templates from separate individuals (mother and child), distinct thermocyclers and undertaking amplification on different days, ≤4 wk apart (fig. 2). These results not only indicate that LSSP-PCR can be used as an identity test based on the simultaneous and direct comparison of two specimens but also raise the eventual possibility of establishing databases, if the D-loop signatures can be appropriately digitized.

The high sensitivity of LSSP-PCR is illustrated by the remarkable variability of D-loop signatures in unrelated individuals (figs. 3–5), some of which may differ by as

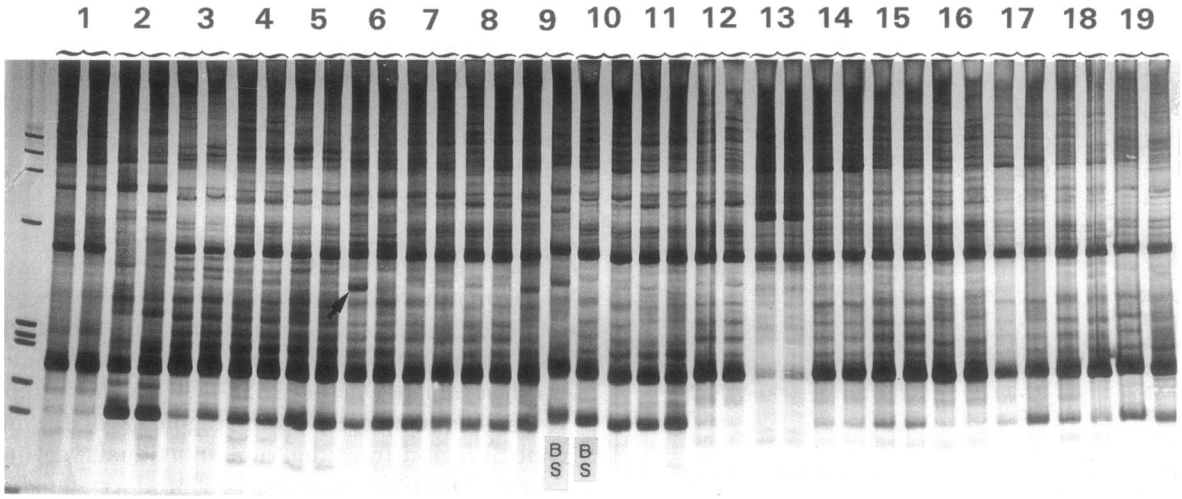

Figure 5 LSSP-PCR and identity testing. A silver-stained 6% polyacrylamide gel showing the D-loop signatures obtained by LSSP-PCR of the control region of mtDNA of 19 mother-child pairs (1–19). Primer L15996 was the driver for LSSP-PCR. All mother-child pair signatures were identical, except in the sixth pair (lanes 12–13), where the mother had an extra artifactual band (lane 13, arrow), which, however, did not prevent proper pairing. One of the individuals of pairs 9 and 10 had a band shift (BS). In the first lane (left to right) are the molecular size markers (ϕX174 DNA digested with *Hae*III): from bottom, 194, 234, 271, 281, 310, 603, 872, 1,078, and 1,353 bp.

little as a single base (fig. 4). To account for the variations observed, we must conclude that the interactions under the very low stringency conditions used are weak and unstable and that potential priming sites compete for available primers. This renders the amplification reaction exquisitely sensitive to alterations in template sequence so that the test becomes an extremely powerful and rapid means of sequence comparison in fragments up to the order of 1,000 bp. The observation that many bands are considerably larger than the template suggests that the PCR products of the first few cycles may themselves act as primers in further rounds of amplification. This can be seen figure 1*a*, where some low-molecular-weight bands clearly visible at 30 cycles subsequently disappear. Likewise, although small products are discernible on the gel from cycle 20 onwards, products of >1 kb appear only after 30 cycles. The PCR product primers may interact with the initial template or with other PCR products, the latter becoming increasing likely as their concentration builds with successive cycles. However, after 40 cycles no further alterations in the signature occur, probably due to depletion of reaction components or enzyme inactivation. It is clearly of importance in this application of PCR to drive the reaction well into the plateau to achieve the desired reproducibility. The great sensitivity of LSSP-PCR to initial conditions allied to its observed reproducible behavior are features of systems with self-organized criticality that evolve on the edge of chaos (Bak and Chen 1991).

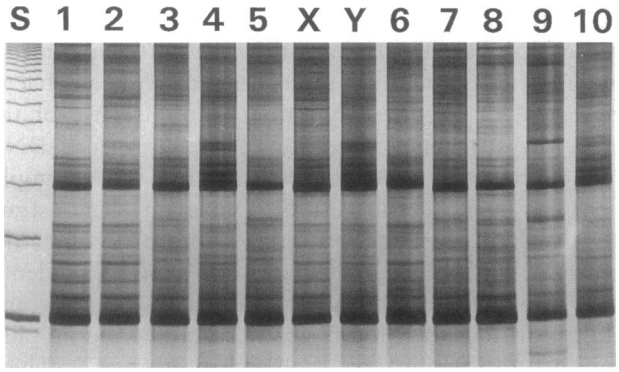

Figure 6 LSSP-PCR and identity testing. DNA from 10 different individuals was prepared from hair samples, and D-loop signatures were produced for all 10 specimens with primer L15996 as the driver (lanes 1–10). DNA was also isolated from blood drawn from two of these subjects, and D-loop signatures were likewise produced (lanes X and Y). The test was to identify blindly the individual origin of the two blood samples. It is clear that X and Y are individuals 1 and 4, respectively. On lane S are the molecular size markers (123-bp ladder [Gibco-BRL], starting at 246 bp).

As indicated in figure 6, the oligonucleotide driver used for LSSP-PCR need not bind to any of the two extremities, although in effect only the portion of the template 3′ to the primer appears to be effectively probed. Thus, any template can be analyzed with a very large number of possible primers, each of which will produce a distinct signature. We have not systematically searched for the most effective drivers for probing the human D-loop fragment, and our choice of L15996 was made empirically, on the basis of the fact that it produced more informative signatures than did H408.

The size of the DNA template being probed represents a further variable within the system. We show here that

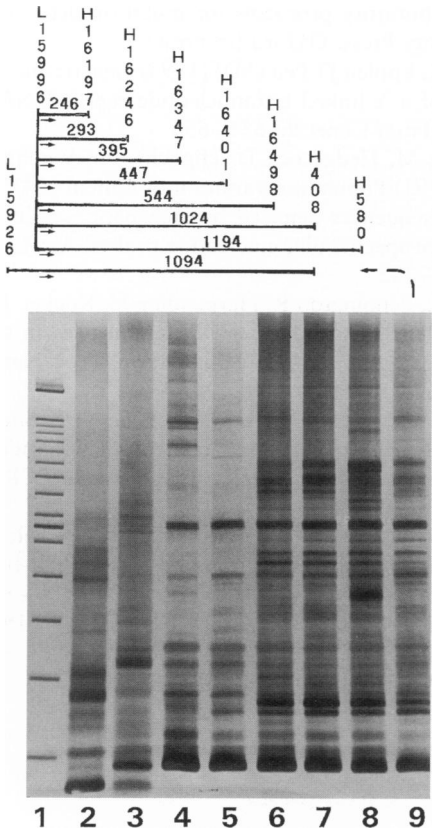

Figure 7 Effect of template size on D-loop signatures. D-loop fragments from the same individual were specifically amplified with L15996 and the lower primers indicated below especially designed to produce different product sizes as follows. Lane 2: L15996-H16197; product with 246 bp. Lane 3: L15996-H16246; product with 293 bp. Lane 4: L15996-H16347; product with 395 bp. Lane 5: L15996-H16401; product with 447 bp. Lane 6: L15996-H16498; product with 544 bp. Lane 7: L15996-H408; product with 1024 bp. Lane 2: L15996-H580; product with 1,194 bp. In lane 9 the initial amplification was performed with primers L15926 and H580, giving a product of 1,094 bp. All fragments, including the latter, were gel purified and subjected to LSSP-PCR with primer L15996, followed by electrophoresis in 6% polyacrylamide gels and silver staining. In lane 1 are the molecular size markers (100-bp ladder) starting at 200 bp.

the D-loop LSSP-PCR protocol that we developed for a 1-kb template appears to be highly adequate as a comparative identity test and that with the 447-bp region I fragment we can efficiently detect single base changes (fig. 4). Further reduction in size of the template results in some simplification and alteration of the subsequent LSSP-PCR signature (fig. 7). We presume that increased signature complexity corresponds to increased sensitivity to mutation detection. Therefore, length reduction may result in some loss of sensitivity, which would have to be balanced against the increased applicability of shorter fragments with degraded forensic samples.

Two different technical approaches have been explored in the past to exploit mtDNA D-loop variability for identity testing: DNA sequencing (Ginther et al. 1992) and allele-specific hybridization (Stoneking et al. 1991). Sequencing is without doubt the most informative procedure, but it is unfortunately still too cumbersome and expensive for routine use, except in sophisticated laboratories. Besides, the whole D-loop is too large for rapid analysis by present-day sequencing technology, since both strands of hypervariable regions I and II have to be sequenced separately. Allele-specific hybridization probes only predetermined variable positions in the D-loop and are also cumbersome, depending on the synthesis and radioactive labeling of 23 different oligonucleotide probes and on delicate stringency control of hybridizations. Compared to these technical approaches, LSSP-PCR is considerably simpler and very easily applied in any laboratory for testing purposes. For instance, in the identification of mortal remains in airplane crashes and other disasters, LSSP-PCR will quickly permit multiple comparisons to be made. Thus far we have limited our analysis of LSSP-PCR signatures to visual comparison. Although admittedly subjective, this criterion has worked well in several blind experiments such as those presented in figures 5 and 6. Of course, matches made on the basis of identical or almost identical mtDNA signatures can be further tested by DNA sequencing.

In conclusion, LSSP-PCR D-loop signatures are simple and inexpensive to produce, highly matrilineage specific, and reproducible. As such, they exhibit the characteristics required for sensitive and cost-effective mtDNA-based comparative identity testing.

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