Complete discrimination of six individuals based on high-resolution melting of hypervariable regions I and II of the mitochondrial genome

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BioTechniques 47:671-678 (August 2009) doi 10.2144/000113197
Keywords: high-resolution melting; HRM, mitochondrial DNA; mtDNA; genotyping; forensics

Supplementary material for this article is available at www.BioTechniques.com/article/113197.

Analysis of mitochondrial DNA in forensic samples is routinely carried out by direct sequencing of hypervariable regions within the non-coding displacement loop. Although the accuracy and sensitivity of this method cannot be questioned, it is both time-consuming and labor intensive. Finding a way to rapidly pre-screen forensic samples—prior to sequencing, to reduce the number of samples that need to be sequenced—would greatly benefit forensic laboratories. Herein, we describe an assay for discrimination of DNA from different individuals based on high-resolution melting analysis of the two hypervariable regions HVI and HVII of the mitochondrial genome. By clearly distinguishing the DNA melting curves of six different individuals, we show that this assay has the potential to function as a rapid and inexpensive pre-screening method for forensic samples prior to DNA sequencing.

Introduction

The identification of forensic material is routinely carried out by analysis of short tandem repeats (STRs) within nuclear DNA (1). However, in cases where the DNA is degraded or only available in scarce amounts, analysis of mitochondrial DNA (mtDNA) is often useful due to its high copy number per cell (2). Most of the mtDNA sequence variation is condensed within the hypervariable regions I and II (HVI and HVII) (3) of the non-coding displacement loop (D-loop). Forensic mtDNA identification is carried out by Sanger sequencing of HVI and HVII allowing detection of the single nucleotide polymorphism (SNP) variation occurring within these regions (4). Although useful and reliable, this method is laborious, time-consuming and expensive. A simple method to rapidly pre-screen crime scene DNA (to exclude suspects and reduce the number of evidence samples that need to be sequenced) would facilitate the process of forensic mtDNA analysis. Although methods for rapidly genotyping mitochondrial polymorphisms have been reported, these techniques are either destructive and open-tube (5), or require the use of costly allele-specific probes (6) or expensive mass spectrometers (7).

High-resolution melting (HRM) analysis is a novel, closed-tube method for rapid analysis of genetic variation within PCR amplicons (8). A wide range of HRM applications have been reported, such as SNP genotyping, mutation discovery, and DNA methylation analysis (9–11). The HRM technique was initially thought to require the use of a new generation of saturating DNA dyes (12); however, it was recently shown to work equally well with SYBR Green I, a non-saturating dye (13–15). In HRM-designated instruments, the decrease in fluorescence caused by the transition of dsDNA to ssDNA with increasing temperature is carefully monitored and, with the aid of tailor made analysis software, different genetic variants can be discriminated by their characteristic melting curves.

SuperConvection is a novel technology to minimize thermal heterogeneity within samples by inducing enhanced mass-transport in the reaction mixture (U.S. Patent no. 6783993) (16). We have implemented this technology into a new real-time PCR instrument, QuanTyper-48 (AlphaHelix Molecular Diagnostics AB, Uppsala, Sweden), along with a sophisticated in-tube temperature measurement system, which enables an exact control of sample temperature to further increase the sensitivity and accuracy of HRM.

Herein, we present superconvective HRM as a method for screening mtDNA variation in forensic samples prior to sequencing. Our objective was to develop an assay that allows for the exclusion of non-matching forensic material, thereby reducing time and cost of mtDNA sequencing, by discriminating between different individuals based on their characteristic high-resolution melting curves. This is a proof-of-concept study and more work will be done to further test the limits of the procedure.

Materials and methods

DNA samples

Genomic DNA. Genomic DNA from six anonymous Swedish blood donors—denoted B14, B15, B18, B20, C2, and C5—was used in the study. Blood samples were collected after informed consent. The DNA was isolated from whole blood using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). DNA concentration was measured using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and the samples were diluted to a final concentration of 10 ng/µL. The DNA samples were sequenced with respect to HVI and HVII using the Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

A/T SNP template DNA. Two oligonucleotide pairs, corresponding to the Y-chromosomal region NCBI36:Y:20327149–20327198, were synthesized to represent an A/T and a T/A genotype and were used as templates in the A/T SNP PCR.

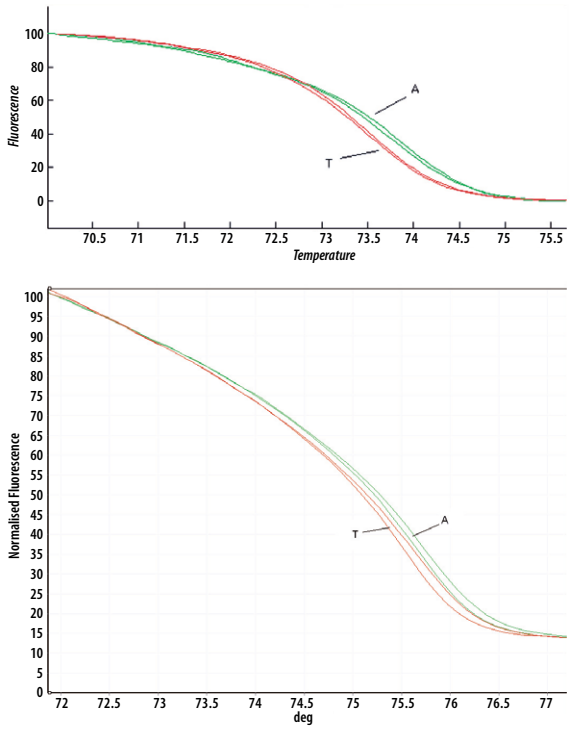

Figure 1. Discrimination of two amplicons differing by a single A/T conversion on two high-resolution melting (HRM) instruments. HRM curves resulting from amplicons containing either an A (shown in green) or a T (shown in red) genotype on the QuanTyper-48 (upper panel) or the Rotor-Gene 6000 (lower panel). Each genotype was analyzed in duplicates.

Table 1. PCR primer sequences

PCR	Primer name	Sequence	Amplicon length (bp)
A/T SNP	M45 F	AAATTGGCAGTGAAAAATTATA	50
	M45 R	AAGCTCCTTCTGAGGTCC	
HVII	II 45 F	ATGCATTTGGTATTTTCGTCTG	242
	II 287 R	TTGTTATGATGTCTGTGTGGAAAG	
HVI	I 16105 F	TGCCAGCCACCATGAATA	243
	I 16348 R	GACTGTAATGTGCTATGTACGGTAAA	

Primer design

A/T SNP PCR. Primers were designed to flank the Y-chromosomal SNP position NCBI36:Y:20327175 and amplify a 50-bp amplicon from the A/T SNP template DNA. The primer sequences are depicted in Table 1.

HVI/HVII PCR. HVI and HVII primer design is detailed elsewhere (17) and the primer sequences are summarized in Table 1. Briefly, primers were designed to flank highly polymorphic regions of HVI and HVII. The HVI amplicon is 243 base pairs in length and spans from position 16105 to 16348 of the mtDNA genome (18), while the HVII amplicon is 242 base pairs long and spans from position 45 to 287. HVII and HVI amplicon SNP sequences for each of the six individuals are summarized in Tables 2 and 3.

PCR reaction mixtures

PCR was performed in 20-µL reactions containing 1× PCR reaction buffer; 2.5 mM $MgCl_2$; 0.04 U/µL Platinum *Taq* Polymerase; 2 mM SYTO-9 fluorescent DNA dye (all from Invitrogen, Carlsbad, CA, USA); 0.4 µM each of forward and reverse primer (biomers.net GmbH, Ulm, Germany); 200 µM dNTPs (GE Healthcare, Uppsala, Sweden); and 10 ng genomic DNA (for the HVI/HVII PCR) or 1 nM of A/T-oligonucleotide (for the A/T SNP PCR).

PCR and HRM conditions

A/T SNP PCR. Real-time PCR was carried out either in a QuanTyper-48 superconvective thermal cycler or a HRM-enabled Rotor-Gene 6000 thermal cycler (Corbett Research, Mortlake, New South Wales, Australia). A program consisting of an initial denaturation of 95°C for 2 min, followed by 30 cycles of 95°C for 0 [5] s; 60°C for 3 [10] s; and 72°C for 8 [15] s was carried out (numbers within brackets are hold times for the Rotor-Gene 6000). The samples were subsequently subjected to a 65°C isotherm step for 10 [90] s followed by a temperature gradient from 65°C to 85°C heating 0.1°C/s continuously (QuanTyper-48) or 0.1°C per step with a 2-s wait at each step (Rotor-Gene 6000). Total run times (including amplification) were 19 min for the QuanTyper-48 and 72 min for the Rotor-Gene 6000. Each genotype was analyzed in duplicates and the experiment was repeated on two separate occasions with consistent results.

HVI/HVII PCR. Real-time PCR was carried out in a QuanTyper-48 or a Rotor-Gene 6000 with an initial denaturation of 95°C for 2 min, followed by 35 cycles of 94°C for 0 [5] s, 56°C for 3 [10] s and 72°C for 9 [15] s (numbers within brackets are hold times for Rotor-Gene 6000). The samples were subsequently subjected to a 70°C isotherm step for 10 [90] s followed by a temperature gradient from 70°C to 90°C heating 0.1°C/s continuously (QuanTyper-48) or 0.1°C per step with a 2-s wait at each step (Rotor-Gene 6000). Total run times (including amplification) were 22 min for the QuanTyper-48 and 80 min for the Rotor-Gene 6000. Each sample was run in triplicates for the HVII PCR and in duplicates for the HVI PCR. The experiments were repeated on two separate occasions with consistent results.

HRM curve analysis

The HRM data was analyzed using QT analysis software Version 1.03 and Rotor-Gene 6000 series software Version 1.7, respectively. Raw fluorescence data was subjected to normalization and temperature shifting in order to remove background fluorescence, make up for sample-to-sample variation, and aid visual interpretation and automatic grouping of similar melting curves (temperature shifting is not implemented in the Rotor-Gene 6000 software, thus Rotor-Gene data was only normalized). Normalization intervals of 2°C were set in linear regions before and after the melting transitions and the curves were rescaled from 0% to 100%. Automatic grouping of similar melting curves was done using a shape-matching algorithm within the analysis software.

Results and discussion

HRM analysis with QuanTyper-48 versus Rotor-Gene 6000

Since no HRM studies have previously been published for the QuanTyper-48, we wanted to benchmark its performance against one of the leading HRM instruments (19) and investigate the beneficial effects of the SuperConvection technology (U.S. Patent no. 6783993) (16), implemented in the instrument, on HRM.

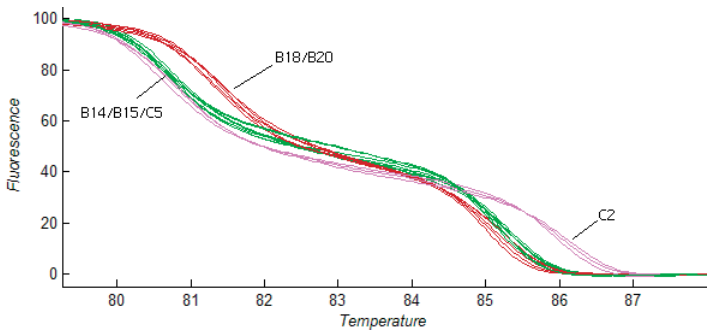

Figure 2. Discrimination of individuals based on the characteristic appearance of their HVII HRM curves. HVII type I curves representing individuals B14, B15, and C5 are shown in green; type II curves, representing B18 and B20, are shown in red; and type III curves, representing individual C2, are shown in pink. Each sample was analyzed in triplicates.

Table 2. Grouping of the six individuals based on HVII type I, II, and III curves and the HVII variation

HVII type	Individual	HVII nucleotide positions referring to revised Cambridge reference sequence										
		72	73	89	93	150	152	195	199	204	207	250
I	B14	T	G	T	A	T	C	T	T	T	G	T
	B15	T	G	T	A	C	T	T	T	T	G	T
	C5	T	G	T	A	C	C	T	T	T	G	T
II	B18	T	G	T	A	C	C	T	C	C	A	C
	B20	T	A	T	A	C	T	C	T	T	G	T
III	C2	C	A	C	G	C	T	C	T	T	G	T

HVII amplicon mtDNA sequences for each of the six individuals included in this study. Deviations from the revised Cambridge reference sequence (rCRS) (21) are highlighted.

The increased intra-sample homogeneity resulting from high-speed centrifugation and directional heating (collectively referred to as SuperConvection), along with unique technical features such as an in-tube temperature measurement system and a continuous heat gradient (contrasted by the stepwise heat gradients available in all other HRM instruments) could potentially enhance the quality of the analysis.

One of the most challenging tasks for an HRM-enabled instrument is the discrimination of amplicons differing by a single A/T SNP (9). The difference in melting temperature between A/T and T/A genotypes is approximately 0.1°C, and very precise instrumentation is required in order to separate their melting curves.

We compared the QuanTyper-48 to the Rotor-Gene 6000 using short amplicons containing either an A or a T variant that were subjected to HRM on both instruments (Figure 1). The two A/T and T/A variants could clearly be discriminated on the QuanTyper-48, showing a slightly better separation compared with the parallel result obtained on the Rotor-Gene 6000, indicating that superconvective HRM has a sensitivity and resolution that could improve HRM analysis.

Discrimination of six individuals based on HRM analysis of HVI and HVII amplicons

To test our hypothesis that DNA from different individuals could be distinguished based on the melting curves generated by the HVI and HVII amplicons, DNA samples from six human individuals were first sequenced with respect to the HVI and HVII regions and then used as templates in the HVI and HVII PCR reactions. All samples were readily amplified under the PCR conditions described, with cycle threshold (Ct) values of ~15–18 for the HVI PCR and ~18–22 for the HVII PCR, giving distinct single bands when examined by agarose gel electrophoresis (data not shown).

At first the HVII fragment was analyzed, as this region displayed a larger number of variable positions than HVI among the six samples we studied (variation in 11 positions for HVII in contrast to variation in 8 positions for HVI). The HRM curves derived from the HVII amplicon on the QuanTyper-48 were grouped into three distinct 'HVII types' by the HRM software (Figure 2). All curves contained two separate melting transitions, indicating that the amplicon consists of two distinct melting domains. HVII type I curves (represented by individuals B14, B15, and C5) and HVII type II curves (represented by individuals B18 and B20) could be distinguished from each other by the difference in the first melting transition. The HVII type III curve (represented by individual C2) could be distinguished from types I and II by its late second melting transition.

To be able to discriminate between individuals with HVII type I and type II curves, we analyzed the melting curves derived from the HVI amplicon (Figure 3). All three individuals that exhibited HVII type I melting curves could easily be resolved by their characteristic HVI profiles by the HRM software. The two individuals with HVII type II melting curves could equally easy be resolved by their respective HVI melting profiles. Grouping of the six individuals, based on HVII HRM curves, and their respective HVII and HVI SNP sequences, is detailed in Tables 2 and 3.

HRM analysis of the HVII amplicon alone allowed exclusion of 50–100% of the individuals (depending on what sample was considered the reference) with 11 out of 15 possible sequence combinations being discriminated against (Table 4). The combined use of HVI and HVII resulted in 100% exclusion of all six individuals. In a study by Divne et al. (20), DNA sequencing of HVI and HVII resulted in an exclusion rate of 70% in a material consisting of 90 forensic samples involving 16 cases, based on two or more base pair differences. Assuming that our HRM assay can discern two or more base pair differences (which it did in 11 out 13 cases; see Table 4) we anticipate roughly the same success rate, but this requires further testing. Regarding the correlation between nucleotide sequence and HVII curve type, it is apparent that multiple SNPs within an amplicon can compensate for each other, as is the case for individuals B14 and B15 having a T or a C, respectively, at position 150, followed by a C or a T, respectively, at position 152. As a result, they present identical HVII melting curves. It is also obvious that SNP variations in certain parts of the amplicon are more important than others in determining curve shape. Variations in the extremes of the amplicons presumably have a smaller effect on the melting curve shape compared with sequence variation in the more central part of the amplicon. Individuals B18 and B20 showed similar HVII melting curves even though they differ at seven SNP positions toward the ends of the amplicon. We assume that this somewhat limited resolution of HVII is

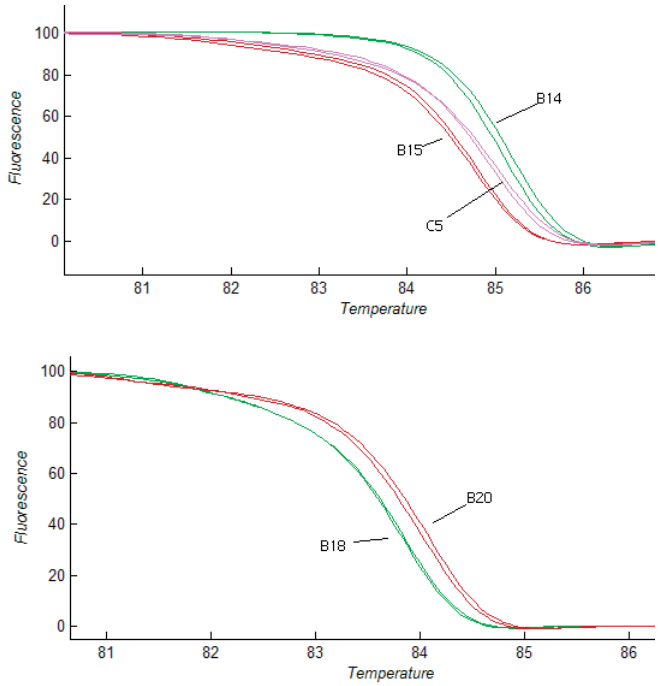

Figure 3. Further discrimination of HVII type I and II individuals based on the appearance of their HVI HRM curves. The upper panel shows melting curves of individuals B14 (shown in green), B15 (shown in red), and C5 (shown in pink). The lower panel shows melting curves of individuals B18 (shown in green) and B20 (shown in red). Each sample was analyzed in duplicates.

Table 3. HVI variation among five individuals

HVII type	Individual	HVI nucleotide positions referring to revised Cambridge reference sequence							
		16129	16132	16153	16223	16224	16298	16311	16347
I	B14	G	G	G	C	C	T	C	A
	B15	G	A	G	C	T	T	T	A
	C5	G	A	A	C	T	C	T	T
II	B18	A	A	G	T	T	T	T	A
	B20	G	A	G	C	T	T	T	A

HVI amplicon mtDNA sequences for each of the five individuals not excluded by HVII HRM analysis. Deviations from the rCRS (21) are highlighted. I and II (left column) refer to the HRM software assisted grouping of sequences, based on the HVII amplicon, into 'HVII types.'

an intrinsic property primarily of the 3′ domain of the HVII amplicon, rather than of the HRM technique. Nevertheless, resolution is achieved in one out two cases where the samples differ by two SNPs at HVII (Table 4). Furthermore, the HVI amplicon resolves individuals B18 and B20 based on a difference in two SNP positions.

Amplicons used for HRM should be long enough to contain sufficient sequence variation for discrimination but still be short enough to avoid overly complex melting behavior. It is possible that the resolution of the HVII amplicon could be improved by splitting it into two parts so as to divide the two melt domains into separate amplicons. Besides amplicon size, GC content also affects melting behavior, which is evident for HVI as well as the second (but not the first) melt domain of HVII, where higher GC content results in a melting transition at a higher temperature.

When analyzing mtDNA, it is important to consider heteroplasmy (i.e., when different mtDNA genome variants are present within one individual as a result of mutations), since it might complicate the interpretation of analysis results, for HRM as well as DNA sequencing. Length heteroplasmy is common in the 'poly-C stretch' of HVII. Thus, we have used a primer pair for HVII (Table 1) that does not include the poly-C stretch. In most cases, point heteroplasmies occur as single base pair differences at hot spots, but can be found at essentially any place within the mtDNA genome. Consequently, a careful primer design cannot circumvent this type of

Table 4. Number of SNP differences at HVII between the six different individuals

HVII	B15	B18	B20	C2	C5
B14	2	5	4	7	1
B15		5	2	5	1
B18			7	10	4
B20				3	3
C2					6

The color indicates whether the difference was (green) or was not (red) resolved by HRM.

heteroplasmy. We think, however, that this could be avoided if the assay is developed to have a detection threshold of ≥2 SNPs, which would not allow the detection of single base pair differences. Our HVII amplicon did not allow discrimination of individuals based on a single difference in two out of two cases (Table 4). This will be further optimized and evaluated on a larger number of individuals; if the threshold is ≥2 SNPs, we think it is fair to say that point heteroplasmy is not a problem. It is important to find a detection threshold that excludes as many non-matching samples as possible and that ensures that absolutely no matching samples are falsely excluded. As all matching samples are sequenced, false inclusions will be easily identified. The strategy of using at least two differences to exclude samples is also in line with interpretation guidelines for mtDNA sequence analysis in forensic genetics (21).

Our objective was to develop an assay for rapid pre-screening of mtDNA-variation in forensic samples prior to sequencing, allowing exclusion of non-matching samples. The results presented here indicate that the combined sequence variation occurring in HVI and HVII regions of the mtDNA does indeed generate satisfying differences in melting curve shapes for the discrimination of DNA from different individuals. Our study also shows the potential to increase the discriminatory power of HRM by combining information from two amplicons; this is highlighted by individuals B15 and B20, who share the same HVI sequence and could thus never have been separated by the HVI amplicon alone. This indicates a high resolving power of HRM at a low price and without much effort, compared with other techniques (5–7). However, since it is a proof-of-concept study, additional work is needed to further test the limits of our approach.

Prior to this study, three different approaches have been described for rapid genotyping of mtDNA sequence variation. The first one (5) combines restriction enzyme digestion of PCR amplified variable regions with ordinary melting curve analysis. This method is capable of

resolving amplicons differing by 3°C, a 30-fold lower resolution compared with the method we present here. The open-tube nature of this technique also increases the risk of contamination, which is particularly troublesome in forensic investigations. The second approach is based on the use of costly allele-specific hybridization probes (6,20). In one case, one probe is required per SNP and each SNP is analyzed as a separate PCR reaction (6), which represents neither a rapid nor non-laborious method. Probes can also be attached to a solid support such as in the case of the linear array assay for mtDNA variation (20). This assay is a rapid and informative test but requires 2–3 h post-PCR analysis. A third approach (7) relies on mass spectrometry (MS) and may work well as a screening method, but requires DNA amplification in a PCR instrument as well as expensive MS equipment on the range of from $125,000–400,000 USD. A combined qPCR and HRM instrument like QuanTyper-48 is in the range of $40,000–45,000 USD. Total hands-on time and analysis time differ: HRM analysis is done, without delay, directly after the amplification and takes 3–4 min in the QuanTyper-48. MS analysis requires a substantially longer time since the samples have to be transferred and loaded into the MS instrument, followed by the MS run and subsequent data analysis and interpretation. In the assay described here, several SNPs were studied per PCR reaction through the use of an intercalating dye, saving both time and money.

As a control, parallel runs were performed on a Rotor-Gene 6000. The curve shapes we obtained using a Rotor-Gene 6000 were quite similar, but grouping of HVII melting curves was not possible due to a somewhat larger spread between replicates on the Rotor-Gene 6000 (Supplementary Figure S1). HVI melting curves corresponded well between instruments (Supplementary Figure S2). The QuanTyper-48 features in-tube temperature measurement and increased intra-sample temperature homogenization through SuperConvection. As a result, the instrument allows for a continuous (non-stepwise) heating of the samples while continuously measuring fluorescence data at a high acquisition rate. We believe this is why the QuanTyper-48 exhibits a somewhat greater HRM resolution compared with the Rotor-Gene 6000.

HRM analysis represents a simple and cost-effective screening method for the detection of genetic variation within PCR amplicons. Since PCR amplification is a necessary part of analysis of forensic DNA samples, a HRM-based pre-screening assay could easily be integrated into the laboratory routine. Using HRM, a large number of crime scene samples can be screened simultaneously for identification of samples of interest for further DNA sequencing analysis. As this is a proof of concept study, more work will be done to further test the limits of the procedure.

Acknowledgments

The QuanTyper-48 is manufactured by AlphaHelix Molecular Diagnostics, Uppsala, Sweden. L.E. and M.M. are employed by AlphaHelix Molecular Diagnostics AB.

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Received 1 October 2008; accepted 29 April 2009.

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