Molecular Ecology Notes (2001) **1**, 325–328

TECHNICAL NOTE

Electrophoresis artefacts — a previously unrecognized cause of error in microsatellite analysis

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Abstract

While microsatellite genotyping has found wide application in many fields, a number of causes that could lead to error in microsatellite genotyping have been previously reported. Here we report another cause of error that we term 'Electrophoresis Artefacts' (EA), which arise when high concentrations of polymerase chain reaction (PCR) products are electrophoresed in ABI 377 automated sequencers. We describe the phenomenon of EAs, characterize PCR and gel running conditions that cause them and suggest a simple method of overcoming this problem.

Keywords: artefacts, error, gel-electrophoresis, genotyping, microsatellite, PCR

Received 15 December 2000; revision received 8 March 2001; accepted 26 April 2001

Microsatellite genotyping is prone to error through allele nonamplification from primer binding site mutation ('null alleles'), sampling stochasticity ('allelic dropout') and amplification artefacts ('false alleles'), resulting in false homozygotes and false heterozygotes (Schlotterer & Tautz 1992; Callen *et al*. 1993; Gagneux *et al*. 1997). We report another cause of microsatellite genotyping error that we term 'Electrophoresis Artefacts' (EA), which occur when high concentrations of polymerase chain reaction (PCR) products are electrophoresed in ABI 377 sequencers. As EAs are caused by high PCR product concentration, their occurrence is related to primer sensitivity and optimization of amplification conditions. EAs can cause two classes of genotyping error: (i) false identification of true homozygotes as heterozygotes (Fig. 1a); and (ii) incorrect allele assignment in true heterozygotes (Fig. 1b).

Class 1 errors are more common. In PCR amplification of DNA extracted from elephant blood, using 1 μL undiluted DNA extract and 25 cycles of PCR (see below for PCR conditions), we detected the occurrence of genotyping error from EAs in four of five loci analysed (Table 1). We have also detected EAs in a study of gibbons, using human microsatellite loci (unpublished data), suggesting EAs are not taxon dependant.

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Here we characterize PCR and gel electrophoresis conditions that produce EAs and introduce a simple method of overcoming them. We illustrate the problem using one tetranucleotide and two trinucleotide microsatellite loci in elephants, which were chosen in preference to dinucleotide loci, to eliminate confounding effects of false alleles (Taberlet *et al*. 1999).

DNA extraction from elephant blood followed a phenol/chloroform/isoamyl-alcohol and Qiagen column protocol (Fernando *et al*. 2000). DNA concentrations were estimated using a fluorometer (Hoefer Scientific Instruments TKO-100). Forward primers were labelled with fluorescent dye markers (Biosynthesis). Reactions were amplified in a Perkin-Elmer 9700 thermocycler at the appropriate temperature profile for each primer pair (Table 1), using 1 μL DNA extract, 2 μL 100 mg/mL BSA, 2.5 μL 10× PCR buffer (100 mM Tris-HCl pH 8.4, 500 mM KCl, 15 mM $MgCl_2$), 2.5 μL 8 mM dNTPs, 0.5 μL 10 μM primers, 0.1 μL *Taq* DNA polymerase (Perkin-Elmer), and 15.9 μL water. Reactions were amplified for 25 cycles of 1 min each at: the appropriate annealing temperature; 72 °C extension; and 92 °C denaturation. Cycles were preceded by a 93 °C 3 min incubation step and followed by a 72 °C 15 min extension step. Amplified products were electrophoresed on 5.3% 0.2 mm urea-acrylamide gels in an ABI 377 machine. Gels were polymerized for a minimum of 1.5 h. One μL amplification product and 1.0 μL formamide loading buffer were mixed with 0.4 μL TAMRA 500 internal size standard (Perkin Elmer), heated

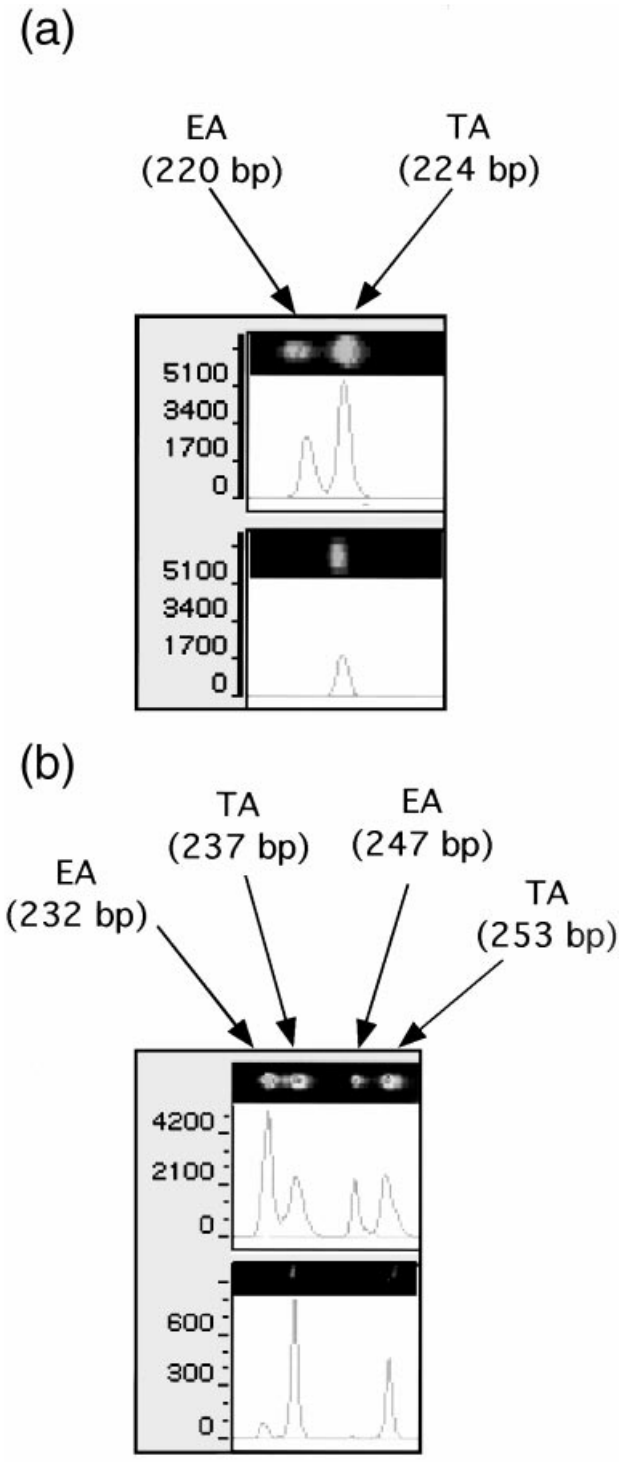

Fig. 1 Electrophoresis artefacts in two individuals. Insert of gel image — lanes correspond to peaks in electropherogram. Upper panel; undiluted polymerase chain reaction (PCR) product. Lower panel; 1 in 20 dilution of the same PCR product, electrophoresed under identical conditions. EA: electrophoresis artefact, TA: true allele. (a) Individual SLPI-01, locus EMX-2. Electrophoresis artefact leading to the identification of a false heterozygote. (b) Individual SLPI-15, locus EMX-3. Electrophoresis artefact leading to incorrect allele assignment in a heterozygote.

at 98 °C for 2 min and flash cooled on ice. Gels were pre-run for 1 h at 1 KV prior to loading 1 μL of the sample mix into each lane. Samples were electrophoresed at 3 KV for 2.5 h. Allele sizes were analysed using GENESCAN Version 3.02 (ABI). The criteria employed in scoring alleles were the default settings in GENESCAN, which included a Peak amplitude threshold of 50 and a Minimum peak half width of 3. A peak more than one third the height of the highest peak in the electropherogram was considered to represent an additional allele.

We conducted the following experiments to demonstrate the occurrence of EAs:

1 Amplification of a set of three samples over a range of template DNA concentrations (0.5, 1, 5, 10, 50, 100, 500 and 1000 ng/100 μL PCR reaction) and PCR cycles (20, 25 and 30) for each locus. A different set of samples were analysed for each of the three loci, for a total of 216 PCR reactions; and
2 Re-running 1 in 5, 1 in 10 and 1 in 20 dilutions of PCR products that previously produced EAs.

DNA template concentration and PCR cycle number. The 'optimal window' (detectable PCR product, no EAs) was related to template DNA concentration and PCR cycle number in that increasing cycles shifted the 'optimal window' towards decreasing DNA template concentrations (Fig. 2). For a given number of PCR cycles, template DNA concentrations corresponding to the 'optimal window' varied between loci and to a lesser degree between samples. Eliminating EAs by manipulating DNA template concentration or PCR cycle number requires locus-specific and possibly sample-specific optimization, hence it is an inefficient option in large-scale studies. Pre-PCR optimization of DNA template concentrations is also not possible where template DNA quality varies between samples and in the presence of nontarget DNA, as with suboptimal DNA sources such as dung.

Re-running dilutions of PCR product. One in 10 or greater dilution of PCR products that produced EAs when undiluted, eliminated or decreased EAs to levels that enabled accurate genotyping. Although PCR product dilution could be used to overcome EAs, it requires re-running gels and hence is an inefficient option in large-scale studies.

By empirical testing, we found pre-running gels at 3 KV for 1 h eliminated EAs or reduced them to levels that enabled accurate genotyping. All genotype calls from gels prerun at 3 KV matched those within the 'optimal window' and product dilution for the same individual. The physical properties of the gel and the concentration of labelled product electrophoresed likely determine the fraction of product in EA bands. Pre-running gels at 3 KV instead of 1 KV possibly causes physical changes in the gel that eliminate or reduce EAs.

Based on our observations, in the presence of high PCR yields we recommend pre-running gels at 3 KV for one

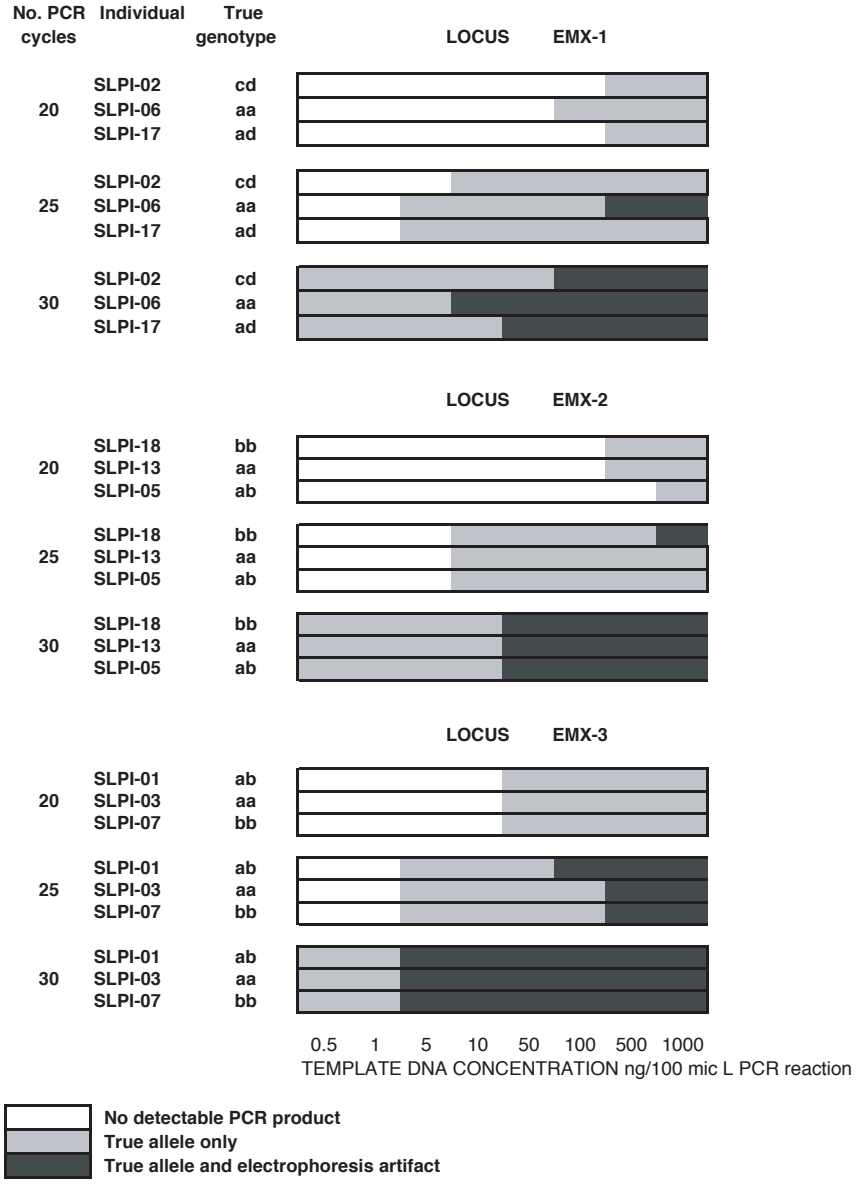

Fig. 2 Occurrence of electrophoresis artefacts at three loci, in three different individuals at each locus, with varying DNA template concentration and polymerase chain reaction cycle number.

Table 1 Prevalence of genotyping error due to 'Electrophoresis Artefacts' at five loci

Locus	Primers	T_a (°C)	No. samples analysed	No. samples yielding incorrect genotypes
EMX-1	F: AGGACTTATTTGCTTAGATGG	64	20	06
	R: AGGCAATGTTTCGTTCTGT			
EMX-2	F: CCCATGAGTCGGAATCCACTT	70	20	04
	R: CCATAGGGTTGCCAAGGAATG			
EMX-3	F: CATGGTTAACTCATTGCTTGC	64	20	05
	R: GTGTTCCCTCCCTCTCATCAT			
EMX-4	F: AGTTCGTGTCTCGGTGCTGTA	61	20	00
	R: GTATGCTGATGGAAATGTCTA			
EMX-5	F: AAATAGGAAAAGTCTGAGGTT	59	20	08
	R: CCCCTGGATTTTCTTCACCTG			

T_a, annealing temperature.

hour prior to sample loading when microsatellite genotyping with ABI 377 sequencers.

Acknowledgements

We thank Chandana Rajapakse, Ashoka Dangolla and LKA Jayasinghe for assistance in obtaining samples from Asian elephants, Senarath Gunasena and National Zoological Gardens Sri Lanka for permission to obtain samples from the Pinnawela Elephant Orphanage Sri Lanka, Jeff Hatcher, Beatriz M. Perez-Sweeney, Zoltan Takacs and Ximena Valderrama for samples and discussion, Danielle J. Whittaker for data on gibbons and Michael E. Pfrender and Per J. Palsbøll for comments on previous versions of the manuscript. This work was supported by grants from WWF US, WWF International, and USFWS to PF and DJM, and additional support from the Center for Environmental Research and Conservation (CERC).

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