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TECHNICAL NOTE

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Characterization of the *N*+3 Stutter Product in the Trinucleotide Repeat Locus DYS392

ABSTRACT: Stutter products generated during DNA amplification by the polymerase chain reaction (PCR) may complicate mixture interpretation. The PCR amplification of the DYS392 locus typically results in three distinct detectable PCR products: the true allele product (*N*), a stutter product three bases smaller (*N* − 3), and a reproducible low-level product, three bases larger (*N*+3). Sequence analysis of the *N*+3 product demonstrated that its sequence is one TAT repeat longer than the true allele product. Our experiments demonstrated that the quantity of both *N* − 3 and *N*+3 stutter increased as the allele number increased. The percent stutter also increased as the magnesium concentration was increased in the reaction, as well as when the amount of input DNA was decreased. As both stutter products behave in a similar and reproducible fashion, the same rules that apply to the interpretation of *N* − 3 stutter products in short tandem repeat analysis, can be applied to *N*+3 stutters. The characterization of the DYS392 *N*+3 product is the first detailed published study of a stutter product larger than the true allele.

KEYWORDS: forensic science, PCR amplification, short tandem repeat, stutter, Y-STR

The amplification by the polymerase chain reaction (PCR) of multiplexed short tandem repeats (STRs) is a widely used approach in forensic DNA analysis because it yields highly discriminating results with very small quantities of sample. However, one of the factors that can impact allele calling, particularly in mixtures with a minor component, is the presence of stutter products and their relationship to true STR alleles (1–4). Stutter products are artifacts generated during PCR amplification of STR loci and are usually one repeat unit smaller than the main allele peak (5,6). It has been proposed that the stutter peak is a result of *Taq* DNA polymerase slippage during DNA synthesis allowing more opportunity for misalignment of the repeats of both strands during PCR (5–8). In general, stutter products decrease as the number of base pairs in the core repeat unit becomes longer. Thus, stutter is displayed more so with di- and trinucleotide repeat loci, and to a lesser degree with larger repeat loci such as tetra-, penta- and hexanucleotide repeats (3,4,9,10). Because stutter products are primarily one repeat unit smaller than the expected main allele peak it can be difficult to differentiate an allele belonging to a minor contributor from a stutter product of an allele of the major contributor (2,11,12).

DYS392 is a polymorphic, Y-chromosome trinucleotide STR locus featured as part of the European minimal haplotype marker set (DYS19, DYS385a/b, DYS389I, DYS389II, DYS390, DYS391, DYS392, and DYS393) (13,14) and SWGDAM core Y-STR loci (15). Previous studies on the DYS392 locus (3,10) have reported the presence of an additional peak three nucleotides larger (*N*+3) than the expected main allele peak (*N*). In this study we confirmed the nucleotide sequence of this amplification product and characterized the behavior of this *N*+3 product relative to the true allele (*N*) and the *N* − 3 stutter product.

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Materials and Methods

DNA Samples

Anonymous DNA samples were purchased from Seracare Life Sciences (Oceanside, CA). The male control DNA 007 was purchased from the Serological Research Institute (Richmond, CA). DNA quantity was determined using the Quantifiler™ Y Human Male DNA Quantification Kit (Applied Biosystems, Foster City, CA) following manufacturer's recommendations (16).

PCR Amplification

Samples were amplified in 1 X AmpFℓSTR® PCR reaction mix (Applied Biosystems, Foster City, CA) (1.25 mM $MgCl_2$) supplemented with additional $MgCl_2$ to 1.60 mM. The 25 µL reactions also contained 2.5 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems) and 1 µM final primer concentrations. For all experiments, 1 ng of DNA was used per reaction unless otherwise indicated. Samples were amplified in MicroAmp® reaction tubes (Applied Biosystems) in the GeneAmp® PCR system 9700 with a gold-plated silver or silver block (Applied Biosystems). The standard thermal cycling conditions in the 9600 emulation mode consisted of enzyme activation at 95°C for 11 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°Cfor 1 min, and extension at 72°C for 1 min. A final extension was performed at 60°C for 45 min with a 4°C temperature hold if the PCR product was to remain in the thermal cycler for an extended period. Four PCR replicates were prepared for each sample.

Sequencing of DYS392 Bands

Male control 007 genomic DNA (10 ng) was amplified as described above using the unlabeled DYS392 primer 5′-AAAAGC CAAGAAGGAAAACAAA-3′ and Y392R51 (Table 1), resulting in a 113 bp fragment. After PCR, 5 μL of the amplified product were heat denatured at 70°C for 3 min in 2X TBE Urea Novex Sample buffer (Invitrogen, Rockville, MD), and then subjected to electrophoresis at 150 V on a precast 6% TBE-Urea Novex polyacrylamide gel (Invitrogen). DNA was detected with 1X Vista Green Dye (GE Healthcare, Milwaukee, WI). The main band and the regions immediately above and below it were carefully excised from the gel. The gel fragments were transferred to 500 μL of TE buffer and heated at 85°C for 10 min to elute the DNA. One microliter each of the eluates was reamplified with the same DYS392 primers described above and purified by another round of gel electrophoresis and gel elution. The eluted purified PCR products were cloned using the TOPO TA cloning® kit for Sequencing (Invitrogen,) as described by the manufacturer. The bacterial transformation yielded numerous colonies which were diluted 10^{-6}-fold in water, arrayed in a 96-well plate format and screened with the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). The plasmid DNA content of these colonies was amplified with primers Y392F51 and Y392R51 (Table 1). Two representative colonies containing products for each size ($N-3$, N, $N+3$) were selected for plasmid DNA isolation using a QIAprep Spin Miniprep kit (Qiagen, Valencia, CA). The sequence of the inserts of the purified plasmid DNA was determined in both directions with T3 and T7 primers using the Big Dye® Terminator v 1.1 kit (Applied Biosystems). The sequencing reaction was performed as described in the kit using *c*. 200 ng of plasmid DNA as input. Unincorporated dye terminators were removed using the DyeEx 2.0 Spin kit (Qiagen). The eluted DNA was vaccum-dried and resuspended in 20 μL deionized Hi-Di™ Formamide (Applied Biosystems). Samples were electrophoresed on the ABI PRISM® 3100 Genetic Analyzer using POP-6™ polymer on a 50 cm capillary array. The matrix file was generated using the E_BigDyeV1 set with the BigDye® v1.1 Terminator Sequencing Standard (Applied Biosystems). The samples were injected for 22 s at 1 kV and subjected to electrophoresis at 12.2 kV for 5000 s with a run temperature of 50°C. The data were collected using the ABI Data Collection software application v1.1. The sequences were analyzed using the DNA Sequencing Analysis software v5.1 (Applied Biosystems).

Primer Studies

PCR amplifications were performed as described above with four previously characterized DYS392 primer sets (Table 1) in which the forward primer was labeled with NED™ dye. The relative proportion of stutter product to the main allele peak was studied using DNA from six individuals selected based on their DYS392 repeat number differences (allele repeats ranged from 11 to 16). In addition, the NED dye label for all four primer sets in Table 1 was switched to the reverse primer and in the case of the Y392F50 primer sequence only, changed to a different dye (6-FAM™, VIC®, PET®), and the proportion of stutter formation was calculated. The experiments in Figs. 2–5 were performed by PCR amplifying each DNA four times.

Sample Electrophoresis and Data Analysis

Amplification products were separated and detected on the ABI PRISM® 3100 Genetic Analyzer. The matrix file (spectral calibration) was generated using a 5-dye system (6-FAM™, VIC®, NED™, PET®, and LIZ® standards). Sample preparations and electrophoresis on the ABI PRISM® 3100 analyzer occurred as follows: 1 μL of the amplified product and 0.3 μL of GeneScan™-500 LIZ® size standard were added to 8.7 μL of deionized Hi-Di™ Formamide (Applied Biosystems), denatured at 95°C for 3 min, and then chilled on ice for 3 min. Samples were injected for 10 s at 3 kV and subjected to electrophoresis at 15 kV for 1500 s in performance optimized polymer (POP-4™ polymer) with a run temperature of 60°C as indicated in the GeneScan36vb_POP4-DyeSetG5Module. The data were collected using the ABI PRISM® 3100 Data Collection Software application v1.1. Electrophoresis results were analyzed using ABI PRISM® GeneScan® Analysis Software v3.7.1.

Magnesium Chloride

The role that $MgCl_2$ concentration plays on stutter formation was studied at concentrations (1.25–1.95 mM) encompassing most Y-STR multiplex amplification protocols (3,10,17,22–24,26,27).

DNA Concentration

Twofold serial dilutions of male control DNA 007 were made from 1 ng to 125 pg. The analysis of the $N+3$ product at 125 pg was performed with a peak amplitude threshold of 25 RFUs (instead of 50 RFUs) because of the overall low peak heights.

Statistical Analysis

The proportion of the stutter product relative to the true allele was calculated by dividing the stutter peak height by the height of the true allele peak. The results are expressed as a percentage of the true allele peak height. Graphs are presented as mean ± standard deviation and when necessary analyzed using unpaired Student's *t*-tests assuming equal variances. $p<0.01$ was considered statistically significant.

The variation due to primer sequences and allele repeat number was analyzed by analysis of variance (ANOVA) using the statistical software Design-Expert v.6 (Stat-Ease, Minneapolis).

TABLE 1—*DYS392 primer sets and their characteristics.*

Primer Set	Primer Sequences	Range for 6–17 Repeats (bp)	Reference
Y392F50	NED-TCATTAATCTAGCTTTTAAAAACAA	233–266	(17–21)
Y392R50	AGACCCAGTTGATGCAATGT		
Y392F51	NED-AAAAGCCAAGAAGGAAAACAAA	92–125	(20,22,23)
Y392R51	AAACCTACCAATCCCATTCCTT		
Y392F52	NED-TAGAGGCAGTCATCGCAGTG	289–322	(20,24)
Y392R52	GACCTACCAATCCCATTCCTT		
Y392F51	NED-AAAAGCCAAGAAGGAAAACAAA	151–184	(25)
Y392R57	CAGTCAAAGTGGAAAGTAGTCTGG		

Results

Sequencing

Each amplification of the male control DNA 007, with the different DYS392 primer sets studied (Table 1), produced three products: the expected true allele and two additional minor products ($N-3$ and $N+3$) (Fig. 1). The range of minor products observed for 1 ng of male DNA in this study was *c*. 4–10% for $N-3$ and 1–3% for $N+3$, relative to the allele product. DNA sequence analysis of the main amplification band (N) determined the product to be a 113 bp sequence containing 13 TAT repeats. The DNA sequence of the $N-3$ band was 110 bp in size with 12 TAT repeats, and the $N+3$ band was 116 bp in size with 14 TAT repeats. No other sequence variation was detected between the main band and the minor products other than the change in repeat number. The flanking sequences were identical to previously described DYS392 sequences (Genbank AF140638).

Primer Studies

The amount of stutter product generated increased with allele repeat number regardless of the primer set used (Table 1, Fig. 2). The amount of $N-3$ stutter produced (panel A, Fig. 2) as a result of the PCR amplification was 2.5–4.4-fold higher than the $N+3$ stutter (panel B, Fig. 2). The statistical significance of variations due to primer set and allele number were measured with an ANOVA test using the stutter percentage as input. Table 2 shows the significance levels of the two factors but not the significance levels of the interaction terms because the values were not statistically significant (p values greater then 0.1). Both primer set and allele number were highly significant ($p<0.01\%$). From the calculated F-variance ratio, the $N-3$ allele number had the greatest influence on the stutter percentage (*c*. 4.2 times the effect of the $N-3$ primer set). The influence of allele number and primer set on variance was approximately equal in the $N+3$ stutter percentage. The stutter percentage for the Y392-F51+Y392-R51 primer set was *c*. 0.78% (in $N-3$) and 0.54% (in $N+3$) higher than the average percent stutter resulting from all the primer sets. This amount while statistically significant was a modest increase relative to the other primer sets. Similar trends were observed when the fluorescent dye label was switched to the opposite primer for all four primer sets in Table 1 resulting in a labeled reverse strand (data not shown). The effect of several fluorescent dyes (FAM™, VIC®, NED™, and PET®) on the forward primer sequence in the Y392-F50+Y392-R50 primer set was studied for $N+3$ and $N-3$ stutter formation but no statistically significant differences were detected (data not shown).

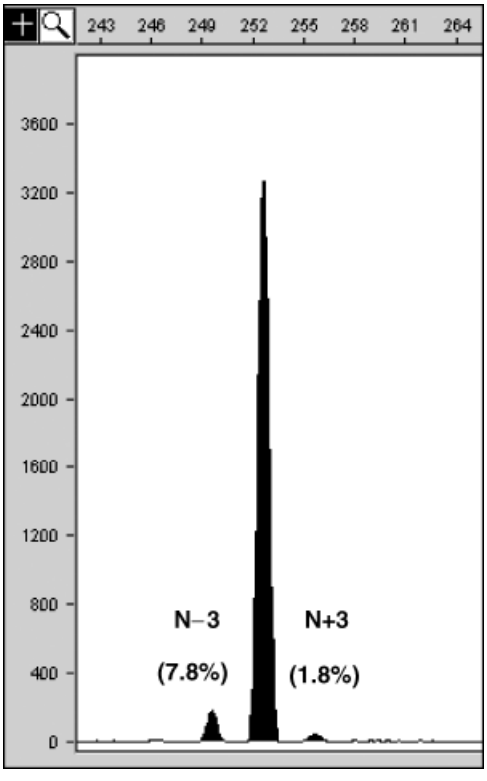

FIG. 1—*Representative electropherogram indicating the position of the $N-3$ and $N+3$ stutter products relative to the true allele product (N). Y392F50 and Y392R50 primers were used to amplify 1 ng of the male control DNA 007 as described in the Materials and Methods. The stutter percentages for this electropherogram are shown in parentheses.*

PCR Variables ($MgCl_2$ and DNA Concentration)

Magnesium concentration plays an important role in the fidelity of the *Taq* polymerase (28). As shown in Fig. 3, the amount of $N-3$ and $N+3$ stutters (as % peak height of the true allele) increased in a direct relationship within the range of magnesium chloride concentrations examined. Similarly, DNA input affected stutter amounts. However, an inverse relationship was noted between the input DNA and the stutter percentage (Fig. 4).

Discussion

The experiments described in this report aim to address the identity and conditions for generation of the DYS392 $N+3$ product. The characterization of the DYS392 $N+3$ product is important because this stutter could complicate interpretation of results, particularly from DNA mixtures that can occur with forensic casework samples. Furthermore, DYS392 appears to be the only STR in widespread use which reproducibly generates a stutter product larger than the true allele. DNA sequencing analysis of the $N+3$ product revealed that it contains one additional TAT repeat relative to the main PCR product, supporting that this is a stutter artifact. A mechanism involving a deletion caused by forward slippage has been proposed for the formation of stutter products smaller than the true allele (5,29). Similarly, although less frequently, an insertion caused by backward slippage could generate stutter products longer than the true allele (30).

The conditions for generation of the stutter products in the DYS392 locus were characterized by examining the potential influence of primer sequences, fluorescent dye labels, repeat length, $MgCl_2$ concentration, and amount of DNA input. Our results indicate that the proportion of N–3 and $N+3$ stutter relative to the true allele was primarily affected by the latter three variables with a very small effect produced by primer sequence. Consistent with previous STR studies, the stutter percentage increased proportionately with an increase in repeat length (3,5,10). Similarly, the proportion of stutter relative to the main allele product increased when the magnesium concentration was increased in the reaction. These results are consistent with a study (31) that described an increase in *Taq* polymerase slippage with an increase in magnesium concentration. The amount of DNA template amplified has direct bearing on the peak height of the final main product of the PCR. Because the stutter product is not present in the input DNA, the variation in proportion of stutter relative to the true allele was monitored as the input DNA was varied. Interestingly, the pro-

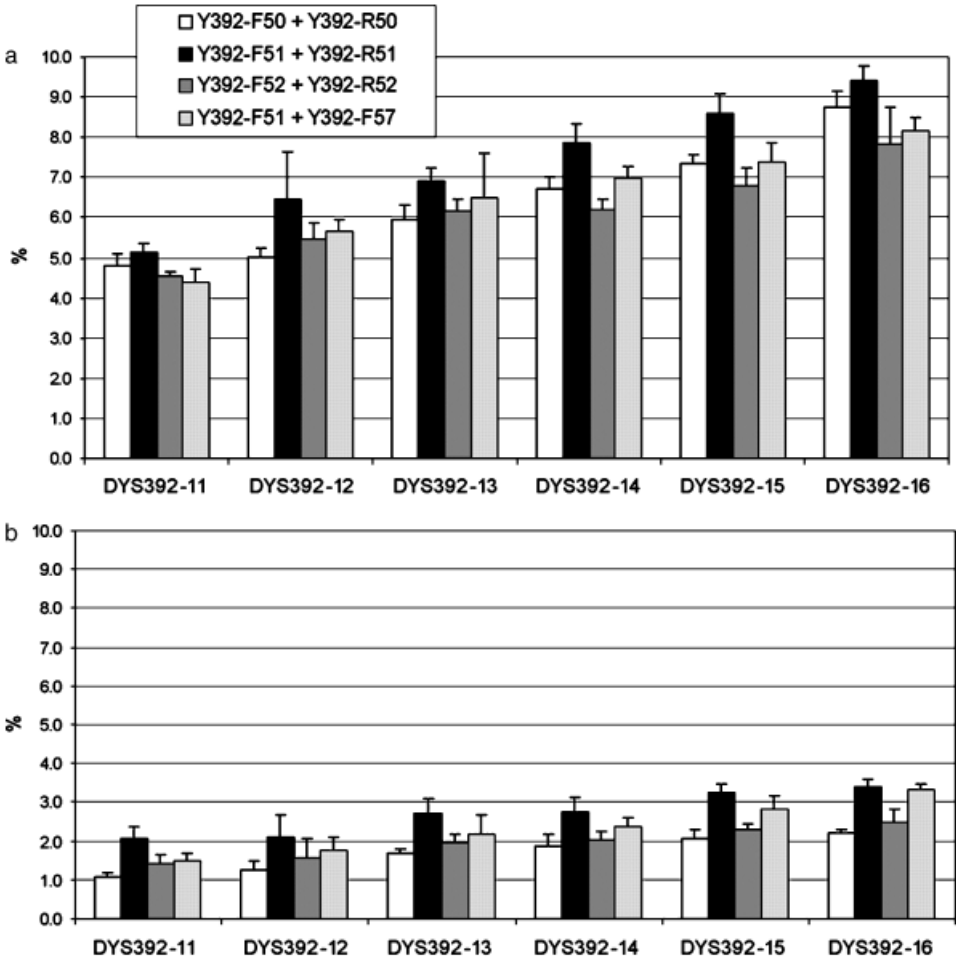

FIG. 2—*Comparison of stutter percentages observed for six DYS392 alleles each amplified with four different primer sets (Table 1). Panels A and B describe N − 3 and N+3 stutter, respectively. Y-axis units are shown as stutter amounts based on the percentage of the stutter peak height relative to the allele peak height. The values are expressed as the mean ± SD (N = 4).*

portion of stutter increased as the amount of input DNA in the reaction was decreased. A similar increase in the allelic stutter proportion has been noted (32) during amplification of low copy number amounts of DNA. Possibly, higher DNA concentrations sequester free $MgCl_2$ decreasing slippage of the *Taq* polymerase, therefore lowering stutter formation.

In conclusion, our results indicate that the formation of the $N+3$ product is reproducible and displays similar behavior to the $N-3$ stutter product, although its percent stutter is lower than that of the $N-3$ product. Because both stutter products behave in a similar and reproducible fashion, the same rules that apply to the interpretation of $N-3$ stutter products can be applied to $N+3$ stutters. Manufacturers of STR amplification kits and analysis software often include stutter filter thresholds to provide guidance for data analysis interpretation. However, each laboratory should conduct internal validation studies with single source samples to establish levels for mixture interpretation to verify that the filter threshold settings in the software analysis program are appropriate for their particular data analysis needs (33).

TABLE 2—*Variation attributable to primer set and allele number following an ANOVA test using stutter percentage as the variable factor.*

Effect	SS	df	MS	*F*	*p*
N+3 primer set	13.58	3	4.53	55.12	<0.0001
N+3 allele number	20.93	5	4.19	50.96	<0.0001
N–3 primer set	20.42	3	6.81	24.66	<0.0001
N–3 allele number	144.28	5	28.86	104.58	<0.0001

SS, sum of squares; df, degrees of freedom; MS, mean square; *F*, ratio of variances; *p*, probability of obtaining a specific result, given the null hypothesis.

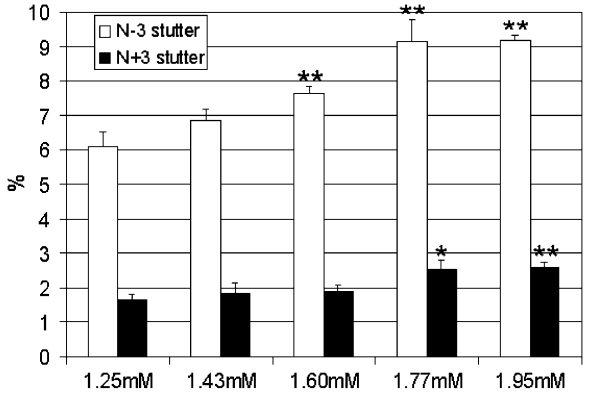

FIG. 3—*Increased magnesium chloride concentration affects stutter formation. Data are from DYS392-13 amplified from the male control DNA 007. Y-axis units are the stutter amount, expressed as a percentage of the allele peak. Values are expressed as mean ± SD (N = 4). $^{*}p<0.01$ and $^{**}p<0.001$ compared with 1.25 mM $MgCl_2$.*

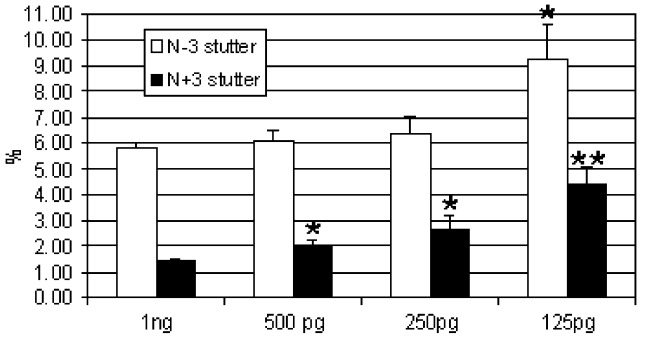

FIG. 4—*Decreased DNA input affects formation of stutter. Twofold serial dilutions were made of the male control DNA 007. Y-axis units are the stutter peak amount, expressed as a percentage of the allele peak. Values are expressed as mean ± SD (N = 4). $^{*}p<0.01$ and $^{**}p<0.001$ compared with the 1 ng template.*

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