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TECHNICAL NOTE

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Fungal DNA Challenge in Human STR Typing of Bone Samples*

ABSTRACT: The present study focuses on possible cross-reaction of fungal DNA with human STR primers that may affect subsequent forensic DNA analysis of forensic samples. Specificity of human STR markers namely HUMAMEL, HUMCSF1PO, D8S306, HUMTH01, HUMvWA, HUMFES/FPS, HUMF13A01, HUMDHFRP2, HUMFGA and HUMTPOX was tested using DNA of 24 different filamentous fungal isolates obtained from exhumed bone samples.

The specificity of these ten STR markers for human DNA was demonstrated. Presence of non-human DNA in five bone samples analyzed did not alter scoring of detected alleles. Notably, amplification was inhibited in the presence of a high proportion of fungal DNA compared to human DNA (1000 ng: 1 ng) in DNA mixture experiments. The results of the present study underscore the importance of carefully analyzing the presence of non-human biological contaminants that may affect DNA typing of environmentally challenged forensic samples to avoid spurious data interpretation.

KEYWORDS: forensic science, DNA typing, validation, short tandem repeats, fungal challenge

There are several methods used to type human DNA. Currently, short tandem repeat (STR) typing is the most accepted procedure for DNA profiling among forensic laboratories in various countries. These tandem repeats are highly variable in terms of the number of repeating units found in an individual, e.g., repeating units vary from 4 to 26 units depending on the genetic locus being analyzed. Short Tandem Repeats (STR's) also called microsatellites, are widely dispersed in prokaryotic and eukaryotic genomes and show extensive length polymorphism, hence they are widely used in DNA fingerprinting and diversity studies.

Previous validation studies tested the specificity of human STR systems against other animal and bacterial systems. These studies demonstrated that microbial DNA, mostly on bacteria and yeast could act as non-specific templates affecting human PCR based DNA typing (1–3). Moreover, the invading microorganism may excrete staining and fluorescing secondary metabolites into the bone (4) which could similarly have an adverse effect on downstream DNA analysis.

The present study focuses on possible cross-reaction of fungal DNA using human STR primers. This is the first reported study to isolate fungi from bone samples exposed to conditions of burning, burial and exhumation and to investigate possible interferences of fungal DNA from filamentous microfungi in subsequent DNA analysis of human samples.

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Because of the ability of fungi to inhabit many environmental conditions, reproduce asexually and sexually through spore formation and regenerate by means of filamentous segments, this group of eukaryotic microorganisms presents a major concern when analyzing forensic case samples, especially those taken from abandoned crime scenes and burial sites. Hence, possible non-amplification due to interference of fungal DNA in low or high concentrations and non-specific binding of human STR primers to fungal DNA targets require investigation.

We validated our current DNA procedures by testing the specificity of ten sets of human STR primers for human DNA against fungal DNA. For this purpose, we isolated, identified major fungal genera from this type of environmentally challenged sample that could possibly interfere with human DNA typing and subsequent analysis. A gender test marker (HUMAMEL) and nine autosomal markers namely, HUMCSF1PO, HUMDHFRP2, D8S306, HUMFES/FPS, HUMFGA, HUMF13A01, HUMTH01, HUMTPOX and HUMvWA, were used based on the availability of data on genetic polymorphisms of the Philippine population (5,6). Bone samples were taken from a fire tragedy and were used because of the following considerations: availability of post-mortem bone samples that were exposed to extreme environmental insults, e.g., high temperature and humidity, that enhanced sample decomposition and microbial growth and availability of preserved biological material of victims prior to the fire (victims' ante-mortem reference samples) and blood samples collected from living relatives (relative reference samples).

Methods

DNA Analysis and Data Interpretation

Twenty four fungal isolates were obtained from bone samples of five exhumed remains. To extract fungal DNA, a loopful of the

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mycelia of pure isolates was incubated in 300 μL cell lysis buffer (10 mM Tris-100 mM NaCl-39 mM DTT-10 mM EDTA-2% SDS) and 36 μL 20 mg/mL Proteinase K (Gibco-BRL, Gaithersburg, MD). DNA was purified using phenol/chloroform/isoamyl alcohol and concentrated using cold absolute ethanol, following the method of Sambrook (7). Isolation of DNA from human sources (bone, tissue and blood samples), singleplex DNA amplification at ten STR markers including HUMAMEL, fragment analysis and data interpretation were performed as described previously (8). DNA was amplified using 0.72 μM of unlabeled (Gibco-BRL, Life Technologies, Gaithersburg, MD) and Cy5-labeled fluorescence primers (GenSet Oligos, Singapore) using previously published primer sequences (9), 60 ng/μL bovine serum albumin (Promega, Madison, WI), 50 μM dNTPs (Invitrogen, Carlsbad, CA), 0.025 U/μL of and Amplitaq Gold® Polymerase (Applied Biosystems, Foster City CA). Amplification was carried out in a Biometra UNO thermal cycler (Biometra, Göttingen, Germany) following these conditions: For HUMFES/FPS, HUMF13A01, HUMTH01, HUMvWA, D8S306, HUMDHFRP2 and HUMAMEL, initial denaturation was at 92°C for 12 min, followed by 40 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1.5 min, then a final dwell of 72°C for 10 min. For HUMCSF1PO, HUMFGA and HUMTPOX, initial denaturation was at 92°C for 12 min, followed by 10 cycles of 94°C for 1 min, 64°C for 1 min, 70°C for 1.5 min, then 20 cycles of 90°C for 1 min, 64°C for 1 min and 70°C for 1.5 min with a final dwell of 72°C for 30 min. Amplified products were detected with the ALFExpress™ sequencer and ALFwin™ and Allelelinks™ version 1.01 software (Amersham Pharmacia Biotech, Uppsala, Sweden) using automated fluorescence technology.

Simulation Experiments

Effects of fungal DNA on subsequent DNA analysis were investigated. To test for specificity, 20 ng of DNA extracted from 24 fungal isolates namely *Aspergillus niger, A. flavipes, A. glaucus, A. nidulans, A. janus* var. *brevis, A. ustus, A. clavato-flavus, A. tamarii, Curvularia lunata, Dactylella* sp., *Geotrichum candidum, Cladosporium* sp., *Phialocephalla* sp., *Helicomyces* sp., *Trichosporon* sp., and nine *Penicillium* isolates were amplified using the same set of ten STR markers including HUMAMEL. The amount of DNA template used was equivalent to concentrations used in routine casework analysis in our laboratory.

Two representative fungal isolates, *C. lunata* and *Penicillium* sp. 3, were selected for mixture analysis. Preliminary analysis of pure *C. lunata* DNA showed the presence of non-specific peaks at two STR loci (HUMDHFRP2 and HUMF13A01). *Penicillium* sp. 3 on the other hand is a representative isolate of the most abundant genus present in bone samples included in the present study (10). Fungal DNA was mixed separately in various proportions with known concentrations of human DNA (Table 1). Two sources of human DNA were used namely K562 genomic DNA (Promega, Madison, WI) which was derived from a female cancer cell line and an internal laboratory standard DNA (NSDNA 3967) extracted from blood samples of a male source.

TABLE 1—*Concentrations of human and fungal DNA.*

	Human DNA (ng)	Fungal DNA (ng)*
a) simulating conditions of low fungal contamination	0	20
	5	15
	10	10
	15	5
b) simulating conditions of high fungal contamination	20	0
	1	10
	1	100
	1	1000

* *Curvularia lunata* or *Penicillium* sp. 3 in separate DNA mixture analyses.

To determine human DNA concentration, QuantiBlot® human DNA quantitation kit (Applied Biosystems, Foster City CA) utilizing D17Z1 α-satellite probe was used. Preparation of reagents and DNA standards, immobilization of DNA or slot blotting, hybridization and detection procedures strictly followed manufacturer's instruction. Immobilization of DNA on nylon membranes was done using the Convertible® slot blot apparatus (Gibco-BRL, Gaithersburg, MD). Non-human DNA were estimated using UV spectrophotometry. Samples for quantification were prepared by diluting 2 μL DNA extract to 500 μL with sterile deionized water (1/250 dilution). The entire solution was transferred to a quartz cuvette and the absorbance read at 260 nm on a GeneQuant® RNA/DNA calculator (Amersham Pharmacia Biotech, Uppsala, Sweden).

To test whether any effect on PCR may be due to the presence of high concentrations of non-human DNA rather than inherent properties of fungal DNA, human DNA (1 ng) was separately mixed with 1000 ng of bacterial DNA (*Salmonella* sp.), equine DNA (*Equus caballus*) and cattle DNA (*Bos indicus*).

Casework Application

Numerous genetic profiles were generated during the entire casework. Of these, five cases were selected to study factors affecting forensic DNA typing procedures. DNA profiles obtained from bone samples of fire victims were compared with genotypes obtained from relatives or by directly matching with DNA profiles obtained from victims' ante-mortem samples (8).

Results and Discussion

Species Specificity

Since microsatellites or short tandem repeats (STR's) are densely interspersed in eukaryotic genomes, the species specificity of ten sets of human STR primers including the HUMAMEL sex determining marker used in forensic DNA typing was investigated. In particular, the specificity of these STR primers for human DNA in the presence of low and high level of contaminating fungal DNA was studied. In the first stage of the present study, possible binding of human STR primers to pure fungal DNA templates was investigated. To test specificity of PCR primers for human DNA, fungal DNA from 24 pure isolates were amplified at ten STR loci including the HUMAMEL marker. Most fungal strains did not yield distinct peak signals within the standard criteria for assignment of true alleles at all STR loci tested, with the exception of *C. lunata* at HUMDHFRP2 and HUMF13A01. The observed peaks were within the size range of known human alleles at the STR locus HUMDHFRP2 (Fig. 1*A*) but outside the size range of known human alleles at HUMF13A01 (Fig. 1*B*).

Quantitative information of the signals present was analyzed by comparing peak areas [11–13]. It was observed that PCR products generated using fungal DNA templates are non-reproducible and indistinct, e.g., low peak areas and aberrant peak morphology. These PCR products exhibited different mobility compared to amplified products from human DNA and are consistent with stochastic effects rather than amplification of specific PCR products. DNA searches using primer sequences used here and published fungal sequences (14) did not show any significant match. Both observations support the absence of specific binding sites on fungal DNA for the ten STR primers included in the present study.

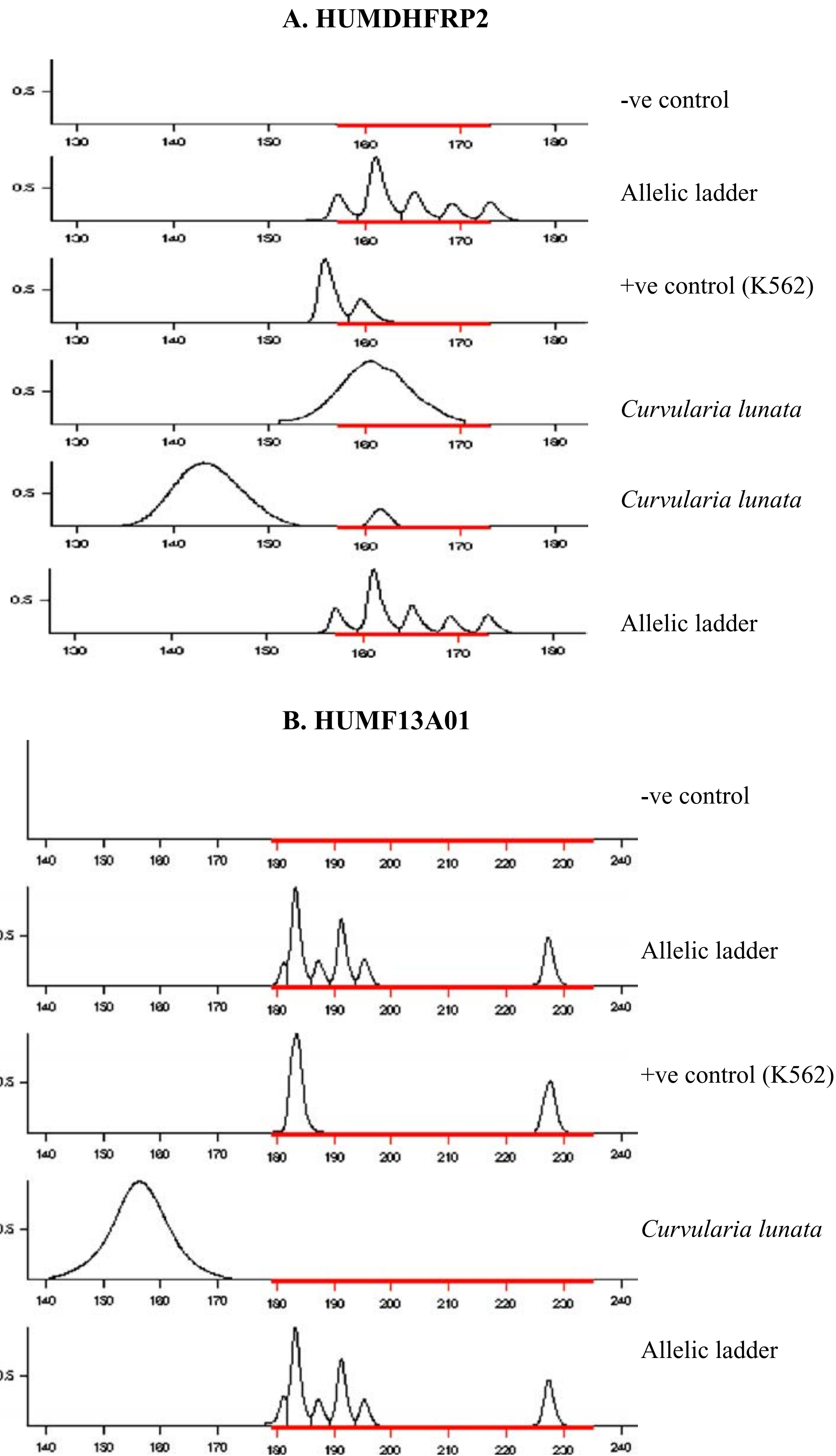

FIG. 1—*Electropherograms showing non-specific artifacts amplified using Curvularia lunata DNA at A. HUMDHFRP2 B. HUMF13A01. The x-axis is scaled into number of bases and the y-axis represents the peak area of the curve relative to the largest peak area within the lane.*

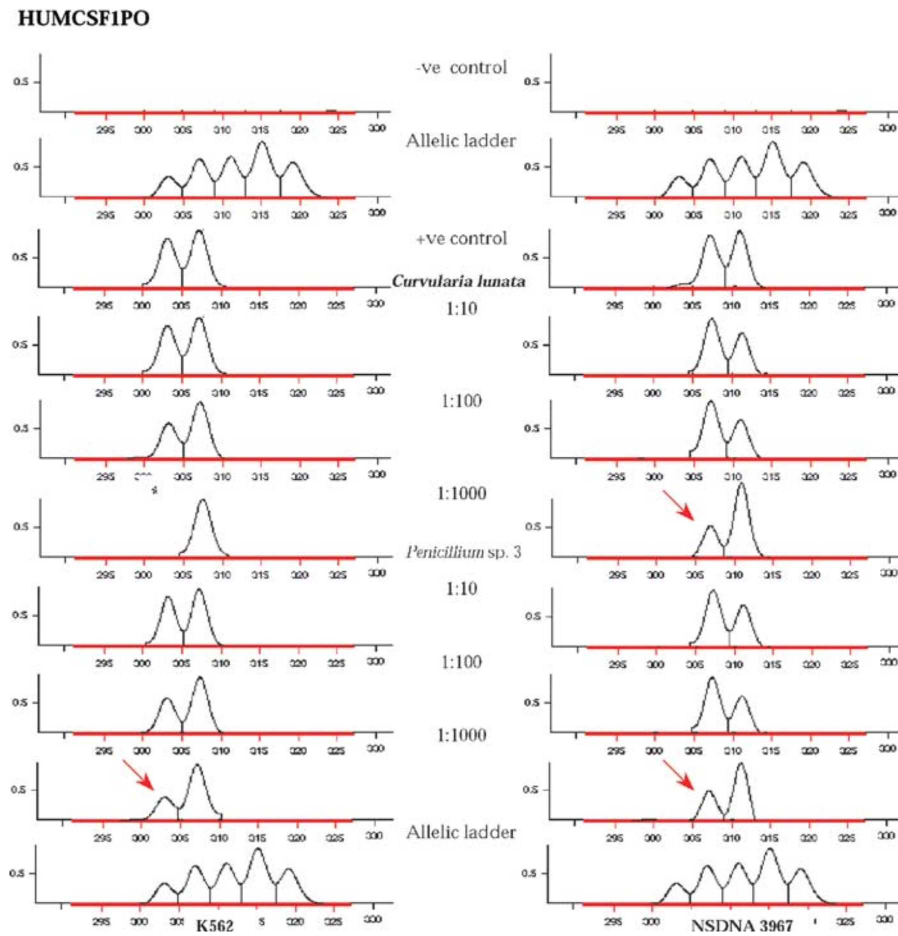

FIG. 2—*Electropherograms of amplified products at HUMCSF1PO using 1ng human DNA (male and female sources) and various concentrations of DNA extracted from two fungal isolates simulating conditions of high fungal contamination. An arrow (↓) indicates reduction in amplification signals with increasing fungal concentration and an asterisk (*) marks loss of allele observed when fungal DNA was increased to 1000 times the amount of human DNA.*

Mixture Experiments

In addition, the presence of contaminating non-human DNA in forensic samples may interfere or inhibit PCR. DNA of *Curvularia lunata* and *Penicillium* sp. 3 were separately mixed in various proportions with known concentrations of human DNA. Fungal DNA did not prevent amplification of human DNA using the following human: fungal DNA mixture ratios namely 15:5, 10:10 and 5:15 ng units at ten STR loci tested (Table 1). Non-specific fragments that were previously detected in HUMDHFRP2 and HUMF13A01 were not observed when *Curvulaia lunata* DNA was mixed with human DNA source (data not shown). It is likely that the initial amplification using pure fungal DNA may be attributed to stochastic effects. However in the presence of human DNA which contains binding sites for HUMDHFRP2 and HUMF13A01, only specific PCR products from the amplification of human DNA templates were observed.

However, with increasing concentrations of fungal DNA (10 ng to 1000 ng) mixed with a fixed human DNA concentration (1 ng), lower amplification signals and loss of allele leading to false homozygous scores were observed in some markers e.g., HUMCSF1PO locus (Fig. 2), HUMFES/FPS (Fig. 3) and HUMF13A01 (Fig. 4). Interference with human STR profile was more pronounced when 1000 ng of fungal DNA mixed with 1ng human DNA was used. The presence of 1000 ng fungal DNA resulted in complete PCR inhibition at HUMFGA (Fig. 5). Notably, the incidence of allelic dropouts is more frequent when using K562 DNA compared to the in-house laboratory control NSDNA 3967. K562 DNA was derived from cancer cells and is known to exhibit heterozygous peaks with marked peak imbalance in the HUMFGA marker and an abnormal profile on chromosome 21 (11).

The imbalance in the proportion of fungal DNA to human DNA reduced the efficiency of the amplification process. Therefore, it is likely that the presence of excess fungal DNA molecules in the reaction physically interferes with primer binding to human DNA template, and other components of the amplification reaction. e.g., primers, dNTP's and *Taq* polymerase. The effect of high concentrations of fungal DNA (1000 ng) on the efficiency of the PCR process could not be overcome by doubling the concentrations of each of the PCR components in the reaction mixture. PCR was also inhibited when high amounts (1000 ng) of non-human DNA other than fungi, e.g., *Salmonella,* horse and cattle DNA, were mixed with 1 ng human DNA (data not shown). This observation was consistent with non-specific inhibition of PCR in the presence of large amounts of contaminating non-human DNA.

Results from the present study underscore the need for repeated analyses of samples, particularly when handling highly contaminated samples. This is to avoid spurious data interpretation e.g.,

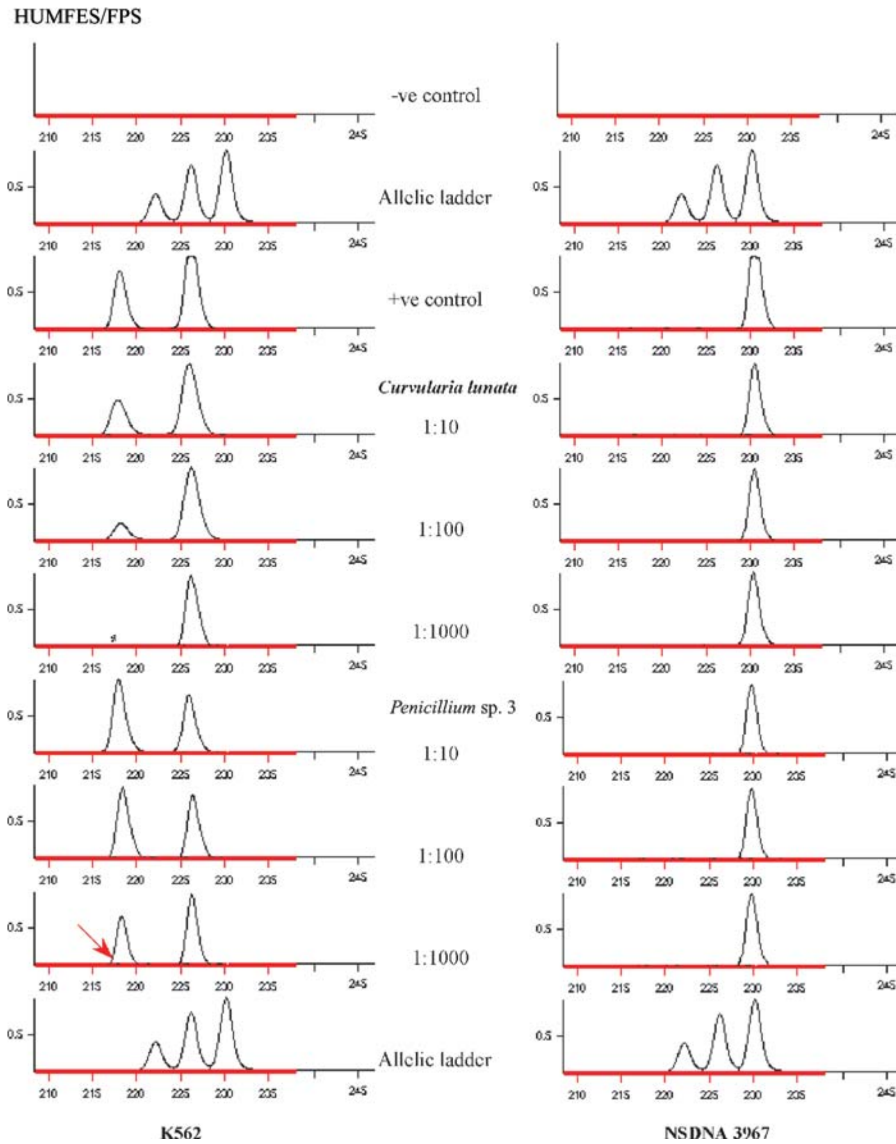

FIG. 3—*Electropherograms of amplified products at HUMFES/FPS using 1ng human DNA (from male and female sources) and various concentrations of DNA extracted from Culvularia lunata and Penicillium sp. isolate 3 simulating conditions of high fungal contamination. Allelic loss (*) was observed when the human:fungal DNA ratio was equal to 1:1000.*

HUMF13A01

-ve control

Allelic ladder

+ve ctrl

Curvularia lunata

1:10

1:100

1:1000

Penicillium sp. 3

1:10

1:100

1:1000

Allelic ladder

K562 **NSDNA 3967**

FIG. 4—*Electropherograms of amplified products at HUMF13A01 using 1 ng human DNA (male and female source) and various concentration of DNA extracted from Curvularia lunata and Penicillium sp. isolate 3 simulating conditions of high fungal contamination. Loss of allele (*) was observed when the human:fungal DNA ratio was brought to 1:1000.*

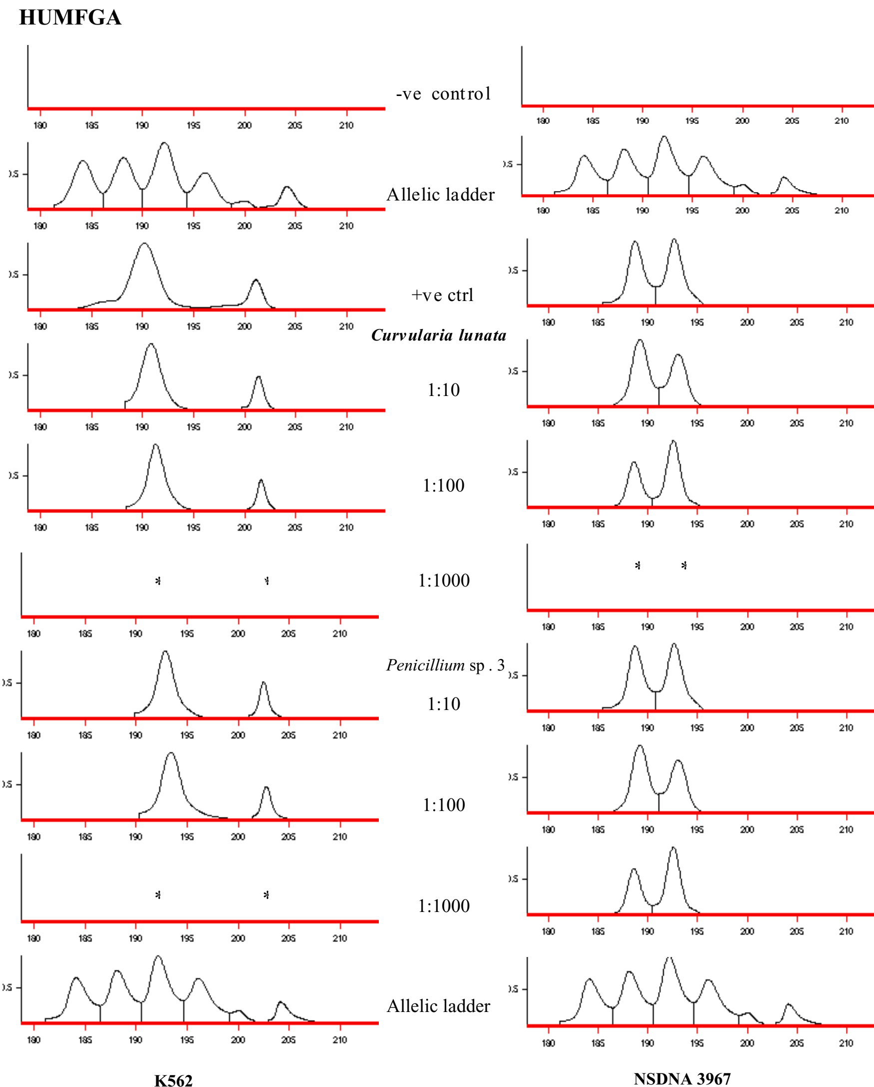

FIG. 5—*Electropherograms of amplified products at HUMFGA using 1ng human DNA (male and female sources) and various concentration of DNA extracted from Curvularia lunata and Penicillium sp. isolate 3 simulating conditions of high fungal contamination. Non-amplification of both alleles* were observed when the human:fungal DNA ratio was brought to 1:1000.*

false homozygotes due to incomplete amplification, and negative PCR results. Fungi are highly invasive and may be present in large quantities in exhumed bones. Knowledge of the extent of fungal contamination of forensic evidence is vital for proper decontamination, DNA analysis and subsequent interpretation of DNA test results. Dematiaceous fungi have been observed to thrive inside devitalized bones (15) and fungal survival in extremely dry conditions is well documented.

The STR system used in this study proved to be robust and the presence of non-human DNA in bone samples did not alter possible genotypes provided the amount of contaminating DNA is less than 1000 times that of target human DNA. The five bone samples exhumed three months after internment and included here present situations similar to low fungal DNA contamination (10). DNA profiles were generated in all five bone samples tested. The effect of non-specific PCR inhibition is more pronounced in samples with higher amounts of contaminating non-human DNA.

The results of the present study highlight the importance of carefully analyzing factors affecting DNA typing of environmentally challenged samples, e.g., exhumed remains, to avoid spurious data interpretation. This is of utmost importance when dealing with environmentally challenged case samples such as those collected from abandoned crime scenes and exhumed graves.

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