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Increasing the Forensic Discrimination of Mitochondrial DNA Testing through Analysis of the Entire Mitochondrial DNA Genome

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The principal limitation in forensic mitochondrial DNA (mtDNA) testing is the low power of discrimination that is obtained when common "mtDNA types" are involved in a case. Currently, an "mtDNA type" refers to the sequence within hypervariable regions I and II (HV1/HV2) of the control region, ~610 bp. In Caucasians, the most common HV1/HV2 type is found in ~7% of the population and there are 12 additional types found at greater than ~0.5% (ignoring HV2 C-stretch polymorphism). We are performing large scale sequencing of the entire mtDNA genome (mtGenome), ~16,569 bp, of individuals who have common HV1/HV2 types. Of 31 individuals with the most common HV1/HV2 type, only 3 still match after mtGenome sequencing. Similar high discrimination is seen in other common HV1/HV2 types. The sites that discriminate the various common HV1/HV2 types are generally not those that are known to vary widely in more diverse population samples. This indicates that complete mtGenome sequencing of selected HV1/HV2 types may stand as the best way for identifying maximally useful single nucleotide polymorphism sites outside of the control region. Our strategy for identifying SNP sites is useful in resolving U.S. Caucasian, Hispanic, and African American mtDNAs is discussed. We also discuss the development of homogeneous fluorogenic polymerase chain reaction assays that target phenotypically neutral sites for practical use in casework.

Key words: *DNA, mitochondrial; fluorescent probes; forensic medicine; polymerase chain reaction; polymorphism; polymorphism, restriction fragment length; United States*

Mitochondrial DNA (mtDNA) has found a vital niche in forensic DNA testing, and its use is expanding very rapidly both in terms of volume of casework and the number of laboratories performing mtDNA analysis. Mitochondrial DNA exists within cytoplasmic mitochondria as a separate small genome of ~16,569 base pairs. The principal advantage of mtDNA is that it is present in ~500- 2000 copies per cell. This abundance correspondingly increases the chances that some copies of mtDNA will survive in highly degraded forensic samples. Nuclear DNA, despite its great power for identification, is present in only two copies per cell. Mitochondrial DNA testing permits typing a great range of samples that would otherwise be inaccessible. Such samples include highly degraded stains, bones, saliva, fingernails, and hair shafts. Another advantage of mtDNA is that it is maternally inherited, so even distant maternal relatives can provide a comparative reference sample with an expectation of a match. The attributes, population genetics, and forensic applications of mtDNA are well established, and recently reviewed in Holland and Parsons (1). References for general mtDNA characteristics discussed above and below can be found therein.

Current mtDNA testing most often involves polymerase chain reaction (PCR) amplification and complete sequence determination of two hypervariable regions (HV1 and HV2) in the control region. The control region is the only significant portion of mtDNA that does not code for genes. Together, HV1 and HV2 encompass roughly 610 bases of information. These regions are highly variable in the population, due to a very high evolutionary rate of mtDNA, and the fact that the non- coding regions are subject to diminished functional constraint. The evolutionary rate of the control region is approximately ten times that of the gene-coding region, so variation is very much concentrated in HV1/HV2. However, a point that has until now been ignored by forensics is that the coding region is fifteen times larger than the control region, so *the greatest portion of total mtDNA variation occurs in the coding region*.

Mitochondrial DNA typing does not provide definitive identification. Firstly, an individual is expected to match maternal relatives. Secondly, mtDNA is a single linked molecule, so one cannot multiply the probabilities of individual polymorphisms along the mtDNA molecule – the product rule does not apply to frequencies of individual polymorphisms in the mtDNA sequence. Hence, with mtDNA there is an appreciable chance for a random match in the population, although many mtDNA types are so rare that they have been seen only once in large databases. In order to as-

sess the significance of an mtDNA match, reference must be made to the frequency with which that particular mtDNA sequence, as a whole, has been observed in a relevant population. Therefore the significance of mtDNA typing when previously unobserved types are encountered is currently database-limited. Fortunately, databases are growing very rapidly, and the significance of a match for an mtDNA type that has not been previously seen in any database is quite substantial (2).

However, there are some quite common mtDNA types in all populations studied to date. In U.S. Caucasians, fully 7% of the population share the most common HV1/HV2 type (this ignores length variation in the HV2 C-stretch that is not generally considered a stable character for the purposes of forensic exclusion). There are 13 additional HV1/HV2 types that occur at levels of 0.5% or greater in the population (within a current international mtDNA database of Caucasian sequences: MitoSearch Version 2.1, FBI Laboratory). However, the overall distribution of mtDNA types is highly skewed toward rare types: 982 of 1175 different mtDNA types are unique in the database. *Therefore, the greatest limitation for mtDNA testing lies with the small number of common types for which the power of discrimination is low.*

This limitation may be the most systematically troublesome in applications where non-mtDNA information is relatively limited. In the Armed Forces DNA Identification Laboratory (AFDIL), our work to assist in identification of missing soldiers from the Korean War often involves comparison of sequences from skeletal remains to a large database of reference HV1/HV2 sequences from families who have a member missing from the Korean conflict. For the various common HV1/HV2 mtDNA types referred to above, this database has multiple families who match. Therefore the mtDNA evidence cannot discriminate among these families (although it can still greatly focus investigations relating to non- mtDNA evidence). Such database comparisons are not restricted to the AFDIL laboratory. The International Commission on Missing Persons (ICMP) is conducting a similar approach in a massive effort to assist in identification of thousands of individuals missing from the ethnic conflicts in the former Yugoslavia (3). Additionally, the Federal Bureau of Investigation (FBI) and the State of California are establishing missing persons reference databases to include mitochondrial DNA data, for comparison to sequences from questioned samples.

There is a large potential for additional variation residing outside of the HV1 and HV2 regions of mtDNA to be used in discriminating individuals or lineages who match for HV1 and HV2. With this in mind, our laboratory has begun a large scale effort to analyze the entire mtDNA genomes (mtGenomes) in order to identify sites that – to as large an extent possible – alleviate the problem of low discriminatory power when common HV1/HV2 types are encountered. While various types of mutation detection screens might be employed with some utility (4,5) and there does exist much published literature relating to mtDNA variation outside of the control region, we decided to pursue a "brute force" approach of sequencing the entire genome of multiple individuals who match various common HV1/HV2 types. In this way, we generate a complete data set that is most specifically directed toward the problem at hand, and that can be used in the future to guide any type of detection assay for mitochondrial polymorphisms. We do not envision that sequencing the entire mtGenome will be practical in application to actual forensic casework. Rather, our efforts will be directed toward identification of specific sites that can be targeted for single nucleotide polymorphism (SNP) analysis in a highly directed manner (see below).

There is an abundance of data on genetic variation from the entire mtDNA genome that comes from restriction length fragment polymorphism (RFLP) analysis in studies of human evolution and population genetics (6). However, the battery of 13 restriction enzymes used standardly in high resolution RFLP studies can be expected to cover only ~30% of the nucleotide sites in the mtDNA, overlooking much potentially useful variation. An even more significant limitation is the fact that this information comes from a diverse collection of randomly selected individuals for whom control region sequences are usually not available. The relative rates of evolution per site of the control region (fast) and the coding region (slow) determines the nature of our search for coding region sites that discriminate among individuals that are not distinguished by control region mutations. It is a bit of a needle in the haystack search. Coding region sites that vary widely in the human population could very well provide little resolution among individuals who are identical in HV1/HV2. Even the recent publication of many entire mtGenome sequences from a diverse population sample (7) does not reveal sites that can be counted upon in advance to discriminate among individuals that share common HV1/HV2 types.

The amount of mtGenome sequencing that needs to be done to identify discriminatory sites for many of the common HV1/HV2 types in various populations is unknown. If a particular common HV1/HV2 type can only be distinguished by a set of coding region polymorphisms private to that type, then the question has to addressed independently for each common HV1/HV2 type. Alternatively, some coding region sites may be useful for multiple common HV1/HV2 types, with the greatest likelihood of this occurring among common types that are closely related. The extent to which forensically informative coding region sites that distinguish a particular common HV1/HV2 type will be useful for other common HV1/HV2 types depends on: 1) the relatedness between the mtDNA types in question, 2) the evolutionary rate of the coding region sites that were found to resolve, and 3) the particular timing of the resolving mutation in evolutionary history.

The most common HV1/HV2 type in the Caucasian population is 263G, 315.1C (with reference to the Cambridge Reference Sequence, ref. 8). Throughout this paper we will refer to this HV1/HV2 type as "H1". For H1, we are searching for sites in the slowly

evolving coding region of the mtDNA genome that have acquired mutations even when none have occurred in the fast-evolving control region. These coding region sites could have the property of being unusually fast-evolving despite residing in the coding region. Little is known about the extent of mutation rate heterogeneity in the coding region, but in the control region, different sites have hugely different rates of evolution (9). There may be substantial rate heterogeneity and mutational "hotspots" in the coding region as well. Alternatively, informative coding region sites may prove to be slowly evolving, but have mutations that simply happened at a fortuitous point in time in population genetic history.

European mtDNA types have been classified into a number of "haplogroups" based on the evolutionary relatedness of the sequences (10,11). Haplogroups are clusters of closely related mtDNA lineages, and forensically-informative coding region sites will have the highest chance of being useful among those mtDNA types that are most closely related to the mtDNA type in which they were discovered. The most common HV1/HV2 type in Caucasians (H1) belongs in the European haplogroup "H". Additionally, examination of the forensic mtDNA sequence database indicates that six common HV1/HV2 types belonging to haplogroup H comprise 12% of the entire U.S. Caucasian population. The most useful SNP sites to target will be those that resolve other H-group types as well a H1. At the outset, the prospects for identifying such sites seem good. All of the common H-group types differ by only a single base in HV1/HV2, and the differences are at very fast- evolving sites. Therefore, these are extremely closely related sequences that might well be resolved by the same coding region sites. This issue will be resolved by comparing mtGenome sequences from multiple representatives of these different HV1/HV2 types (initial data suggest some coding region sites will be widely useful, while others are specific to particular common HV1/HV2 types; data not shown).

It is less clear that other Caucasian haplogroups will be resolved with the sites that are informative for haplogroup H, as these have substantial evolutionary divergence. To test this, we intend to sequence the mtDNA genome of multiple individuals that share common mtDNA types from other haplogroups. Haplogroups J, T, K, and V all have HV1/HV2 types that are common in the population, and we will target each of these groups, beginning with the most common J type. Haplogroup J is evolutionarily distant from H, and the third most common HV1/HV2 type in the Caucasian population (1.5% of the total population) is from haplogroup J.

African and Hispanic mtDNA sequences from the U.S. have a frequency distribution similar to that of Caucasians, and the same approach to identifying informative sites will apply. In Hispanics, there are six haplogroups that manifest common HV1/HV2 types in the population; the four most predominant are the prevalent founder Amerindian haplogroups A, B, C, D (12), and a fifth actually belongs in the European haplogroup J, indicating the admixture in Hispanics of European and Native American populations. Common HV1/HV2 types in African American populations likewise derive from five different highly diverged haplogroups (13). We will sequence multiple individuals from each of these common African American and Hispanic groups.

Progress to Date

We have developed an efficient, robotics-based strategy for sequencing the entire mtDNA genome. Based on a subset of primers published for entire mtGenome amplification (14), the mtGenome is amplified in twelve overlapping PCR fragments of 800 bp to 1.8 kbp each. Each amplicon is then sequenced with six to twelve sequencing primers. The 90 total sequencing reactions provide sequence confirmation on both strands, with enough redundancy for highly confirmed sequence. The process, from fragment amplification to cycle sequencing is automated on the MWG RoboAmp 4200 platform (MWG Biotech, High Point, NC, USA). Sequence data is obtained either on an ABI 3100 or 377 automated sequencer (Applied Biosystms, Foster City, CA, USA). This automated process frees personnel time for the rate limiting steps of sequence assembly, editing, and databasing.

As of March 2001, we have sequenced 70 mtGenomes, with a current output of seven to ten per week. 31 of the mtGenomes correspond to "H1" individuals (with the most common Caucasian HV1/HV2 type). The other mtGenomes we have completed come from a variety of common HV1/HV2 types from Caucasian haplogroups J and T, along with others from haplogroup H. We are still studying this data to develop an optimal strategy for targeting SNP assays, so the details of the sequence data and their application will be published elsewhere in the near future. Here we will summarize some of the general observations to date.

The 31 non-related H1 individuals belong to haplogroup H according to the classification of Torroni et al (10). Among these we identified 75 polymorphic sites outside of HV1 and HV2. Each site had only two nucleotide variants. The majority of the polymorphic variants (56 of 75) were novel compared to a compendium of known mtDNA polymorphisms (MITOMAP: *http://www.gen.emory.edu/mitomap.html*). The sites were distributed as follows: 56 in protein coding regions, 6 in tRNA genes, 5 in rRNA genes, 6 in the coding region (outside of HV1 and HV2), and 2 polymorphic sites were in non-coding "spacer" regions between coding regions. The average number of differences from the revised Cambridge Reference Sequence (CRS, ref. 15) was 3.6 (ranging from 1 to 9), and among the H1's the maximum number of differences was 16.

Of the 31 H1 individuals, only three still match after sequencing the entire mtGenome. This divides the 31 individuals into 29 different mtDNA types, all of which differ at two or more sites. A majority of the sites that distinguish these individuals were private polymorphisms, ie, unique in the group of 31. Such private polymorphisms are far from optimal as SNP target sites as they not very likely to vary among other

H1 individuals. However, the data indicate 21 sites where non-CRS variants are shared among multiple H1 individuals, indicating that these sites are polymorphic at a level that would make them suitable as SNP assay sites. This information indicates clearly that variation in the coding region can almost completely solve the problem of mtDNA types that occur at high frequency in the population.

We plan to attack the problem of resolving many of the common mtDNA types in a step-wise fashion, coordinating mtGenome sequencing with SNP development and testing. We will likely be able to decrease the amount of mtDNA genome sequencing that is required by testing candidate SNP assays as they become available, to survey the variability of target sites in other common mtDNA types. For example, SNP assays for the variable sites we have already identified for H1 types allow us to test if these also resolve common types from the other Caucasian haplogroups. Thus, we envision the project to proceed in a coordinated stepwise manner, where sequencing guides SNP assay development, and assays are then used to assess the utility of the sites for resolving common types in other groups. Ultimately, the discrimination potential of the fluorescent SNP probes will be determined for the general population comprising three major ethnic groups present in the United States (Caucasians, African-Americans, and Hispanics). Once a final battery of non-HV1/HV2 SNP assays is available, we will establish a population database of 1,000 individuals, to indicate the rarity of the mtDNA types in the population. Coding region SNP assays are envisioned to complement sequence data from HV1 and/or HV2, or to function as a standalone homogeneous assay system in concert with SNP assays that target control region sites.

Avoiding Polymorphisms with Disease Phenotypes

There are many pathological conditions known to be due to mutations in mtDNA coding genes (reviewed in 6). To avoid situations where forensic identity testing unintentionally becomes genetic disease screening, it is desirable to develop assays only for silent or neutral positions within the mtDNA genome. These include, predominantly, silent polymorphisms at third codon positions in protein coding genes. These have no potential to affect phenotype, and therefore can be considered in the same manner as HV1/HV2 polymorphisms that are currently used in forensic analysis. Other neutral polymorphisms would include those that occur in the short segments of non-coding DNA that occurs between particular genes in the coding region, and polymorphic control region sites that reside outside of HV1 and HV2. The latter have been shown in our laboratory and others to add substantially to the discrimination potential of mtDNA (for example, position 16,519 is the most highly polymorphic site known in mtDNA, but is not usually tested because it is outside HV1/HV2). Another set of polymorphic sites that we consider acceptable candidates for forensic testing are sites that, while they theoretically have potential for phenotypic effects, are already known to segregate widely in the population and are assuredly neutral (with reference to the "neutral theory" of molecular evolution; ref. 16). The use of these sites should be minimized, but, for example, we have found one site in the 16S RNA gene that is extremely useful for resolving H1 types. Since it is a widely reported polymorphism never associated with any disease, we suggest that it is an acceptable candidate site.

Because of the concerns relating to genetic disease, our research involving entire mtDNA genome sequencing is performed on samples that have been anonymized. Multiple individuals that have the same common HV1/HV2 types have been selected from database samples in hand. Within the common types, samples have been stripped of identifiers and shuffled prior to sequencing so that there is no chance for genetic information identifiable to source individual to be generated. The potential for uncovering genetic information with medical significance is another primary reason (in addition to ease and speed) why we believe forensic testing outside of the control region should be performed by SNP assay only. In this way, one is assured of obtaining information only from carefully selected sites, and can avoid issues relating to medical genetic privacy, disclosure, and responsibility.

Fluorogenic Single Nucleotide Polymorphism Assays

The information compiled from complete mtDNA genome sequencing can be utilized for any genetic detection assay, and some type of microchip based system will probably be developed eventually. However, as an initial approach to SNP assays that will permit development and application in the immediate future, we have chosen to pursue homogeneous fluorescent PCR assays. Homogeneous assays are those that do not subject amplified products to post-amplification manipulations such as sample clean-up, cycle sequencing, and electrophoresis. A primary motivation is speed and ease, but in the present context contamination avoidance is a major advantage of homogeneous assays as well. PCR products themselves are a principal danger for contamination in mtDNA testing, and assays that do not require the PCR tube to be opened after amplification could significantly reduce the complexity of contamination avoidance procedures and laboratory design in forensic mtDNA typing.

Homogeneous fluorescent PCR assays have been developed for a wide variety of applications. The relevant application for our purpose is most often referred to as "allelic discrimination" where base variants are typed at a particular nucleotide position. The most widely used approach employs "TaqMan" chemistry, where two internal fluorescently labeled probes are included in a standard PCR (17-22). The probes are complementary to a binding site that spans the base position of interest, and each probe is specific for one of the two variants, so that one probe will match and the other will have a single mismatch. During PCR the Taq enzyme digests any bound probe, releasing a

fluorescent moiety from a linked quencher, causing signal to be detected by the instrument. Each variant probe has a different fluorescent label, enabling the variant present in the PCR product to be identified, and can be modified with minor groove binding moieties to improve specificity. With the TaqMan approach, assays for two different sites can be multiplexed in the same reaction. Initial development of TaqMan allelic discrimination for mtDNA SNP detection has shown great promise (data not shown, W. Parson and H. Neiderstaetter, personal communication).

Conclusion

Our studies have demonstrated the potential for variation in the mtDNA coding region to provide discrimination within the common HV1\HV2 types in the Caucasian population, and to greatly decrease the single biggest limitation of forensic mtDNA testing. As in studies of human molecular evolution and population genetics (7), we believe that mitochondrial DNA genomics will constitute the next major step forward in forensic mtDNA analysis. It may be some time before stand-alone systems that assay variation over the entire mtGenome become the norm, but in the near future we anticipate that particular SNP assays will be available to increase the strength of mtDNA testing in frequent cases where common HV1/HV2 types are encountered. At this point, it is important to roll up our sleeves and do the heavy lifting of entire mtDNA genome sequencing to sample the information that we will rely upon in the future. Subsequently, as with the mtDNA control region, large population databases will need to be established.

With a large battery of well characterized SNP sites available for use, it may be possible to tailor forensic mtDNA testing in a manner that maximizes the information obtained from limited DNA extracts. For example, if the most common Caucasian type is encountered after sequencing HV1, a practitioner may choose to target a battery of specific SNP sites that are most likely to provide additional resolution among individuals with this HV1 type. Optimal mtDNA typing systems of the future will likely be based on basic mtGenome sequence information that we are beginning to produce at present.

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