REPORT OF THE

DEFENSE SCIENCE BOARD

TASK FORCE

ON

THE USE OF DNA TECHNOLOGY FOR IDENTIFICATION OF ANCIENT REMAINS

JULY 1995

OFFICE OF THE UNDER SECRETARY OF DEFENSE
FOR ACQUISITION & TECHNOLOGY
WASHINGTON, D.C. 20301-3140

This report is a product of the Defense Science Board (DSB). The DSB is a Federal Advisory Committee established to provide independent advice to the Secretary of Defense. Statements, opinions, conclusions and recommendations in this report do not necessarily represent the official position of the Department of Defense.

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OFFICE OF THE SECRETARY OF DEFENSE
WASHINGTON, D.C. 20301-3 140

DEFENSE SCIENCE
BOARD

16 JUL 1995

MEMORANDUM FOR UNDER SECRETARY OF DEFENSE (ACQUISITION & TECHNOLOGY)

SUBJECT: Report of the Defense Science Board (DSB) Task Force on the Use of DNA Technology for Identification of Ancient Remains

I am pleased to forward the final report of the DSB study on the Use of DNA Technology for Identification of Ancient Remains. The report focuses on a new and exciting area of life science technology that can have a dramatic impact on the Department's ability to resolve current and future issues concerning the fullest possible accounting of prisoners of war and missing in action (ROW/MIAs).

The report's recommendations could serve to support broad policy adjustments for the Department of Defense on issues concerning identification of ancient war remains. I concur with the observations and recommendations of the Task Force, and recommend that you forward the report to the Secretary of Defense.

Craig I. Fields
Chairman

OFFICE OF THE SECRETARY OF DEFENSE
WASHINGTON, D.C. 20301-3140

DEFENSE SCIENCE
BOARD

MEMORANDUM FOR CHAIRMAN, DEFENSE SCIENCE BOARD

SUBJECT: Report of the Defense Science Board (DSB) Task Force on the Use of DNA Technology for Identification of Ancient Remains

Attached is the report of the DSB study on the use of DNA Technology for Identification of Ancient Remains. This DSB Task Force was formed to study the issues and provide findings concerning the use of DNA comparison techniques for ancient remains identification. The primary purpose was to determine the feasibility of utilizing DNA techniques to identify unassociated ancient remains from past conflicts.

The Task Force heard presentations from a wide range of scientific and medical experts from within and outside the Department of Defense. We also reviewed written information from published and unpublished sources that was pertinent to our terms of reference.

The DSB Task Force has found that mitochondrial DNA (mtDNA) sequencing currently offers the best means of identifying those skeletal remains that cannot be identified through traditional means. The Task Force finds that current DNA identification efforts are supported by sufficient scientific evidence to proceed, in particular with application of mtDNA sequencing to ancient remains from the Korean conflict.

The Task Force also finds that operations at the Armed Forces DNA Identification Laboratory (AFDIL) are conducted appropriately such that families can rely on the results generated.

Joshua Lederberg
Task Force Chairman

Report
of the

DEFENSE SCIENCE BOARD TASK FORCE
ON
THE USE OF DNA TECHNOLOGY
FOR IDENTIFICATION OF ANCIENT REMAINS

January 20, 1995

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REPORT SUMMARY

The Defense Science Board (DSB) was requested by the Defense POW/MIA Office (DPMO) to address key issues arising from efforts to identify skeletal remains using new DNA testing technologies (Annex A and B). The Armed Forces DNA Identification Laboratory (AFDIL) of the Armed Forces Institute of Pathology (AFIP), a Department of Defense organization, is currently performing this testing under a Memorandum of Agreement for the U.S. Army Central Identification Laboratory, Hawaii (CILHI) of the U.S. Army Casualty and Memorial Affairs Operations Center (CMAOC) with funds from the U.S. Army Deputy Chief of Staff for Personnel (DCSPER).

A priority of the United States government has been the recovery and identification of the remains of American servicemembers from Southeast Asia, the Cold War era, and Korea. Currently, there are over 2,200 servicemembers from Southeast Asia, 132 servicemembers from the Cold War period, and over 8,100 servicemembers from the Korean conflict, whose remains have not been recovered and/or identified.

Current remains testing by the AFDIL involves mitochondrial DNA (mtDNA) sequencing, a new technology used only by a few laboratories in the world for forensic identification purposes. The Assistant Secretary of Defense (Health Affairs) [ASD(HA)] with input from civilian organizations developed a Quality Assurance Program for mtDNA testing of ancient remains. An important component of this program is the formation of an oversight committee composed of civilian technical consultants.

MtDNA testing is currently performed on repatriated remains with a name association, primarily from Southeast Asia. It is estimated that 500 cases from Southeast Asia will require mtDNA testing over approximately 5 years.

It is estimated that 3,000 remains could be repatriated from North Korea. This is in addition to the 200 remains repatriated by North Korea already at CILHI and the 865 unidentified American remains from Korea interred in the National Memorial Cemetery of the Pacific, Honolulu, Hawaii. The vast majority would require

mtDNA testing due to lack of adequate dental and medical records. These remains would not generally have name associations. Accordingly, a database of family reference mtDNA sequences would be constructed and mtDNA sequences of these remains would be compared to it.

The DSB Task Force finds that mtDNA sequencing currently offers the best means of identifying those skeletal remains that cannot be identified through traditional means. The Task Force finds that operations at the AFDIL are conducted appropriately, such that identification of military remains using mtDNA technology, is defendable and that families can rely on the results generated in these **cases.** The Task Force finds that mtDNA sequencing in conjunction with other nonDNA evidence could provide identifications on the unassociated Korean remains. The cost of this program over the next 12 years would be approximately $2 million annually over existing funding levels.

Principal Conclusions:

The Task Force finds that current DNA identification efforts are supported by sufficient scientific evidence to proceed, in particular with application of mtDNA sequencing to identify ancient remains from the Korean conflict.

The Task *Force* supports the Assistant Secretary of Defense (Health Affairs) in the creation of a scientific advisory board composed of civilian technical consultants.

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INTRODUCTION

Throughout United States history, the military services have to the best of their ability attempted to recover and identify it's deceased military personnel. In 1981, President Reagan placed the issue of accounting for American servicemembers from Southeast Asia as a matter of highest national priority. This position has been reaffirmed by all Presidents since. The Department of Defense has been tasked to investigate and account, to the greatest extent possible, for the "unaccounted for" Americans and repatriate, identify, and return the remains to their families. Today, there are over 2,200 servicemembers from Southeast Asia, 132 servicemembers from the Cold War era, and over 8,100 servicemembers from Korea, whose remains have not been recovered and/or identified (POW/MIA Fact Book, Department of Defense, October 1992).

The United States Army Central Identification Laboratory, Hawaii (CILHI) of the U.S. Army Casualty and Memorial Affairs Operation Center (CMAOC) is responsible for the recovery, identification, and processing of human remains from previous conflicts. CILHI uses traditional forensic odontological and anthropological methods, as well as other state of the art methods to identify human remains, including the Computer Assisted Post Mortem Identification (CAPMI) dental system and computerized craniofacial superimposition. CILHI uses state of the art photographic, microscopic, and radiographic equipment to accomplish their mission. The availability of records, the passage of time, and the environment to which remains have been exposed are obstacles to traditional identification efforts. **Emerging** technologies Offer new opportunities.

In 1991, the Army contacted the Armed Forces DNA Identification Laboratory (AFDIL), a division of the Office of the Armed Forces Medical Examiner (OAFME) at the Armed Forces Institute of Pathology (AFIP) to apply mitochondrial DNA (mtDNA) technology to

the identification of human remains. The mtDNA analysis and other corroborating evidence has since successfully identified the remains of Americans recovered in Southeast Asia. This technology now offers the prospect of identifying remains in the absence of name association, dental, medical, fingerprint, or circumstantial evidence.

The AFDIL has been in the world-wide vanguard of activities to identify remains through the use of DNA technology, and thus carries on the fine tradition of military biomedical research. From the first command-directed immunization program, inoculation for smallpox in President Washington's Army, up to and including the present time, many military and civilian medical scientists continue to make seminal contributions to military and general medicine. Among the many contributions, are Beaumont's studies of digestion in 1824; the founding of the first American School of Preventive Medicine and Public Health in 1893; and Walter Reed's proof that mosquitoes transmit yellow fever in 1900; anti-malarial drugs such as mefloquine halotantrine; vaccines such as VEE, typhoid, hemorrhagic fever, adenovirus and meningococcus; plasma and albumin blood products, and CPDA-1, AS-1 and AS-3 blood preservatives. The fields of burn therapy and emergency medicine have their roots in the military and are largely patterned after developments in military medicine. Through the Advanced Research Projects Agency, the military funded efforts that led to the development of the CAT scan and MRI. (Dora Strother, Army Science Board, 50 Years of Accomplishments in Army Research and Development, Social & Scientific Systems, Inc. Bethesda, MD)

The benefit of this military initiative and leadership can now be seen in the specialized area of DNA analysis of human remains. The use of mtDNA identification on remains by AFDIL continues that legacy of pioneering advances in medicine that contribute to society at large. This national resource can be used to assist in the identification of remains not only of servicemen and women who died in battle, but also to other disaster related deaths such as aircraft mishaps, earthquakes, explosions, and fires.

Despite difficulties in extracting mtDNA from ancient remains, the AFDIL has been successful in positively correlating mtDNA extracted from skeletal remains to their maternal relatives in

many CILHI cases. In addition, the AFDIL has performed the mtDNA sequence analysis on the skeletal remains and awaits family blood reference specimens for comparison in numerous other CILHI cases.

In one case, "X-6", conflicting results were received from the mtDNA analysis of remains analyzed by the AFDIL and a laboratory of the University of California-Berkeley. The source of the discrepancy has not been determined. This discrepancy case caused the Department of Defense to re-examine the use of mtDNA and to take several actions.

First, uncertainty concerning the efficacy of mtDNA technology created by the discrepancy in case "X-6" caused the U.S. Army on February 3, 1994, to suspend using mtDNA to determine the identity of war remains without corroborating evidence. The Department of Defense supported that position.

Second, the Army recommended that the Department of Defense Science Board (DSB) establish a task force to examine the issues associated with using mtDNA to identify remains. In May 1994, the Defense POW/MIA Office (DPMO) accepted responsibility to be the Department of Defense sponsor for this Task Force. On June 20, 1994, the Under Secretary of Defense requested the Chairman of the Defense Science Board establish a Task Force on the use of DNA technology for identification of ancient remains. The issues (Annex A) for the members of the DSB Task Force (Annex B) were incorporated into a set of Terms of Reference (TOR).

Third, the military reviewed the standards used to perform mtDNA testing and noted that a set of formally recognized and widely accepted technical and quality assurance standards did not exist specifically for mtDNA testing of ancient skeletal remains. It has become well-understood that minuscule levels of contaminants can lead to erroneous results and that extraordinary measures of quality control are needed. The ASD(HA), with input from the forensic and genetics communities, developed a quality assurance program for the Department of Defense that would have credibility and **acceptability** within the scientific and legal communities, to the families, and to the general public (Annex C) .

Fourth, portions of the contested remains were sent to the British Forensic Science Service (FSS, also known as the British Home Office) for analysis. Results confirmed AF'DIL's findings

(Annex D). Furthermore, when subsequently and unknowingly challenged by CILHI with skeletal remains of the same case, the AFDIL twice more obtained the same mtDNA sequence result.

On January 6, 1995, the Army concluded that they had confidence in the AFDIL testing results. The Department of Defense then gave approval to respond to all Army requests for analysis and to consider that Army requests have had a waiver, at Army level, to the Army imposed moratorium (Annex E).

Meanwhile, the issue of repatriation and identification of remains from the Korean conflict has come to the forefront. On August 24, 1993, the Korean People's Army (KPA) signed an agreement with the United Nations Command (UNC) for cooperation in the recovery, repatriation, and identification of UNC remains located north of the Demilitarized Zone (DMZ). Since 1990, the North Koreans have repatriated 208 coffins containing purported American remains. Because of the condition of these remains and the paucity of relevant personal, medical, and dental records for servicemembers serving in the Korean action, mtDNA analysis offers the best prospect to identify these remains.

The TOR represent the issues that needed to be addressed before the military proceeded with mtDNA testing of skeletal remains, particularly "unassociated" Korean remains. Paramount is assurance that the technology is cost effective and that families can depend on the methods of identification used.

I. FEASIBILITY

DSB TOR: **To determine the feasibility of using DNA techniques for identification of ancient remains as evidenced, in part, by success in identification efforts thus far.** ***[Is the conceptual basis for mtDNA identification of ancient skeletal remains workable? Is the discriminatory potential of mtDNA as currently obtained, and with or without other identification data, sufficient for individuation of skeletal remains from Southeast Asia and Korea?]***

Nuclear DNA typing has the capacity to be used for identification because DNA is different among all individuals, except identical twins. The potential exists for DNA tests to provide identifications which cannot be made in any other way. Any portion of skeletal remains could potentially be useful for DNA

identification. Since reference specimens for DNA comparison can be obtained from family members, it can be useful in situations even though premortem specimens are not available. In contrast, premortem records must be available for conventional identification methods using medical, dental or anthropological comparisons.

A. Forensic DNA Identification

Molecular techniques have revolutionized the biological sciences. Procedures for rapid DNA sequencing were developed in the 197Os, the Southern blot technique for DNA fragment sizing was developed a few years later, and the polymerase chain reaction (PCR) for DNA fragment amplification in 1985. These techniques have become well established and now are at the heart of innumerable research efforts in the biologic sciences. Molecular biologic techniques have long since moved from the research laboratory to the clinical service laboratory. The revolution created by this new technology has spread to the forensic sciences where DNA typing is taking its place alongside fingerprinting in terms of its impact on the criminal justice system. The basic molecular biologic principles are at this point well established and documented.

The Office of Technology Assessment released its report on the forensic uses of DNA typing in 1990. They concluded that "no scientific doubt remains that technologies already available can accurately detect genetic differences between humans? Similarly, the National Research Council (NRC) of the National Academy of Sciences issued a report in 1992, confirming the capability of DNA testing as a new and important technology to identify the origin of biologic trace evidence. Traditional serologic testing is based on genetic differences that are best characterized at the DNA level.

Courts of law have generally embraced the new DNA technology. The passage of the DNA Identification Act as a part of the 1994 Crime Bill by Congress, to spur creation of a national network of state DNA databases of convicted sex offenders and other felons, is a recognition of the value and validity of this DNA identification technology.

Application of DNA typing to the identification of human remains is obvious. Identification of tissue origin is being performed by many crime labs around the world. The AFDIL has been a leading laboratory devoted to the identification of human remains, and assisting other Federal Government agencies.

However, most DNA identification efforts have thus far focused on the typing of nuclear DNA of relatively recent vintage. Identification of ancient skeletal remains through mtDNA sequencing presents new issues which have not been a significant part of the larger discussions of the application of molecular biology to forensic identification.

B. MtDNA Sequence Identifications

Dr. Mary-Claire King first employed mtDNA to identify the Argentina "disappeared? In these cases, Dr. King would match the mtDNA sequence of children, whose parents were killed for political reasons, to that of purported maternal grandmothers. In 1991, Dr. Mark Stoneking described the use of mtDNA sequencing for the identification of a skull found in the Mojave desert, approximately four years after a 3 year-old girl was reported missing. In 1994, Dr. Peter Gill described the use of mtDNA to identify the Russian Romanov Tsar Nicholas II and his family.

In 1991, the AFDIL first successfully employed mtDNA sequencing to identify the skeletal remains of a servicemember killed in the Southeast Asian conflict; a report of this case was published in the ***Journal of Forensic Sciences*** in 1993. Subsequently, AFDIL has performed testing which has led to other identifications.

C. Mitochondria

Human mitochondria are thought to have evolved through incorporation of an intracellular symbiont (parasite) into early life forms (Lynn Margulis, Symbiosis and Cell Evolution, **1981,** Freeman Publishing). This intracellular symbiont, similar to a primitive bacterium, had its own DNA. The symbiont flourished within the cytoplasm in harmony with the host cell. Mitochondrial symbionts would pass into the daughter cells of every dividing cell. In time, cells came to depend on the efficient energy utilization mechanisms of this symbiont. This theory of endosymbiotic origin explains many of the peculiar features of this intracytoplasmic organelle.

Mitochondria are the primary means of oxidative respiration of the cell. They are critical to the utilization of oxygen from the air to generate usable energy in the form of phosphorylated compounds for the cell. Hence, mitochondria are considered to be the "powerhouses of the cell".

D. Mitochondrial DNA

Human mtDNA is a circular DNA "particle", 16,569 base pairs in length. The complete sequence from a composite of individuals was published in the journal ***Nature*** in 1981 by Anderson, et. al. An MboI restriction site within the major noncoding region was arbitrarily designated as the origin, and the base pairs are numbered sequentially proceeding clockwise (Figure 1). This published sequence is by convention used as a reference sequence in studies of human mtDNA variation, with polymorphisms usually indicated as differences from this reference sequence.

The mtDNA genome contains 37 genes, including 13 protein-coding genes, 2 ribosomal RNA (rRNA) genes, and 22 transfer RNA (tRNA) genes (Figure 2). The protein-coding genes include two ATP synthetase subunits, seven NADH dehydrogenase subunits, three cytochrome oxidase subunits, and cytochrome b. These proteins are all involved in electron transport and cellular respiration, the primary function of the mitochondria. All of the remaining several hundred proteins necessary for mitochondrial function (including those required for replication, transcription, and translation of mtDNA) are encoded in the nucleus, and hence must be imported from the cytoplasm.

The two complementary single DNA strands that comprise the double-stranded human mtDNA genome have an asymmetric distribution of guanine and thymine residues, and can be separated as heavy (H) and light (L) strands via ultracentrifugation. Most of the mtDNA genes are transcribed from the H strand, with only one protein-coding gene and eight **tRNA** genes transcribed from the L strand.

One of the most striking features of the human mtDNA genome is the extreme paucity of noncoding sequence. Only 7% does not encode proteins, rRNA, or tRNA, and the coding regions do not contain intervening sequences. By contrast, it is estimated that at least 90% of the nuclear DNA genome is noncoding, and large

and frequent intervening sequences are the rule for nuclear genes. Intergenic regions in mtDNA are usually less than lObp in length, and for some of the genes polyadenylation of the mRNA transcript is required to form the termination codon. Approximately 90% of the noncoding mtDNA consists of the control region (displacement loop or D-loop), an 1,100 base pairs region that includes the H-strand origin of replication and origins of transcription for both strands. The L-strand origin of replication is located in a noncoding segment of 31 base pairs, located about 5,700 base pairs from the control region.

Cells typically have several hundred to several thousand copies of DNA, each with one to ten mtDNA molecules, whereas most nuclear genes exist in just a single paired complement per cell. Thus, for ancient remains where there may be very small amounts of surviving DNA that is highly degraded, the probability of obtaining a DNA type is greater for mtDNA. For some types of remains, such as telogen (shed) hairs or keratinized skin, nuclear DNA appears to be absent while mtDNA is still present.

Human mtDNA is strictly maternally inherited. Accordingly, the DNA in mitochondria is not present as pairs of genes, one maternal and one paternal, as is the nuclear DNA of chromosomes. Thus, despite the high'number of copies, only a single sequence is found and recombinational events do not occur.

Spermatozoa have about 50 to 100 mitochondria in the midpiece, which provides the energy for the spermatozoa to swim. While the midpiece does penetrate the egg upon fertilization, it is not clear what subsequently happens to the paternal mitochondria. It may be that the paternal mitochondria are preferentially sequestered and destroyed. Prior to fertilization the maternal mitochondria increase to about 100,000 to 200,000 in the oocyte (egg). Maternal inheritance may simply reflect this greatly enhanced abundance of maternal mtDNA relative to paternal mtDNA in the egg. In addition, a bottleneck theory has been proposed in which only a few copies of the oocyte mtDNA are actually replicated, excluding the paternal copies from proliferating. Some studies have reported a low level of paternal mtDNA inheritance in Drosophila flies and in mice on the order of 0.01 to 0.001% per generation. However, these studies utilized interspecies crosses and could therefore reflect the peculiar nature of the hybrids. Regardless of the mechanism involved, no exception to maternal inheritance has ever been reported in

humans.

In the absence of new mutations, maternally-related individuals should have identical mtDNA types. For the purposes of individual identification, any maternal relative can therefore serve as a reference. The maternal line includes biological mothers, siblings, maternal aunts and uncles, children of sisters, and children in the case of an unidentified deceased female servicemember. The family relation can be distant. For example, fifth generation maternal relatives were used successfully to identify the remains of the last Tsar of Russia and the Royal family.

Critical for identification purposes, mtDNA is highly polymorphic, differing between most individuals.

E. Polymorphisms

The highly streamlined nature of the vertebrate mtDNA genome initially led to the expectation that it would be highly conserved evolutionarily. In the late 1970's it was postulated that mtDNA evolves, on average, 5-10 times more rapidly than nuclear DNA. Further studies in the laboratories of Dr. Douglas Wallace and Dr. Allan Wilson showed that there were high levels of mtDNA polymorphism within humans. These studies were based on analyses of restriction fragment length polymorphisms (RFLPS) across the entire human mtDNA genome. RFLP analyses have continued to be a valuable source of information concerning human mtDNA variation, evolution, and disease. However, for individual identification purposes, attention has focused instead on DNA sequence analysis of the control region which is known as the displacement loop or D-loop (Figures 3 and 4).

The control region is the major noncoding segment of human mtDNA, and is the most polymorphic segment as well. It is a region of greater than one thousand base pairs (16,024-576), which is instrumental in the regulation and initiation of synthesis of the gene products and replication of the mtDNA. Elsewhere, the mtDNA sequence is highly conserved. Consequently, the level of polymorphism in the coding region is about one third that of the

control region.

Sequence analysis of the entire control region demonstrates that variability is not distributed at random, but rather is concentrated in two hypervariable HV) segments of about 400 base pairs each, with the first segment (HVI) having about twice as much variability as the second segment (HV2) (Figures 5 and 6). Virtually all subsequent studies of control region sequence variation have therefore analyzed either HV1 alone or both HV1 and HV2.

These studies have revealed much higher levels of mtDNA sequence variation than nuclear DNA sequence variation. Dr. Peter Gill of the Forensic Science Service, sequenced HVl and HV2 from 100 British Caucasians and found the level of mtDNA nucleotide diversity to be l.l%, while the level of nuclear DNA nucleotide diversity has been estimated to be at most about 0.11%; thus, the amount of mtDNA nucleotide diversity in the control region is about ten times the amount of nuclear DNA nucleotide diversity.

It is not clear why mtDNA evolves so rapidly. The rate of evolution is a function of the rate at which new mutations arise, and the rate at which mutations become fixed; there is reason to suspect that both of these factors are elevated for mtDNA. Highly mutagenic by-products of respiration, such as free radicals, are known to be present in mitochondria, and while some repair of mtDNA damage apparently does occur, it does not seem to be as efficient as DNA repair in the nucleus. The rate at which mutations become fixed in a population is inversely related to the effective size of the population, and because of the maternal inheritance of mtDNA, it has a smaller effective size than nuclear DNA. Still, further work is required to understand how mtDNA mutations arise and spread before a general theory relating mtDNA polymorphism, mutation rates, and evolutionary rates can be developed.

F. Ancient DNA

In order for DNA to be useful to the identification of individuals, the DNA must remain sufficiently intact and capable of extraction.

A new field of scientific endeavor has emerged which seeks to extract and amplify DNA from very old biological materials, such

as woolly mammoths, moas, sabre-toothed tigers, and mummies. The goals of these endeavors are diverse, but focused on evolutionary studies or population migrations and origins. This discipline is known as "molecular archaeology", "molecular anthropology" or "molecular paleontology", and those in this field study "ancient DNA".

Books have been written on the subject of ancient DNA and the community has organized itself. An informal newsletter is distributed and two international conferences on ancient DNA have been held (Nottingham, England, 1991; Washington, D.C. 1993). ***Jurassic Park*** was written by Michael Crichton from the machinations of the Extinct DNA Study Group at the University of California-Berkeley, a founding group of this community.

There is a background of important scientific work from which to draw in performing DNA analyses of ancient skeletal remains. The common ground between all scientific efforts in the field of ancient DNA and military skeletal remains identification of the Vietnam and Korean conflicts is the extraction of DNA information from samples in which the DNA is so extensively broken into small fragments and extensively damaged. The techniques and concerns of molecular anthropologists in the analysis of ancient DNA are **applicable to** military skeletal remains identification efforts.

G. Polymerase Chain Reaction

DNA from skeletal remains is degraded or broken down into very small fragments. This is particularly true of ancient DNA, where the average fragment size may be less than one hundred bases. Furthermore, most of the original DNA has been destroyed or washed away so that very low quantities of DNA are present and recovering DNA sequence information from old skeletal remains poses a significant technical challenge.

The polymerase chain reaction (PCR) is a method to amplify a target region of DNA. PCR is an enzymatic reaction resulting in the exponential production of copies of a given DNA segment, where the copies produced can be themselves copied. A million-fold increase in the number of copies of target DNA is often accomplished by PCR, permitting further analysis. Accordingly, PCR-based testing is exquisitely sensitive. This PCR **amplification** technique has revolutionized DNA testing and all molecular biology. PCR is of such significance that Dr. Kary

Mullis was awarded the 1993 Nobel Prize in Chemistry for its discovery. DNA testing of minimal and degraded DNA of ancient skeletal remains is made possible by the PCR method of amplification.

H. Military Skeletal Remains Identification

Substantial medical and/or dental records and nonDNA evidence exist to identify the majority of remains recovered from Southeast Asia using traditional methods; mtDNA testing would not be required in most cases where intact remains can be recovered. It is estimated by CMAOC that approximately 500 cases would require DNA tests, however, the number is unavoidably speculative. The AFDIL is currently resourced to perform mtDNA testing at a rate of ten cases per month. These skeletal remains involve cases with name associations and living family members are known in nearly all cases. The AFDIL will normally obtain the bone mtDNA sequences from the remains and then compare them to family reference blood specimens. Many families have already donated blood specimens in hopes that they may be useful for identification of their family member. The identification efforts using mtDNA have been highly successful and are anticipated to continue for the next five years.

The United Nations Command Military Armistice Commission (UNCMAC), representing the 16 nations that supported the South Korean government in the Korean conflict, has continued to press for the repatriation of the remains of UNC servicemembers since the termination of hostilities in 1953. During Operations Little Switch and Big Switch in 1953, a total of 3,748 U.S. POWs (out of a UNC total of 13,457) were repatriated. In 1954, the remains of 1,868 U.S. servicemen (out of a UNC total of 4,023) were repatriated in Operation Glory. Of these remains, 866 are declared unknown and 865 were buried in the National Memorial Cemetery of the Pacific (Punchbowl), Honolulu, Hawaii.

The total of over 8,100 U.S. servicemembers (out of a UNC total of over 10,200) includes those remains that have not been recovered, and were buried in known UNC cemeteries in North Korea, lost or buried at sea, and others who were unaccounted for with the body not recovered. Also included are 389 personnel (out of a UNC total of 2,233) about whom the Korean People's Army (KPA) and the Chinese People's Volunteers (CPV) should have knowledge. Information gathered from intelligence sources and

POW debriefings suggested that these were individuals who were possibly captured and died under **KPA/CPV** control.

In May 1990, for the first time since 1954, the KPA repatriated remains. Over 200 remains have been turned over in recent years. However, the change of power in North Korea has brought uncertainty into the future of remains repatriation..

The poor condition of remains recovered from Korea, the lack of records necessary to make an identification, and the inability to make joint recoveries have largely impeded CILHI's ability to identify these remains using traditional methods. In 1973, about 80% of the necessary medical and personnel records were destroyed in a fire at the National Personnel Records Center in St. Louis, Missouri. Existing records of that era provide a dearth of medical and dental information. Recent repatriations include many cases in which the midfacial portion of the skull is missing and thus the most useful anthropologic features for identification are absent. Accordingly, mtDNA testing is anticipated to be used in most Korean cases.

The Casualty Data section of CILHI has established preliminary figures for the number of recoveries through joint United States/KPA recovery operations and investigations in North Korea. There is sufficient information currently existing to establish a known location, where the recovery of remains is possible for 2,400 of 6,000 remains located in North Korea. These include 1,612 reported burials in former POW camps with known locations, 181 reported interments in known temporary cemeteries north of the demilitarized zone (DMZ), and 633 known aircraft loss sites north of the DMZ. In an additional 568 incidents, sufficient information is known which may lead to remains recovery. These include 535 reported POW camp burials with no known location, **1** reported interment in a cemetery with no location, and 32 aircraft losses without a fixed or general location. Most cases represent burials by American personnel during advances before reoccupation by the KPA. Accordingly, CILHI estimates the upper limit of the reasonable prospect of the number or individuals that may be recovered through joint investigations and recoveries is about 3,000.

I. NonDNA Identification Evidence

For Korean cases, nonDNA evidence, such as location of recovery

and forensic anthropologic and dental examinations, can provide identifying information, but due to the paucity of antemortem medical and dental records, these will serve primarily to limit the servicemembers considered for a potential mtDNA match. CILHI scientists estimate that they will be able to narrow the possible name associations to an average of 25 from the overall pool of the approximately 8,100 cases based on age, race, and dental characteristics in the vast majority of cases and in some cases to within five individuals.

J. Current Methodology

The process of mtDNA testing ancient remains is technically difficult and demanding, but AFDIL now has considerable experience. The first steps involve the preparation of the sample. A portion of bone (about two grams per extraction) is cleaned to prevent cross contamination and to remove any mineralization that inhibits mtDNA testing. The sample is pulverized; from the bone powder, DNA is extracted. A quick analysis of the quality and purity of the DNA recovered is performed at that stage. The region of the mtDNA to be analyzed is then amplified using PCR. Multiple copies of specific segments of the mtDNA that are to be analyzed are generated until sufficient material for the sequencing reaction is produced. Once this process is completed automated sequencing instrumentation can then read the exact sequence of the mtDNA molecule. Finally, the mtDNA sequence from the family reference specimen is compared to that of the skeletal samples. The amplification and sequencing scheme employs two overlapping sets of primer sets which allow for further confirmation of sequencing results (Figure 7).

The Task Force finds that identification of so-called ancient skeletal remains by a program of mtDNA testing is possible, particularly in association with other information. A few **specimens may remain unresolved. Although contenders may emerge; at this time, mtDNA sequencing technology is the most appropriate technology.**

II. FACTORS

DSB TOR: To evaluate factors that might influence the effectiveness of using mtDNA techniques. *[What factors might effect the utility of mtDNA testing for identification purposes, e.g. age of the skeletal remains, manner of interment, environmental temperature, acidity of soil, limitations of sample availability, commingling, etc? Do environmental factors affect the mtDNA sequence results obtained? How does somatic mutation and heteroplasmy affect mtDNA typing efforts? To* what *extent may the lack of family reference samples impede mtDNA typing efforts? What is the likely average number of bases obtained from Southeast Asian and Korean remains? If this data are not available, what size sampling would be sufficient for establishing reasonable estimates? How will these limitations of mtDNA sequence information available from Southeast Asian and Korean cases affect the ability to identify unassociated remains?]*

Many factors may affect the ability of the military to perform accurate and successful mtDNA identifications on the skeletal remains.

A. Skeletal Remains

Recovered remains from Southeast Asia vary **drastically in their** quantity and quality. Some skeletal remains are virtually complete, where others have completely disintegrated, dissolving in the acidic soil. The integrity of the remains is affected by the manner of interment, length of interment, environmental temperature, and the acidity of the soil. In some cases, a hot environment can preserve by permitting thorough desiccation; in some cases, total submersion can permit preservation. Anthropologists note that even within the same burial site remains may demonstrate great differences in their degree of preservation. Thus, small changes in the environment can cause substantially different rates of degeneration.

Bone samples from Southeast Asia have demonstrated that they harbor only small amounts of mtDNA, and that it is severely fragmented. The variance in the quality of mtDNA within the bone samples is significant and unpredictable. The quality of the bone and the ease of obtaining genetic information from the bone

varies between bones from the same individual. Skeletal remains of the last Russian Tsar, Nicholas II, tested 73 years after his death, yielded relatively abundant quantities of large mtDNA fragments despite their age. The determination of whether or not a sample of bone will yield mtDNA information is simply a matter of trial. A second sampling of bone may yield mtDNA when the first sample did not. Teeth may yield mtDNA when bone will not.

There are indications that extraction of mtDNA from skeletal remains from North Korea may be significantly easier and more productive than from skeletal remains from Southeast Asia. The climate of Korea is far cooler and drier than Southeast Asia. Visual inspection of the quality of remains from Korea reveal that they are in reasonable condition relative to remains recovered from Southeast **Asia.** The AFDIL experience with Korean remains suggests they may not be any more problematic than those from Southeast **Asia.**

Recent repatriation of Korean remains disclose an-80% rate of admixture of different remains or "commingling? This is often easily recognized when, for instance, two right femurs **are** submitted; other times commingling is more difficult to diagnose. Joint recovery operations, which could include the use of more experienced U.S recovery teams could substantially decrease the rate of commingling. Commingled remains may require multiple mtDNA samplings.

B. Family Reference Specimens

A lack of family reference samples may prohibit effective mtDNA identification. If necessary, family references could at least theoretically be obtained from exhumed remains of deceased family members. Occasionally, pre-mortem reference specimens such as locks of hair, paraffin-embedded material from prior biopsies, and neonatal bloodstains can be obtained to compare with the deceased individual. The ability of the military to find appropriate kindred is **a** factor in the success of mtDNA identifications. Unfortunately, the immediate kindred of servicemembers from the Vietnam and particularly the **Korean** conflicts are an aging population and hence the availability of family references is rapidly diminishing. There are indications

of interest by many families, suggesting they will cooperate by donating blood specimens (section V., Family Reference Database, p. 29).

C. Discriminatory Potential

The full discriminatory potential of the D-loop sequence is not yet known, since the region has been sequenced from relatively few people. Samples of sequences from large Caucasian populations have shown no sequence occurring with a frequency of greater than 3%. Sequences occurring with higher frequency would have almost certainly been detected. More frequent sequences have been found within some native African tribal populations. Coincidental matches between unrelated members are therefore likely to arise only a few percent of the time.

The amount of information obtained from family members will quickly allow greater precision to be attached to statements of discriminatory power, as will the sequences from remains. The latter sequences may, however, be of less than the complete hypervariable regions. Once 500 sequences are available from a population, a particular sequence can either be assigned its observed frequency or said (with over 99% confidence) to have a frequency of less than 1% if it is not observed in the sample. However, sufficient data are available to conclude that a high discriminatory power is achievable by current methods and can be used for reasonable estimates of mtDNA sequence population frequencies.

Although, the DNA information from hypervariable regions I and II will permit great discrimination, the sequence information actually obtained from ancient samples may be limited, reducing the discriminatory power. The full mtDNA sequence may not be obtained due to amplification failure by one or more sets of primers, fading of terminal sequence signal, or from internal sequence ambiguities. The AFDIL is very conservative in determination of sequence or "base calling" and hence may not call an ambiguous, though informative base. Accordingly, the lack of full sequence information may result in limitation of the full potential discriminatory power in a given case.

Another potential limiting factor is the occurrence of multiple servicemembers of the same lineage. For example, brothers and maternal first cousins will have the identical mtDNA sequence.

The rate of maternal kindred relations among servicemembers is not known. The rate for servicemembers serving in Korea is likely somewhat higher than current frequencies, due to the larger families during that time period. Regardless, families themselves should typically have some idea of the existence of other kindred servicemembers who might potentially confound an identification.

D. Mutations

The mtDNA sequence is identical throughout the body unless a somatic mutation arises. Investigations by Monnat and Reay demonstrated that the mtDNA control region does not differ among the various tissues of the body. An investigation of 83 retinal cell clones, resulting in 32,000 bases of DNA sequence (from both the D-loop and coding regions), revealed only one mutation (in a tRNA gene).

More extensive age-related changes have however been found in other post-mitotic cells (Annex F). Bone and teeth have not yet been extensively studied. While somatic and germline mutations should be vigilantly looked for, they should not vitiate mtDNA identification in the majority of cases.

The polymorphic nature of mtDNA, especially within the control region, is evidence of mutation, and surveys support a widely held belief that mutations occur at a higher rate in the mitochondrion than in the nucleus. Whatever the mechanism for this elevated rate, it makes the mitochondrion useful for human identification. The disadvantage with high mutation rates is that sequences within the same maternal lineage, especially when separated by several generations, may exhibit occasional differences.

Although no comprehensive studies have been performed, evolutionary studies have estimated that the average fixed mutation rate for the mtDNA control region is one nucleotide difference per every 300 generations, or one difference every 6,000 years. Consequently, one would not expect to observe many examples of nucleotide differences between maternal relatives.

The AFDIL has observed at least two examples of fixed sequence differences between mother and child in approximately 30 maternal relative comparisons evaluated by AFDIL during routine casework.

In one case, there were two nucleotide differences between a mother and her daughter, but not in her two sons. The maternal relationship was verified using 12 DNA markers, including 8 RFLP loci.

The AFDIL surveyed 46 mother-child comparisons in a controlled study of family specimens from the Centre d'Etude du Polymorphisme Humaine (CEPH), showing no sequence differences. The AFDIL is completing an extensive study across three hundred generations which will help to further define the fixed mutation rate. Preliminary data suggest that the observed rate will be closer to one nucleotide difference every 50 generations. Dr. Mark Stoneking has found similar results in a recent study of the resident population of Tristan da Cunha, a small island off the Atlantic coast of South Africa.

This higher than expected rate of mutation may be explained by so-called "hot spots" which have a higher mutation rate than other regions even within HVl and HV2.

E. Heteroplasmy

The condition in which only a single mtDNA sequence (albeit in many copies) is present in a cell is termed homoplasmy. The possibility exists that a subpopulation of mitochondria could harbor a different mtDNA sequence due to mutation or paternal contribution; this is termed heteroplasmy. Heteroplasmy is most often seen in disease states, in which a mtDNA sequence is defective (e.g. deletions, duplications) and hence at a competitive disadvantage. Stable heteroplasmy in humans although rare has been described.

Measuring the fixed mutation rate does not take into account the rate of heteroplasmy during the process of fixing a particular mutation. It is not known whether most mutations are manifested through many generations via heteroplasmy or if they are fixed during a single generation. Examples of each have been observed (i.e., the Russian Tzar was reported to be heteroplasmic, despite the lack of heteroplasmy in other family members). The consensus of the Task Force Committee is the level of heteroplasmy within the control region of humans is generally low.

F. Environmental Damage

Environmental changes do not alter the mtDNA sequence. Long term exposure has resulted in depurination in which Guanines result in Adenines after PCR, but this has been observed only in material thousands of years old. This does not appear to be a problem with the identification of skeletal remains from the Vietnam and Korean conflicts. If environmental damage could be assessed in a rapid and cost effective manner, then that information could be used as indication of the quality of the data obtained or the need for repair of the DNA before amplification.

G. Contamination

The mtDNA present in ancient skeletal remains is minimal. The mtDNA that had existed has largely been enzymatically or chemically broken down into small fragments and much has been leached away. The AFDIL reports that only a few dozen target mtDNA fragments are obtained by their extraction techniques on skeletal remains from Vietnam. Therefore, the methods of mtDNA typing must be very sensitive. The enhanced sensitivity required for this type of testing (PCR-based) also means that it is capable of detecting trace contaminants.

MtDNA contaminants can come from a variety of sources. Contamination through proximity to other remains and through specimen handling in the field is not thought to be a significant source, especially when a laboratory removes the outer surfaces of bone samples prior to mtDNA extraction. Shed skin or exhaled droplets from individuals performing the extraction is another potential source of contaminating mtDNA. The mtDNA sequence of laboratory personnel must be determined so that their sequence types will be recognized as a possible contaminant. The most serious contamination concern is cross-contamination by PCR product within a laboratory. Millions of copies of the mtDNA target are generated during the PCR process. If a minute quantity of this product is allowed to contaminate another mtDNA extract, the contaminating product may mask the true mtDNA type of the extract. When all appropriate precautions are taken, random sporadic contaminants, not attributable to any known source, may be encountered.

Controlling contamination is vital to the success of a PCR-based mtDNA testing program, especially when targeting old skeletal remains. A range of precautionary measures tailored to the specific laboratory are necessary and when anticipating high

volumes of mtDNA testing, further measures must be considered to control the higher potential contaminant burden. Some examples of possible useful precautions are discussed below.

Control of contamination must begin with the physical separation of DNA extraction and amplification set-up activities (pre-PCR) from PCR product analysis (post-PCR). In addition, all steps in the extraction and amplification set-up procedures should be performed in a hood. Areas for extracting low levels of mtDNA from skeletal remains should be separated from high level extraction areas. Anterooms or vestibules may be placed at the entrance of each laboratory where mtDNA testing is being performed to help prevent the transfer of PCR product from one laboratory to another. When possible, mixing of the air circulation between pre-PCR and post-PCR areas should be minimized.

Dedicated equipment should be used for amplification setup and in PCR product areas. Positive displacement or plugged tip pipettors should be used for aliquoting samples and PCR reagents. Use of laboratory coats and gloves are essential. Laboratory coats should be disposable or dedicated to the areas where mtDNA PCR product is being handled. Care should be taken when wearing disposable gloves not to touch any surface which may contain a contaminant such as the surface of the skin, eye glasses, clothing or even a cleaned bench-top. As a common practice, before handling evidence or items which come in contact with evidence, always change gloves or wipe gloves with bleach, allowing the gloves to air dry.

General cleaning practices are important for controlling contamination. The universal cleaning agent for PCR contamination is 10% commercial bleach (7 mM sodium hypochlorite). The bench-tops, hoods, and any surface which comes in contact with the evidence or DNA extract should be washed with bleach frequently. In addition, the floors of each laboratory should be periodically washed with bleach.

To illustrate the impact of proper laboratory design and practices, a representative number of cases were evaluated from the AFDIL laboratory. One set of data represented the conditions under which cases were processed in 1991. The second set represented cases performed in 1994. The number of times PCR product was observed in an extraction reagent blank control or a

PCR reagent blank control was counted for each case. For seven cases processed in 1991, 15% of the extraction reagent blanks and 7% of the PCR reagent blanks showed PCR product following amplification. For five cases processed in 1994, only 8% of the extraction reagent blanks and 3% of the PCR reagent blanks showed PCR product following amplification. These numbers indicate how sensitive mtDNA testing is to contamination, but also illustrate that significant progress can be made to limit the occurrence of contamination with improvements to laboratory design and practices.

Appropriate controls and measures to detect contamination are imperative. Both extraction reagent blank and PCR reagent negative controls should be run in every case to detect the occurrence of contamination. Documentation of contamination will allow for review of the major sources and will assist in developing a comprehensive quality control program for controlling PCR product contamination.

Where a contaminant is detected, testing should be repeated, if possible, unless the sequence from the skeletal remains is otherwise determined to be reliable. There may be instances when contamination prevents the reporting of results.

In general, other than mtDNA testing of skeletal remains, it is uncommon to find amplification product in a reagent blank during any PCR-based testing. Accordingly, the AFDIL rarely finds a positive amplification product in the extraction reagent blank for a whole blood maternal reference.

When contamination does occur, it will typically result in an apparent nonmatch (false exclusion), not a spurious match (false inclusion). Therefore, all exclusions should be carefully scrutinized. The AFDIL procedures preclude a false match due to contamination by a mtDNA blood reference. All blood specimens are tested in a different area of the laboratory. Moreover, the whole blood maternal reference is usually processed after the skeletal remains, and in most cases after the results generated from the skeletal remains have been reported. Only during databasing operations will there be the occasional situation when reference results are generated prior to the skeletal remains results.

An important quality check of the skeletal remains sequence is the independent extraction and testing of multiple bones. Multiple bones are independently extracted in most cases by the AFDIL, unless only a single specimen is available for testing. In addition, the primer sets used for amplification are overlapping, providing further confirmation of the authenticity of the skeletal sequence.

Given the inevitable random contamination inherent in mtDNA testing, redundancy, when possible, at the level of source material is key. Cautionary statements are important where replicate testing cannot be performed.

H. PCR Amplification Ambiguities

Taq polymerase, an enzyme used in current PCR amplification reactions, is known to occasionally and randomly misincorporate erroneous nucleotide bases at a rate of approximately one to ten in 10,000. Generally this is not problematic, because correct sequences will overwhelmingly predominate. However, if the starting target sequence consists of only a few copies, then the chance of a false result from misincorporation during the first few rounds of thermal cycling becomes a possibility.

Nonspecific priming may occur, particularly where the starting conditions are not optimal. This may result in ambiguous or errant sequence when the starting DNA concentration is very low.

During PCR amplification, the polymerase enzyme may stop due to template fragmentation or damage. The partially extended sequence may then anneal to another template fragment in the next cycle. In fact, several fragments may be assembled in this process to recreate the original full length sequence. This process, known as "jumping PCR", may complicate the interpretation of diploid sequences. In mtDNA, where only one sequence is present, "jumping PCR" may have advantages. However, it may also produce errors from incorrect assembly of fragments so small that they have lost their specificity. Duplication of testing results or overlapping sequence data will allow for interpretation of these occurrences.

I. Sequencing Ambiguities

The evaluation of DNA sequencing data is extremely tedious and time consuming. Evaluation of data using manual sequencing methods may result in transcription and reading errors. Automated sequencing methods will minimize reading errors. Nonetheless, sequencing errors do occur in automated analysis; rates have been published for current instrumentation. Most instrumental error can be avoided by limiting the information read to shorter lengths, because the vast majority of errors occur near the end of a sequencing run as the resolution and strength of the base signals diminish. The automated sequencing instrument used by the AFDIL has an error rate of approximately **1 to 2%** for the size templates analyzed (200-300 base pairs). This error rate increases to greater than **10%** when longer templates (greater than 450 base pairs) are analyzed using current

conventional sequencers. The error rate does not represent errors in the sequence reactions, but instead represent errors in the ability of the instrument software to make an accurate base call.

In large scale sequencing operations, where a maximal quantity of sequencing information is emphasized, some errors can be tolerated. This is not the case in the forensic context. Visual evaluation of the data is mandatory to prevent read errors. Moreover, it is standard practice to confirm sequences, usually by checking complementarity of the reverse strand sequence. With the advent of new sequencing technologies, it may be possible to confirm sequences by a second technology which may not have a tendency toward the same systematic errors. At least two qualified individuals must independently evaluate the sequence in order to ensure that the results accurately represent the data, and to catch transcription errors. It should be recognized that redundancy in casework also provides an opportunity to catch errors; a case involves multiple bones and at least two family blood reference specimens where available. These procedures can virtually eliminate sequencing errors.

J. Casework Experience

Since October of 1994 when the AFDIL began full production of ten cases per month, it successfully obtained sequence information from Southeast Asia, Korean, and World War II cases representing 37 individuals. The AFDIL has achieved a success rate for obtaining DNA sequence information of greater than 95% from CILHI casework since late 1992, even for retested cases from 1991 Southeast Asia casework when the original success rate was only 40%. This does not mean that the AF'DIL obtains sequence information from every bone, nor does it mean that the AFDIL obtains full sequence information in every case. The AFDIL obtains sequence results from little more than half of the bones it tests.

CILHI casework is not routine in the sense that, in virtually every case problems are encountered that require retesting, often using modified test conditions. In the first six months of production mtDNA sequencing operations, an average of 3.7 bones were tested per case, 42% of extractions had to be repeated, 37 amplification reactions were required (2.5 times the minimum), and 55.5 sequencing reactions were required (twice the minimum)

[these numbers do not include the number of controls run per case]. Although these numbers are high, they are expected efficiencies for "ancient DNA" testing. Thus, despite difficulties, the AFDIL is now able to obtain mtDNA information from the vast majority of cases. The results from recent Korean cases suggest that Korean war remains may not be any more problematic than those from Southeast Asia, presumably due to better preservation of the remains.

MtDNA identifications from ancient remains is a scientific tour de force, at the cutting edge of today's capabilities. As single DNA molecules can be detected, some degree of cross-contamination is inevitable, especially in a scaled up production facility. The Task Force nevertheless concludes that with appropriate control measures (redundant testing and meticulous lab hygiene) these problems are surmountable, and a good record has been presented in the currently on-going work.

The Task Force finds that the present probability of coincidental matches between mtDNA control region sequences is no more than a few percent. Once sequences from 500 members of a population have been determined, precise statements about the chance of a false association of **a set of remains with a family will be able to be made.** Published data may be of value, but samples will be needed from Southeast Asian populations. **The precision is expected to suffice in the vast majority of cases, given other non-DNA evidence, to effect the mtDNA identification of unassociated Korean remains. It will not be possible to identify every bone. A great deal can be done with anatomical and historical evidence alone.**

The Task Force finds that control of contamination is essential to PCR-based laboratory testing. Some contamination is unavoidable, particularly in mtDNA testing of ancient remains, but it does not preclude reliable casework testing where redundancy, good laboratory practices, and appropriate cautionary language are used and constant oversight is maintained.

The Task Force finds that casework **experience demonstrates capability to type Korean** skeletal **remains.**

III. RELIABILITY

DSB TOR: To evaluate current and emerging scientific evidence concerning the reliability of the techniques when compared with other current and evolving methodologies. *[Is the current MtDNA sequencing identification method reliable? Are quality assurance efforts satisfactory? What further measures would enhance reliability? What studies are necessary to validate a new DNA typing methodology on ancient skeletal remains? What continuing scientific oversight or advisory body should monitor these DNA identification efforts? What quality assurance mechanisms or measures should be implemented?]*

The scientific community believes that current mtDNA sequence identification technology is reliable. Forensic laboratories in the United States and Great Britain have begun to use it in casework, recognizing they must be able to defend the technology in court, if needed.

The component technologies of DNA extraction, PCR amplification, and DNA sequencing are all validated, having been used in research and service orientated molecular biology laboratories throughout the world for many years. Furthermore, their application to so-called ancient DNA is well established in the scientific literature.

The studies generally accepted by the forensic community to validate a new DNA testing technology have been articulated as guidelines by the Technical Working Group on DNA Analysis Methods (TWGDAM) [1] sponsored by the Federal Bureau of Investigation (FBI). Such studies include the following: optimized standard source studies (such as studies on fresh body tissues and fluids, stored tissues and fluids, and samples from different tissues from the same individual); variance analysis (studies on measurement precision from known DNA controls); population studies (studies of population frequency distribution in different racial/ethnic groups); preservation studies (studies on tissues and fluids as would be typically found at a scene investigation, eg. dried stains); time/temperature studies (studies on samples incubated at various time and temperature); environmental exposure studies (studies on effects of various commonly encountered substances); evidentiary source studies (studies on nonprobative evidence from typical crime scenes); non-human studies (studies on tissues and fluids from common non-human sources); on-site **evaluation**

(studies to evaluate methodology transfer to a working forensic laboratory setting). The FBI is completing their validation studies on mtDNA typing in preparation for casework.

The TWGDAM Quality Assurance Guidelines address basic considerations in DNA analysis--such as analyst training, reagent quality control, evidence handling, analytical procedures, proficiency testing requirements, and method validation. Requirements for standard cell line positive controls and extraction controls are included. Requirements specific to RFLP analysis (such as precision of fragment size measurements) and PCR analysis (such as negative reagent blanks and separation of pre and post amplification areas) are also covered. TWGDAM has a subcommittee devoted to the forensic use of mtDNA sequencing and has recently adopted modifications to their guidelines for mtDNA sequence analysis in forensic laboratories. However, they do not concern themselves with application to ancient DNA.

It is not generally appropriate or possible to exhume remains of known individuals which have been buried for 30 years to validate the utility of a DNA typing system. However, indirect evidence of the validity can be found in the contexts presented elsewhere in this text.

Corroboration will ensue from the internal consistency of the findings. In some cases, corroborative evidence for or against an identification may appear from other documentary or physical evidence acquired after the DNA tests have been concluded.

Evidence of the efficacy of skeletal remains identification by mtDNA sequencing can be found in the identification efforts performed to date by the AFDIL. Repeatedly, bones from the same case yield the exact same sequence. The sequences obtained from the bone samples have matched those of the putative family members. Often these sequences are unique, never having been seen before. The AFDIL processes the skeletal specimens before testing the family reference(s), eliminating the possibilities of bias or cross contamination. Controls are run with all cases and the results are always checked against the mtDNA sequence of the staff processing the specimens. In one case, an exclusion was found by mtDNA testing; when CILHI received the test results, they suggested a second name association which was then confirmed by a match of the case sequence to that of the second putative family. The AFDIL duplicates the mtDNA sequencing casework of

new personnel as they begin actual casework--this data has not revealed any discrepancies to date.

The quality assurance requirements for adequate mtDNA testing of ancient DNA are demanding. The molecular anthropology community has reiterated many of the concerns in this area, particularly that of contamination and adequacy of controls.

Proficiency surveys do not yet exist specifically for mtDNA sequence identifications, although the AFDIL personnel have been submitting mtDNA sequence data to the College of American Pathologists (CAP) and Cellmark proficiency surveys. However, interlaboratory exchanges have been initiated by TWGDAM members. Data, to date, demonstrate the reliability of this method. The National Institute of Standards and Technology (NIST) is currently developing mtDNA reference material.

The greatest single potential for a mistaken DNA sequence result is from contamination. Some contamination events during laboratory testing of ancient remains are inevitable. However, contamination can be minimized through proper laboratory design and sample handling procedures and can be detected through the use of appropriate controls. The molecular anthropology community advocates the use of multiple negative controls, including a DNA carrier control. TWGDAM recommends negative extraction and amplification controls as well as a known positive amplification control.

The discrepancy case, "X-6", between the AFDIL and the outside laboratory, reinforces the critical importance of quality assurance measures. The AFDIL's ability to blindly replicate their testing results on the case two subsequent times and independent confirmation of AFDIL's results by the British FSS, demonstrate the reliability of results when proper quality assurance measures are employed.

Ambiguities and errors can occur during amplification and sequencing. Nonspecific priming can be reduced through well designed primer sets used under optimized conditions. Sequences should be visually checked and interpreted cautiously, in accordance with appropriate protocols. Sequences should be confirmed. Sequencing results should be independently verified by a second qualified analyst (double reading).

Redundant specimens or replicate extractions should be tested, where possible, to ensure that a given finding is not a chance result of a contaminant. Where redundancy is not possible, cautionary language should be used in the reporting of the results.

A principle of forensic testing that is applied in the military's urine drug testing program is that, after a test result is obtained, it should be confirmed by a second test employing another method. There is not a second method by which current mtDNA testing can be confirmed. However, new technologies are being developed which, when they become available, could have application in testing skeletal remains.

Mutations in the mitochondrial sequence are not common, but do occur. Mutations raise the possibility of a false exclusion. A single base change should therefore be considered "indeterminate", rather than an exclusion or an inclusion. The AFDIL methodology of requiring two (2) family references has already proven to be useful to resolve mutational events.

Continuing scientific oversight is a significant part of the military's quality assurance program. The AFIP is formally reviewed twice a year through a Scientific Advisory Board (SAB). The SAB reports are distributed to the AFIP Director and its Board of Governors, which includes the Army Surgeon General, the executive agent for the AFIP, the Surgeon Generals of the Navy and the Air Force, the Public Health Service, the Medical Director of the Veteran's Administration, and the ASD(HA), who has policy oversight of the AFIP. One SAB member is dedicated to review of the Department of Defense DNA Registry. Currently, this Board member is Maimon Cohen, Ph.D., Chairman of the Division of Genetics, University of Maryland Medical School.

The ASD(HA) has ultimate responsibility for oversight of quality assurance of all identifications of human remains performed on behalf of the military services. A plan has been published of the minimum standards for military or military-contract laboratories to follow in performing mtDNA sequence analysis of ancient skeletal remains (Annex C). These standards were submitted to the American College of Medical Genetics, the College of American Pathologists, TWGDAM, the American Society of Crime Laboratory Directors and the Department of Defense Clinical Laboratory Improvement Office for review and comment before

promulgation.

The ASD(HA) Quality Assurance Program calls for laboratory accreditation by an outside professional organization, specified additional mtDNA-specific standards, and oversight by an independent board. The Quality Assurance Oversight Committee, composed of civilian technical consultants, will perform reviews of casework, reports of inspections, proficiency test results, and accreditation materials.

There is currently no framework for mandatory proficiency testing and accreditation of independent laboratories, a circumstance beyond the mandate of this Task Force. Substantial voluntary efforts are nevertheless underway, with important leadership from the TWGDAM.

The Task Force finds that appropriate measures must be taken to prevent and control possible contamination in the testing laboratory.

The Task Force finds that current AFDIL protocols, if diligently performed, are capable of generating quality mtDNA sequence identifications.

The Task Force finds that adequate quality assurance requires accreditation and an oversight board.

The Task Force finds that the program for mtDNA **sequencing quality assurance promulgated by the Assistant Secretary of Defense (Health Affairs) for identification of ancient remains is adequate and responsive.**

IV. OTHER DNA TARGETS

DSB TOR: To evaluate the possibility of obtaining useful mtDNA information from skeletal remains through mtDNA outside the control region (D-Loop) or possibly nuclear DNA. *[What other DNA typing could potentially be used to augment current DNA identification efforts of ancient skeletal remains, e.g. noncontrol region mtDNA sequence, multicopy nuclear DNA regions,* etc. ? *Should the government expend funds to investigate other DNA typing possibilities?]*

The discriminatory potential of sequencing the hypervariable regions of the mtDNA control region is powerful, with most sequences in current samples being unique. In particular cases, the sequence found to match between remains and putative family members may not be found in general population samples. In that case, the probability of a coincidental match will be less than one percent. Stronger statements may not be possible because the proximity of the variable sites in the control region may mean that they are correlated.

Polymorphisms in the mitochondrial genome at sites outside the control region may be used to enhance the discriminatory power of current mtDNA identifications. Polymorphisms at other sites have been well-described and may have significant additional discriminatory power.

The discriminatory power could particularly be enhanced if nuclear DNA could be recovered from ancient skeletal remains. Although to date, typable nuclear DNA has not been obtained from remains from Vietnam and Korea, the fact that mtDNA itself can be recovered lends to the theoretic possibility of recovering nuclear DNA. In fact, short tandem repeat (STR) analysis of nuclear DNA was performed with limited success from the 73 year old remains of Tsar Nicholas II. However, in that case the mtDNA was more intact than in remains recovered from Vietnam and Korea, presumably due to the favorable environmental conditions of Russia.

The primary reason given for the ability to recover mtDNA but not nuclear DNA is the hundreds to thousands fold increase in copy number. The circular nature of the mtDNA, its particular sequence, the presence of a mitochondrial membrane, and the absence of intra-organelle degradative enzymes may confer some

additional longevity to mtDNA; however, there is no experimental data to argue for or against such theoretical possibilities.

Some nuclear sequences are present in many copies within the same individual. These multicopy sequences may be particularly likely to be recovered. Ribosomal DNA repetitive regions may also be of significance.

If nuclear DNA testing is possible, then the children and spouse of deceased servicemen could donate a reference DNA sample; where using mtDNA, they cannot usefully today. Thus, a greater pool of potential volunteers for family reference DNA specimen donations could be created. Given the aging population of reference family members for mtDNA markers, this is of very important consequence. Furthermore, nuclear DNA markers, if they could be obtained, would also provide a greater discriminatory power.

The Task Force recommends that the AFDIL investigate the potential to perform DNA typing outside the mtDNA control region.

V. FAMILY REFERENCE DATABASE

DSB TOR: To **determine the degree to which mtDNA matching could be accomplished with reference donors (family members of up to 8,100 unaccounted for in the case of Korea, adequacy for discrimination of individual from such a database, and what alternatives exist if such family donors are deceased.** *[Can the remains without a name association from Southeast Asia and/or Korea be identified using a database of family reference DNA typing information? To what extent could DNA be used in isolation and in combination with other identification evidence to individually identify servicemembers from a panel of over 8100 U.S. family DNA types? To what extent are identification efforts hampered by the lack of family reference specimens from all families? To what extent are identification efforts hampered by the presence of foreign nationals among the unidentified remains? What reference specimens should be sought from families, e.g. from 2 family members, nuclear DNA relations, consanguinity within 3 generations? Could reference specimens be obtained from the exhumed bodies of family members?]*

Since mtDNA is maternally inherited, maternal kindred can be used as sources of reference material. Specifically, references include the biological mother, siblings, maternal grandmother, maternal aunts and uncles, children of sisters, or children of deceased female servicemembers. Unless nuclear DNA can be used in these cases (see above section IV., Other DNA Targets, p. 27), children of deceased servicemen are not useful for reference specimens.

Since mtDNA is not inherited from both mother and father and does not undergo recombination in the same way that nuclear chromosome pairs do, an exact match of mother to son or daughter is expected. A consequence of finding an exact match in the kindred, is that relatives far removed generationally from the deceased member, may be an appropriate reference. For example, Tsar Nicholas II was identified through his great grandniece.

Unlike the situation with servicemembers from Southeast Asia, the U.S. military does not have a comprehensive database for the approximately 8,100 families from the Korean conflict. A substantial effort will be needed to identify and contact eligible family members for mtDNA reference specimen collections. Many families may come forth quickly upon news of an undertaking

to recover and identify remains of soldiers from Korea. Yet these interested family members are now an aging population and may no longer be available to provide this reference.

The consensus of the military staff working this endeavor is the majority of the families are interested in resolution of these cases. The issue of genetic privacy may be raised in this context in the same way that it is raised with the current DNA collections program for Active and Reserve Component servicemembers. It will require sensitivity to the issue, adoption of safeguards, and recognition of the need for educational of the prospective donors.

The POW/MIA Affairs Division, U.S. Total Army Personnel Command, has developed the framework for an outreach program to identify and contact persons authorized to make a decision on the disposition of remains and acquire reference blood specimens from the families of Americans whose remains were not recovered from the Vietnam, Korean, and Cold War incidents (Annex G). It is estimated that half of the families will be contactable and provide blood reference specimens. The outreach program will: 1) identify and contact persons authorized to decide on the disposition of remains; 2) identify and contact prospective DNA donors; and 3) raise public awareness of government efforts to repatriate, and identify the remains of Americans lost in Southeast Asia, Korea, and the Cold War. A military-supervised, contractor-operated operation is contemplated. The contractor will proactively solicit persons authorized to decide on the disposition of remains and eligible mtDNA donors for 2 years or when 70% of the donors/persons authorized to direct disposition of remains are contacted. The cost of the outreach program will include family notification, a computerized database, blood collections, travel, and staffing.

Where a family reference cannot be obtained from living relatives other theoretical possibilities exist. Possibilities, from either deceased kindred or from the deceased servicemember himself, include biopsy samples maintained in a hospital repository, saved deciduous teeth, and locks of baby hair. Reference samples could conceivably be obtained from exhumed familial remains. A mtDNA match has been performed by the AFDIL between skeletal remains from Vietnam and baby hair from 1927. The AFIP itself may have slides and paraffin-embedded specimens stored from servicemembers who served in the Korea conflict.

As with all methods of identification, comparison data are necessary. Only where family sequences for the prospective remains are known or the ambiguities accounted for, can a match provide certainty of identification. Even a complete family reference database will not address the possibility that given remains may be those of other UNC or KPA troops. The power of a mtDNA sequence match can be stated with increasing confidence as the database expands. Databases of 500 population group (ie. Caucasian, Black, Hispanic, Vietnamese, Korean) are desirable. Other evidence of identification will also confer increasing confidence in a mtDNA sequence match. A given sequence may provide certain identification where all sequences are known within a sufficiently characterized subgroup such as the case of those personnel known to be buried at a particular site. With each identification, the overall pool of potential reference sequences will decrease; thus the power of individuation will increase with completion of the database.

The database of family reference sequence information must be largely established before identification of the unassociated remains can proceed, unless non-DNA evidence allows a restriction on the possible families in a specific case. Moreover, the collection of family references is time sensitive; there is some urgency to contact appropriate family members as many of them are now quite elderly. For each family, it will be necessary to identify the members most likely to allow identification, paying attention to the possibility of the future use of nuclear DNA information.

The Task Force finds that, with a reasonable effort, a sufficient proportion of families are expected to provide mtDNA samples to allow identification of many of unassociated remains from Korea through mtDNA testing, and to attach meaningful probability statements in those cases.

The Task Force recommends consideration be given to the collection of DNA reference specimens from maternal and paternal family members in case future technology permits nuclear DNA testing. Collections from nonmaternal kindred members (e.g. children) should be made with full disclosure of realistic expectations.

VI. STATISTICAL DATABASE

DSB TOR: To recommend a statistical database to **be used in calculating the statistical information.** *[What database(s) should be used in casework for determination of statistical inferences? What are the minimal technical requirements to be met before inclusion in the database? Should databases for racial and ethnic populations be constructed and if so which ones? Should reports include the frequency estimates for indigenous Vietnamese populations as well as for Americans? Is the counting method the best and only form of statistical inference for mtDNA? What degree of independence between polymorphisms exists within and without the mtDNA control region?]*

If a remains are from a closed population group or subgroup, the mtDNA sequence and ancillary data need only distinguish among the population pool without reference to statistical inference. If mtDNA is to be used for identification in situations other than those of closed populations, then estimates are needed of frequencies of specific variants to assess the weight attached to matches of sequences from difference sources. Account must be taken of mutation rates to calculate the likelihood of differing sequences being from the same maternal lineage.

The full discriminatory power of mitochondrial sequencing for identification purposes can be determined only through a database larger than currently exists. However, preliminary evidence from combined statistical bases indicate that a very high discriminatory value can be achieved.

Typing both the skeletal remains and family blood references will result in the largest known DNA sequence databases. These databases will provide the means for attaching probability statements to identifications in situations other than those arising from the Korean Conflict. Further studies are needed to establish the extent to which other mitochondrial and nuclear information may be combined with mitochondrial control region information. **A** database of **500** individuals per population group will be large enough to ensure (within a 99% confidence limit) that a variant with a frequency of 1% will be seen.

Before sequences should be entered into a common database, certain criteria should be met. These criteria include confirmed sequence of at least a certain length, using a given

nomenclature, and with certain demographic data attached.

Moreover, to allow for the possibility of non-U.S. remains being typed, and to allow for the genetic differences within the U.S. population, sequences should be collected from the major groups; Caucasian, African-American, Hispanic and Asian. As data accumulate, it will become evident if there are substantial mitochondrial differences among different Asian countries, or within the racial groups.

The Task Force finds that the existing databases, and those anticipated from the family collections, provide an adequate basis for the current mtDNA sequencing efforts.

The Task Force recommends an expansion of the current AFDIL database.

VII. LARGE-SCALE OPERATIONS

DSB TOR: To **ascertain what effects a large volume of remains could have on the identification process utilizing DNA technologies.** *[What are the consequences and potential problems in scaling up to high volume mtDNA typing operations, e.g. risks and preventive measures of contamination, quality of typing services, resource implications, etc.? Are sufficient qualified personnel available for hire?]*

If the military decides to perform mtDNA testing on skeletal remains from the Korean conflict, an order of magnitude in the scale-up of operations will be required. However, it may not be advisable to greatly scale-up operations. A more sensible approach may be to spread the casework over a longer period of time.

The scaling up of operations is not necessarily a simple unit expansion of current efforts. Fixed costs and fixed assets must be differentiated from incremental costs and resources. Infrastructure support is often a discontinuous step function. Economies of scale may come into play. Larger operations may permit restructuring and work to be performed in ways not possible by smaller operations.

The AFDIL assessment is that scale-up for a higher volume operation is possible within the existing AFDIL facility. With existing facilities and current technologies, the AFDIL expects to achieve a 25% increase in efficiency by FY 96 and a 50% increase by FY 97, after further experience is gained by new personnel and through structural changes in the way in which specimens are processed. More fully implementing and improving the computerization of operations would significantly impact the throughput. Future technologies could potentially increase throughput by several fold. Assuming the administrative space shortage could be resolved, the doubling of laboratory personnel could be accommodated within the existing AFDIL facility; and an even larger staff, if a double shift mode is used. Hence, the military could take advantage of existing facilities to achieve the needed throughput.

A concern for a high volume operation is the increased potential for cross-contamination within the laboratory. Existing facilities are capable of meeting this need.

The forensic DNA community is small. Nationally, the number of academic programs conferring degrees in the forensic sciences is a mere handful. However, only approximately one half of the States have DNA testing capabilities and the turnover among current forensic DNA Analyst positions is low. Consequently, a significant although not large group of forensic laboratorians exists from which to draw. The molecular biology community is much larger and represents an alternative pool of personnel which could be tapped for large scale operations. Medical Technologists are specially educated, trained, and certified in laboratory techniques, practices, principles and quality assurance and represent a further pool of potential analysts.

Each of these three major disciplines has its strengths and weaknesses in regard to the backgrounds for performing this testing. **Most** analysts will not possess great expertise in mitochondrial DNA or ancient DNA analysis at the time of hire. On-the-job training would be anticipated. Nonetheless, AFDIL has not had great difficulty in recruiting high quality staff with significant relevant backgrounds and capable of being trained in the particular discipline. Shortages of qualified personnel should not prevent scale-up efforts, although some hiring delays, significant training, and a substantial learning curve should be anticipated.

Due to the large number of potential cases, the military may have to consider contracting the work to civilian laboratories. The mtDNA QA testing standards are in place in anticipation of contract requirements. Contracting options have been favorably viewed by the Federal Government in recent times as generally more expeditious and less costly than in-house programs. They are particularly useful for projects of a temporary nature or of a defined lifespan. When a project requires resources greater than that in government or would require additional facilities, the contracting option may provide a solution.

However, services involving confidential matters and matters which are of particular seriousness are usually kept within Government. Government contractors cannot be held accountable to the public in the same way as Government agencies. In the case of apparent problems, government contractors may simply declare bankruptcy, dissolve, or otherwise go out of business leaving the Government without recourse. Ultimately, the Government will be held accountable to the public whether work is performed in-house

or out. Due to the special status of Government functions, laws, regulations, judicial and agency scrutiny have developed such that nongovernment entities are not covered and not under the same constraints as the Government. Accordingly and significantly, laboratories for law enforcement are overwhelmingly in-house Government laboratories.

In this particular context, quality needs and reliability may override cost economies which might be achievable from the private sector. Further, families may be uneasy to trust such private information to private sources. Thus, for reasons of accountability and sensitivity, the Federal Government should play a large role in any large scale program of mtDNA testing of remains from Korea.

Economies of scale are important to mtDNA sequencing operations, as they are elsewhere. Due to the low volume and high cost of laboratory space and equipment in current testing operations, fixed costs are disproportionately high; thus, economies of scale are particularly striking. A centralized laboratory would be significantly more cost effective than contracting multiple small private laboratories. It is easier to oversee and control the quality of one or a few large laboratories than several smaller laboratories.

The Task Force finds that current mtDNA testing efforts could be augmented for large scale operations. There are strong arguments for a centralization of the laboratory work **for the** sake **of vigorous oversight, quality control, and accountability.**

VIII. RESOURCE REQUIREMENTS

DSB **TOR: To** determine the scientific and other resource implications **of** undertaking large scale mtDNA testing for identification of unassociated remains. *[What are reasonable resource estimates of mtDNA sequencing identifications as currently performed? What are reasonable and likely projected cost estimates associated with performing DNA identifications for Korean War remains? What laboratory personnel are available to perform large-scale mtDNA typing operations?]*

MtDNA sequencing of ancient remains is resource intensive. This is due primarily to the slow and tedious nature of mtDNA sequencing from poor source material, poor quality DNA template, amplification difficulties, and sequencing reactions that must be optimized and repeated numerous times. Furthermore, the forensic nature of evidential testing demands greater care and documentation.

Current cost estimates for DNA sequencing generally are simply not applicable to the mtDNA sequencing operations necessary for **CILHI** casework, because they are based on estimates of high volume sequencing operations, acceptance of low levels of base miscalls, non-forensic DNA testing standards, and on optimal DNA template. Typical costs from subsidized genome project operations are between **$1** and $2 per "finished" base sequenced.

The **AFDIL** currently sequences 613 bases as two sets of overlapping fragments (a total of **1,046** bases), and confirms the sequence by also sequencing in the reverse direction. An openly bid contract for sequencing the same region for population studies resulted in a open market figure of nearly $1,000 per blood specimen. On the other hand, when performing testing on actual casework (including multiple bone and blood specimens), the British FSS charged the Department of Defense over $100,000 for mtDNA analysis of two cases. This is not unreasonable when considering the cost of salaries for several analysts and Ph.D. molecular biologists over a ten month period.

The **DCSPER** has funded the **AFDIL** to perform mtDNA sequencing for remains identification from Southeast Asia. Projected estimates are that 500 cases would require mtDNA testing over a five year period. A case unit for workload projections is four bones and two blood reference specimens. Current mtDNA sequencing

operations cost approximately $17,500 per case at 120 cases per year ¦ excluding the lease cost of the facilities (approximately $9,000 per case). The largest cost components consist of a new laboratory facility, staff (primarily of one DNA Analyst and one DNA Technician per case per month), and equipment and supplies. Fixed costs are quite high for this labor-intensive equipment-driven operation. Retesting accounts for a substantial proportion of the laboratory testing. Recent AFDIL casework has averaged 3.7 bone fragments tested, 5.2 extractions, 37 amplification reactions, and 55.5 sequencing reactions to obtain 1,480 bases of polished/confirmed sequence per case. In a recent AFDIL workload study, approximately 25% of the labor hours were spent in laboratory testing, 50% in data analysis, and 25% in reviewing and reporting the data (Figure 8). Full casework production operations, at ten cases per month, began in October of 1994. Due to the lack of historical data, actual operational costs have not yet been fully established, but appear to be close to projected costs. Due to the immaturity of the program, full operational efficiency has not yet been achieved.

However, the decision to perform DNA testing for unassociated remains from North Korea has yet to be made. There are over 8,100 servicemembers whose remains were not recovered and identified from the Korean conflict. The best information available indicates that no more than 3,000 of the 6,000 remains could be recovered from North Korea. However, it is anticipated that DNA testing would be performed on most cases. Preliminary returns suggest that commingling of remains will be frequent, unless perhaps, joint recovery with U.S. teams is permitted; thus the number of bones tested per case may be increased. Furthermore, a family reference database of mtDNA sequences for all families would need to be constructed for comparison purposes. The total costs for the entire operation would have to include the costs of: 1) recovery and repatriation; 2) documentation review; 3) primary identification processing by CILHI; 4) a family outreach program (Annex G); 5) the family reference mtDNA sequence database; and 6) mtDNA testing of remains.

The Korean workload could be phased in as the Southeast Asia workload ends (Figure 9).

Phase I (Annex H) of a program of DNA testing of Korean remains would require the creation of a database of family reference

mtDNA sequences. It is estimated that 40 to 70% of the 8,100 families of Korean servicemembers from the Korean conflict would be contactable and willing to provide a blood sample for this purpose. The AFDIL protocol normally requires two maternal reference specimens per family. Consequently, the creation of the family reference database is anticipated to require mtDNA sequencing of between 6,480 to 11,340 blood specimens. Some skeletal remains testing could be performed without the full generation of the family database. Blood samples can be sequenced at significantly less cost and higher production rate than ancient skeletal remains. This can be performed over a two to three year period, in current facilities, at a cost of $1.6 to $2.6 million.

Phase II (Annex I) of a program of DNA testing of Korean remains would involve the mtDNA sequencing of up to 3,000 skeletal remains. Given that one laboratory analyst with technical support can process four bone specimens per month, and an average of four bones are tested per set of skeletal remains with the marginal cost for each bone being approximately $4,400 at current efficiency and staff, the projected cost of this program is approximately **$51** million in FY 98 dollars over **10** years; the annual projected cost of $4.9 million per year consists of $1.3 million in fixed costs **and** $3.8 million in marginal costs. Some difficult cases are far more consuming of resources than other cases. The duration of the program could be shortened or lengthened, but no less than eight years if all work is performed in the current AFDIL facility and using current technology. Recovery and preprocessing of remains by CILHI may limit the number of specimens to be tested per year.

This projection assumes a reasonably **expected increase of** efficiency of 50%, but efficiencies could well be much higher with the development of new technologies. Projected increases in efficiency anticipate functional and organizational shifts from case to bone sequence reporting. Improved computer assisted analysis should be in place by the time of this phase of the program; decreasing analysis time by 50% will increase throughput by 25%. The better preserved bones from Korea along with improvements in extraction and amplification could half the rate of retesting now performed in casework. One particular primer set of the four primer pairs used by the AFDIL performs **substantially better than the** other three, suggesting that improvements could be made to the other three systems. These

improved efficiencies are foreseeable without the development of new automated DNA technologies, which will surely come to bear within the time frames projected.

New DNA sequencing and other DNA typing technologies could achieve an order of magnitude faster turn around time for testing, and decreased testing cost. However, it will not eliminate the very labor intensive job of extraction of DNA from bone nor the analysis time.

The most speculative variable significantly affecting the cost estimate is the assumption that four bones are tested per skeletal remains. This projection does not assume all bones are DNA tested, but rather that CILHI is able to successfully segregate some skeletal remains. The number could be substantially higher given the potential for commingling.

The Task Force finds that current mtDNA testing efforts at AF'DIL are funded appropriately for the Southeast Asia mission.

The Task Force concurs with the projections that analysis of Korean War remains could be accomplished over the next twelve years with an increase of funding of approximately $2 million per year over the cost of current operations.

IX. NEW TECHNOLOGIES

DSB TOR: To evaluate other technologies to assist in the automation and reduction of costs associated with DNA testing. *[What alternative technologies might be brought to bear that may* assist *current DNA identification efforts? Could new* technologies *improve DNA typing efforts by improving discriminatory power or enhancing recovery? Could new technologies speed DNA typing efforts? Could new technologies bring down the cost of DNA identifications? Would new technologies replace or confirm current technologies? Would new technologies permit ancillary studies to improve DNA typing efforts, e.g. assessment of original DNA template damage, quantitation of human and other DNA present, etc.? Should the U.S. military fund any investigations, research, or technology development which might enhance cost effective DNA identifications? If so, what are they?]*

Advances in biotechnology are progressing rapidly, particularly as part of the Human Genome initiative. Many will undoubtedly apply to the DNA identification efforts of the U.S. military. Over the next 3 to 10 years, even without expending funds specifically to develop applicable technologies, off-the-shelf technologies are expected to permit far more rapid testing at a fraction of the cost of current testing. However, investment in technologies now will help to accelerate advances in the area of identification and particularly with respect to military efforts to identify ancient remains, such that long term cost savings would surely be realized. A relatively small investment by the military could be leveraged to achieve significant gains applicable to their needs.

Significant improvements could be achieved over the currently employed DNA typing methods and technologies. New methods and technologies could potentially improve the success of DNA typing efforts as well as substantially reduce the turn-around-time and cost of testing. Possible areas for exploration and investment include, but are not limited to, the following:

A. Specimen Preparation and DNA Extraction

The first steps in mtDNA testing of skeletal remains are sample preparation and extraction of the DNA. Significant improvements could be made in enhancing the success, speeding processing, and

making it more cost efficient.

1) Recovery Improvement

The success of mtDNA identification efforts primarily rests on the ability of the laboratory to get useful mtDNA information from the samples of skeletal remains tested. The AFDIL was successful in only half of the cases initially, but has been more successful in most recent cases from Southeast Asia. Several re-extractions and re-amplifications were necessary to accomplish these successes. Not only does this demonstrate significant progress has already been made, but it also suggests that current testing is pushing the limits of technology. It is anticipated that less well-preserved remains will be returned from Southeast Asia. Early testing on Korean remains suggests that the samples will be more challenging. Even marginal improvements in mtDNA extraction will significantly impact the ability of the military to effect mtDNA identifications. Whole genome amplification through a random primer technique, use of alternative polymerases, improved buffer systems and reaction conditions are obvious directions for possible improvements.

2) Automated Specimen Preparation and Extraction

Preparation and extraction of mtDNA from bone specimens are currently labor-intensive and time-consuming processing. Even if subsequent sequencing is speeded, this step may act as a significant bottleneck. Automation of sample preparation and extraction may be possible. As already performed by some in the ancient DNA community, the surfaces of bone samples could be cleaned chemically rather than by grinding, as currently performed at the AFDIL. Then instead of mechanical breakdown of the bone samples, enzymatic dissolution of the bone may be possible, particularly when accompanied by ultrasonic agitation. Cleaned bone samples could be placed into troughs for incubation, and the dissolved extract subsequently robotically manipulated through the next stages of processing.

Automation could also apply to reference blood samples. Typing family reference samples to create a database for identification of unassociated remains will require literally thousands of mtDNA typings. Automated extraction would greatly benefit these efforts. The AFDIL currently transfers liquid blood samples to bloodstains on cards for ease of use and storage. Small punches

from the cards are then used in analyses. The development of a robotic instrument to punch bloodstain cards and then extract the card punches could dramatically speed the process.

Automation will speed processing and free labor to perform other tasks. Labor is by far the greatest cost in mtDNA typing of ancient skeletal remains. Accompanying substantial decrease in operational costs should be realized after the capitalization of the equipment. Automation may eliminate many potential manual errors in repetitive sample handling. Moreover, automation may further decrease the chance of cross contamination. The automated equipment may also be a boon to other AFDIL service casework, which has call for high volume and rapid sample processing.

3) Repair

Ancient DNA is not only degraded but damaged. Strand nicks, cross-links, extraneous molecular attachments, and other sorts of damage may prevent a polymerase from reading through the length of a DNA fragment. If the DNA reparative machinery that is normally found in a cell can be used to repair small defects in DNA target templates, then the ability to amplify such ancient DNA may be greatly enhanced. Enzymes known as ligases are commonly used in molecular biology labs to splice strands together and could repair a nick in one strand of a double-stranded fragment. A polymerase may replace a missing base by matching the complement of the opposite strand. Bacterial cloning maybe useful to repair damaged DNA using the full set of repair machinery of the host organism. Some evidence suggests that repair of the DNA before amplification can indeed assist recovery of sequence information from ancient DNA. A marginal improvement in the success of the recovery of mtDNA information from bone specimens will significantly impact current identification efforts.

B. Specimen Evaluation

Evaluation of the DNA template from which the DNA information is to be derived may permit accurate sample loading, tailored

amplification conditions, an assessment of likelihood of success, and an indication of how hard to try to recover information from the sample.

1) mtDNA Quantitation

Accurate quantitation of the sample DNA target is an important aspect of various methods of mtDNA testing. Quantitation allows accurate amounts of the sample extract to be added to an amplification reaction mixture. An absence of quantitated mtDNA should result in a determination that further testing would be an unwarranted waste of time. Quantification of source mtDNA would permit inferences of susceptibility to sampling and enzymatic errors. Unfortunately, current methods of quantitating mtDNA are not human mitochondria specific and are too insensitive to be of great value in these old skeletal remains cases. A semi-quantitative sample gel electrophoresis is performed which is virtually always negative unless a significant bacterial DNA content is present. A sensitive human mitochondria specific assay could be developed to assist in this casework. Competitive PCR assays, serial dilution assays, and kinetic assays are potential methods, among others, for quantitation.

2) DNA Damage Assessment

The DNA from ancient skeletal remains is severely degraded and damaged. An assessment of the damage to the DNA to be analyzed would be valuable as an indicator of how best to scientifically approach the sample for analysis and whether or not to continue to expend time and effort in reanalysis of the sample. Such an assessment may be useful to determine whether to attempt repair of the damage before amplification.

Environmental damage of DNA takes the form of certain predictable classes of chemical reaction resulting in the formation of certain specific DNA adducts. A new sensitive, rapid, inexpensive, and specific method that has recently been developed involves matrix-assisted laser desorption followed by mass spectrographic analysis.

C. DNA Amplification

Any mtDNA analysis of skeletal remains will require amplification of the few mtDNA template strands present. This is a critical step in the mtDNA testing. The basic PCR amplification process has not changed substantially since it was first introduced.

1) Alternative Polymerases

The PCR reaction involves the polymerase enzyme produced from a microbe which lives in waters of hot springs called Thermus Aquaticus and known as the Taq polymerase. While Taq polymerase is an excellent polymerase for most DNA amplifications; it does have its limitations. The knowledge of DNA polymerases has recently increased and continues to improve. Newer alternative polymerases offer the possibility of greater fidelity, processivity, and ability to process damaged template or tolerate Taq inhibitors.

2) Inhibition

Inhibition of the PCR amplification reaction is often encountered in the processing of old skeletal remains. Currently, an enormous effort is expended in overcoming a variety of technical hurdles encountered with AFDIL cases. Improvements in overcoming inhibition are likely through better optimization of reaction conditions, better DNA extraction methods, and through alternative polymerases, as previously mentioned. Because inhibition directly prevents the success of DNA typing, even marginal increases are likely to be significant.

D. DNA Analysis

Currently mtDNA analysis has been performed using conventional sequencing on conventional equipment. Due to the Human Genome Initiative advances in DNA analysis are in rapid development. Several possibilities exist that could dramatically increase throughput and decrease the cost mtDNA analysis. Complete automation with integration of sample preparation, DNA

extraction, DNA amplification, and analysis is an achievable and desirable goal.

1) Automation of Sequencing Reactions

Current sequencing reactions are performed manually. Automated methods for performing sequencing reactions are available. Automation not only can achieve higher throughput and reduce overall costs, but also may achieve better results. The large number of steps involved in current testing creates the potential for manual errors to be made. Automation will allow dye primer and T4-sequenase chemistries that are too tedious for manual production sequencing casework efforts.

2) Capillary Electrophoretic Arrays

Current sequencing technology is based on gel electrophoresis in which DNA is pulled across a slab of polyacrylamide gel using an electric current. The speed of this system is determined by the electric voltage applied, but the voltage is limited by the heat generated by the current in the gel. If the electrophoresis is carried out in a very fine capillary then a substantially higher voltage can be applied and a faster run time achieved. Run times are measured in minutes instead of hours. Due to the higher resolution, a greater number of bases can be read per run. Furthermore, a capillary can be sample loaded in an automated fashion, obviating the need for pouring and loading a gel. As a result, many DNA samples (up to 36 or 48) can be run simultaneously on a slab gel, but only one DNA sample can be loaded in a capillary per run. Instruments that employ arrays of capillaries are currently in development and could dramatically speed DNA sequencing using conventional sequencing chemistries, with an accompanying reduction in cost.

3) Sequencing Microchips

Another major new technology under development is sequencing by hybridization (SBH) performed on microchips. Microlithography is an engineering tool used in the manufacture of computer microchips. Microlithography will permit the cost-effective generation of arrays of thousands of DNA probes which enables SBH technology. One of the first prototype "DNA chips" will sequence the D-Loop of mitochondria. This microchip can perform sequencing at a fraction of the time and cost of current DNA

sequencing methods. These microchips should be first used to confirm current sequencing technology and perhaps later employed as the primary method of sequence information.

4) Single-Point Polymorphism Assays

An alternative technique to sequencing which captures most sequence data, developed by Dr. Mark Stoneking in conjunction with Dr. Henry Erlich's laboratory, uses hybridization of sequence specific oligonucleotide (SSO) probes to amplified segments of **HVl** and Hv2 that have been immobilized on a membrane. These SSO probes are designed to detect variation at specific polymorphic nucleotide positions to produce a set series of yes/no results known collectively as a "mitotype" This system is more limited in its discriminatory power than full sequencing; but it offers the advantages of being quick, inexpensive, and does not require great technical skills. Preliminary results suggest that it is more sensitive than current sequencing methods. An analysis of mtDNA SSO-type variation in 142 U.S. whites using 23 SSO-probes revealed 99 different types, and an average probability of identity of 1.9%. A commercial dot/blot strip is under development for the typing of the mitochondrial D-Loop. Moreover, a similar method could be used to interrogate sites outside the D-Loop which are so spread out as to make the cost of sequencing for these polymorphisms prohibitive.

Dot/blot technology could be reduced to microchip probe arrays. Site polymorphisms could also be interrogated through the use of oligonucleotide ligation assays and detection systems, which offer the advantages of greater specificity and large scale multiplexing. These various systems for detecting point polymorphisms are highly amenable to automation. Also denaturing gel electrophoresis could be used as a method to screen for polymorphisms. These technologies could be employed as a screening tool to sort bones with cases, as a method of confirming sequencing results as well as a primary mtDNA typing method to increase the discriminatory potential.

5) Computer Software

Currently, far greater time is spent in the analysis of the sequencing results than in sequencing itself. A significant portion of the time is spent appropriately formatting the data and then checking the raw sequencing data and reading. Computer

software could be developed which could perform these functions more quickly, more accurately, and without tedium. A preliminary neural network system already demonstrates a several fold diminution in the rate of instrumental miscalls from the raw output of the Applied Biosystems, Incorporated, model 373a instrument currently used by the AFDIL.

A laboratory information management system (LIMS) could be developed which could greatly improve the efficiency of current efforts. A **LIMS** could integrate robotic systems, capture instrument data and export it into analysis software, generate reports, improve case tracking and perform quality assurance functions.

The idea that new technologies should be developed and explored, should not be construed as implying current technologies are in any way inaccurate or not worthwhile. Current technology is, however, costly and time consuming, and mitigation of these burdens would be welcome.

The Task Force finds that new technologies should be surveyed for the best prospects for increasing the success and efficiency of DNA identification of ancient skeletal remains. Future investments should be guided by the progress of technology and appropriate advice.

X. SCIENTIFIC BASIS

DSB TOR: To determine the degree of scientific experience and expertise **available to support the Task Force findings.** *[Is the current scientific foundation for DSB findings to these questions adequate or is it premature to answer the foregoing questions? Is further investigation and experience necessary or helpful in determining the answers to the above questions? Should the Task Force be reconvened or should the military otherwise revisit the above issues in the future?]*

The foregoing Task Force Report findings have been based on substantial scientific evidence. While it is always true that more information can and will be produced bearing on important scientific questions, these findings do not seem premature. We note that a Quality Assurance Oversight Committee has been created by direction of the ASD(HA), composed of civilian consultants, which will act to ensure that efforts will continue to be performed with the greatest scientific integrity.

The Task Force supports the ASD(HA) in the creation of a scientific advisory board composed of civilian technical consultants.

The Task Force finds that current DNA identification efforts are supported by sufficient scientific evidence to proceed, in particular with application of mtDNA sequencing to ancient remains from the Korean conflict.

CONCLUSION

The Defense Science Board Task Force find that ancient skeletal remains can be accurately and successfully identified through mtDNA testing. The current methods of mtDNA sequencing are scientifically sound and can be performed in a reliable manner. Furthermore, mtDNA can be used to identify unassociated remains through the creation of a database of family reference specimens.

1. FEASIBILITY:

The Task Force finds that identification of so-called ancient skeletal remains by a program of mtDNA testing is possible, particularly in association with other information. A few specimens may remain unresolved. Although contenders may emerge; at this time, mtDNA sequencing technology is the most appropriate technology.

2. FACTORS:

The Task Force finds that the present probability of coincidental matches between mtDNA control region sequences is no more than a few percent. Once sequences from 500 members of a population have been determined, precise statements about the chance of a false association of a set of remains with a family will be able to be made. Published data may be of value, but samples will be needed from Southeast Asian populations. The precision is expected to suffice in the vast. majority of cases, given other non-DNA evidence, to effect DNA identification of unassociated Korean remains. It will not be possible, however, to identify every bone. A great deal can be done with anatomical and historical evidence alone.

The Task Force finds that control of contamination is essential to PCR-based laboratory testing. Some contamination is unavoidable, particularly in mtDNA testing of ancient remains, but it does not preclude reliable casework testing where redundancy, good laboratory practices, and appropriate cautionary language are used and constant oversight is maintained.

The Task Force finds that casework experience demonstrates capability to type Korean skeletal remains.

3. **RELIABILITY:**

The Task Force finds that appropriate measures must be taken to prevent and control possible contamination in the testing laboratory.

The Task Force finds that current AFDIL protocols, if diligently performed, are capable of generating quality mtDNA sequence identifications.

The Task Force finds that adequate quality assurance requires accreditation and an oversight board.

The Task Force finds that the program for mtDNA sequencing quality assurance promulgated by the Office of the Assistant Secretary of Defense (Health Affairs) for identification of ancient remains is adequate and responsive.

4. **OTHER DNA TARGETS:**

The Task Force recommends that the AFDIL investigate the potential to perform DNA typing outside the mtDNA control region.

5. **FAMILY REFERENCE DATABASE:**

The Task Force finds that, with a reasonable effort, a sufficient proportion of families are expected to provide DNA samples to allow identification of many of the unassociated remains from Korea through mtDNA testing, and to attach meaningful probability statements in those cases.

The Task Force recommends consideration be given to the collection of DNA reference specimens from maternal and paternal family members in case future technology permits nuclear DNA testing. Collections from nonmaternal kindred members (e.g. children) should be made with full disclosure of realistic expectations.

6. **STATISTICAL DATABASE:**

The Task Force finds that the existing databases, and those anticipated from the family collections, provide an adequate basis for the current mtDNA sequencing efforts.

The Task Force recommends an expansion of the current AFDIL

database.

7. LARGE-SCALE OPERATIONS:

The Task Force finds that current mtDNA testing efforts could be augmented for large scale operations. There are strong arguments for a centralization of the laboratory work for the sake of vigorous oversight, quality control, and accountability.

8. RESOURCE REQUIREMENTS:

The Task Force finds that current mtDNA testing efforts at AFDIL are funded appropriately for the Southeast Asia mission.

The Task Force concurs with the projections that analysis of Korean War remains could be accomplished over the next twelve years with an increase of funding of approximately $2 million per year over the cost of current operations.

9. NEW TECHNOLOGIES:

The Task Force finds that new technologies should be surveyed for the best prospects for increasing the success and efficiency of DNA identification of "ancient" skeletal remains. Future investments should be guided by the progress of technology and appropriate advice.

10. SCIENTIFIC BASIS:

The Task Force supports the Assistant Secretary of Defense (Health Affairs) in the creation of a scientific advisory board composed of civilian technical consultants.

The Task Force finds that current DNA identification efforts are supported by sufficient scientific evidence to proceed, in particular with application of mtDNA sequencing to ancient remains from the Korean conflict.

The Task Force notes that the technology for mtDNA sequencing will continue to improve and become less costly due to activities both within and outside the military. Military efforts to identify skeletal remains using DNA will greatly impact law enforcement and contribute to the science of molecular biology, and molecular anthropology.

Annex A

Defense Science Board Terms of Reference

THE UNDER SECRETARY OF DEFENSE

3010 DEFENSE PENTAGON
WASHINGTON, DC 20301-3010

ACQUISITION AND
TECHNOLOGY

-m- 2 1 1994

MEMORANDUM FOR CHAIRMAN, DEFENSE SCIENCE BOARD

SUBJECT: Terms of Reference -- Defense Science Board Task Force on the Use of DNA Technology for Identification of Ancient Remains

Request you establish a Defense Science Board Task Force to study the issues and provide findings concerning the use of DNA comparison techniques for ancient remains identification. The primary purpose is to determine the feasibility of utilizing DNA techniques to identify unassociated ancient remains from past conflicts. Secondarily, the Task Force should pursue and provide findings on issues it uncovers that may have an impact on the current use of DNA in the identification of associated ancient remains.

The Task Force evaluation should include, but is not limited to, the following issues.

a. To determine the feasibility of using DNA techniques for identification of ancient remains as evidenced, in part, by success in identification efforts thus far.

b. To evaluate factors that might influence the effectiveness of using DNA techniques.

c. To evaluate current and emerging scientific evidence concerning the reliability of the techniques when compared with other current and evolving methodologies.

d. To evaluate the possibility of obtaining useful Mitochondrial DNA (MtDNA) information from skeletal remains through MtDNA outside the control region (D-Loop) or possibly nuclear DNA.

e. To determine the degree to which DNA matching could be accomplished with reference donors (family members of up to 8,100 unaccounted for in the case of Korea), adequacy for discrimination of individual from such a database, and what alternatives exist if such family donors are deceased.

f. To recommend a statistical database to be used in calculating the statistical information.

g. To ascertain what effects a large volume of remains could have on the identification process utilizing DNA technologies.

h. To determine the scientific and other resource implications of undertaking large scale DNA testing for identification of unassociated remains.

i. To evaluate other technologies to assist in the automation and reduction of costs associated with DNA testing.

j. To determine the degree of scientific experience and expertise available to support the Task Force findings.

This appraisal should be made using a broad spectrum of governmental and civilian scientific sources and studies. All DOD-related elements that possess technical capabilities that can be brought to bear on this analysis should provide support for the effort. Results of this review will be the basis for policy decisions concerning the use of DNA as a primary or supporting means of identification; establishment and magnitude of an outreach program to family members; and manpower, equipment, and other resources for the Central Identification Laboratory, Hawaii (CILHI) and the Armed Forces DNA Registry.

The Deputy Assistant Secretary of Defense (POW/MIA Affairs) will sponsor this effort. Dr. Joshua Lederberg will serve as Task Force Chairman. LCDR Mark Jensen, USN, will serve a Executive Secretary. LTC John Dertzbaugh, USA, will be the Defense Science Board Secretariat representative. The Defense POW/MIA Office will provide funding and other support as may be necessary. An interim report should be provided within 90 days of Task Force membership approval, and the final report 90 days after that.

R. Noel Longuemare

REGIONAL SECURITY AFFAIRS

OFFICE OF THE ASSISTANT SECRETARY OF DEFENSE
2400 DEFENSE PENTAGON
WASHINGTON. DC 20301-2400

50

In reply refer to:
r-94121998

17 MAY 1994

MEMORANDUM FOR CHAIRMAN, DEFENSE SCIENCE BOARD

THROUGH: ASSISTANT SECRETARY OF DEFENSE FOR INTERNATIONAL SECURITY AFFAIRS 20 MAY 1994

FROM Deputy Assistant Secretary Of Defense for POW/MIA Affairs

SUBJECT; Proposed Terms of Reference – Defense Science Board Task Force on the Use of DNA Technology for Identification of Ancient Remains --*ACTION MEMORANDUM*

Attached is the proposed Terms of Reference for a **DASD** (POW/MIA **Affairs)** Sponsored Defense Science Board review on the use of DNA technology **in** the identification of anclent remains. The proposed terms **have** been reviewed by OUSD(P&R), **OASD(HA),** OASA(M&RA) and **AFIP,** and are submitted for your consideration,

LCDR Mark **Jensen,** USN will serve as my point of contact on this issue, (703) 284-0942. ,

Attachment:
A s stated

DEFENSE SCIENCE BOARD

OFFICE OF THE SECRETARY OF DEFENSE
WASHINGTON. D.C. 20301-3140

MEMORANDUM FOR UNDER SECRETARY OF DEFENSE FOR ACQUISITION AND TECHNOLOGY

FROM: EXECUTIVE DIRECTOR, DEFENSE SClCENCE BOARD
Prepared by LTC John Dertzbaugh, x54157

SUBJECT: Defense Science Board Task Force on the Use of DNA Technology for Identification of Ancient Remains.

PURPOSE: ACTION- To obtain USD(A&T) approval of the Terms of Reference for a DSB Task Force,to review the Use of DNA Technology for Identification of Ancient Remains(Tab A):

*DISCUSSION:

In May, 1994 the Deputy Assistant Secretary of Defense for POW/MIA Affairs requested the Defense Science Board review the use of DNA technology in the identification of ancient remains (Tab 8).

• This Tack Force will study the issues and provide findings concerninq the use of DNA comparison techniques for ancient remains identification The primary purpose is to determine the feasiblility of utilizing DNA techniques to identify unassociated ancient remains from past conflicts. The Task Force will also pursue and provide findings on issues it uncovers that may have an impact on the current use of DNA in the identifioation of associated ancient remains.

COORDINATION: ASD (ISA) see Tab B
Chairman, DSB
Dr. Lederberg

RECOMMENDATION : The USD(ACT) sign the Terms of *Reference* at Tab A.

Attachments
Tab A - Proposed *Terms of* **Reference**
Tab B - USD(ISA) Memo

Approved by John V. Ello/ 6 June 1994

THE UNDER SECRETARY OF DEFENSE

3010 DEFENSE PENTAGON
WASHINGTON, DC 80301-3010

ACQUISITION AND TECHNOLOGY

MEMORANDUM FOR CHAIRMAN, DEFENSE SCIENCE BOARD

SUBJECT: Terms of Reference -- Defense Science Board Task Force on the Use of DNA Teohnology for Identification of Ancient Remains.

Request you establish a Defense Science Board Task Force to study the issues and provide Findings concerning the use of DNA comparison techniques for ancient remains identification. The primary purpose is to det er mine the feasibility of utilizing DNA techniques to identify unassociated ancient remains from past conflicts. Secondarily, the Task Force should pursue and provide findings on issues it uncovers that may have an impact on the current use of DNA in the identification of associated ancient remains.

The Task Force evaluation should include, but is not limited to, the following issues

'To determine the feasibility of using DNA techniques for identification of ancient remains as evidenced, in part, by success in identification efforts thus far.

b. To evaluate factors that might influence the effectiveness of using DNA techniques.

To evaluate current and emerging scientific evidence concerning the reliability of the techniques when compared witb other current and evolving methodologies.

d. To evaluate the possibility of obtaining useful' Mitochondrial DNA (MtDNA) information from skeleta]. remains through MtDNA outside the-control region (D-Loop) or possibly nuclear DNA .

To determine the degree to which DNA matching could be l Cco&ehed with refercnoe donors (family members of up to 8,100 unaccounted for in the case of Korea), adequacy for discrimination of individual From suoh a database, and what alternatives exist if such family donors are deceased.

f. To recommend a statistical database to be used in calculating the statistical information.

g To ascertain what effects a large volume of remains could have on the identification process utilizing DNA technologies.

h. TO determine the scientific and other resource implications of undertaking large scale DNA testing for identification of unassociated remains.

i. To evaluate other technologies to assist, in the automation and reduction of costs associated with DNA, testing.

j To determine the degree of scientific experience and expertise available to support the Task Force findings.

This appraisal should be made using a broad spectrum of governmental and civilian scientific sources and studies. All DOD-related element8 that process technical capabilities that can be brought to bear on this analysis should provide oupport for the effort. Results of this review will be the basis for policy decisions concerning the use- of DNA as a primary or supporting means of identification; establishment and magnitude of an outreach program to family members, and manpower equipment, and other resources for the Central identification Laboratory, Hawaii (CILHI), and the Armed Forces DNA Registry.

The Deputy Assistant Secretary of Defense (POW/MIA Affairs) will sponsor this effort. Dr. Joshua Lederberg will serve as Task Force Chairman. LCDR Hark Jensen, USN, vi11 mrve a Executive secretary. LTC John Dertzbaugh, USA, will be the Defense Science Board Secretariat representative. The Defense POW/MIA Office will provide funding rnd other support as may be neccsary. An interim report should be provided within 90 days of Task Force membership approval, and the final report 90 days after that.

Annex B

Defense Science Board Task Force Members

DEFENSE SCIENCE BOARD MEETING
Task Force Members

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Task Force Chairman

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Task Force Executive Secretary

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Annex C

Mitochondrial DNA Standards

DOD QUALITY ASSURANCE PROGRAM FOR MITOCHONDRIAL DNA IDENTIFICATION OF ANCIENT REMAINS

I. Purpose: To provide basis for ensuring quality testing using mitochondrial DNA (mtDNA) sequence analysis for identification of old skeletal remains for the Department of Defense; government or government-contracted laboratories must comply with the following provisions for DoD laboratory approval:

II. Provisions:

1. Laboratory Accreditation:

   A. College of American Pathologists (General and Molecular Pathology sections); or

   B. American Society of Crime Laboratory Directors - Laboratory Accreditation Board.

2. Standards for MtDNA Sequencing of Skeletal Remains to supplement basic accreditation requirements: [in some standards these requirements are found in one or both of the accreditation requirements of ASCLD or the CAP, but are reiterated to ensure requirement compliance.]

   A. Facilities:
      1) Space dedicated to skeletal remains processing.
      2) Access to the laboratory must be controlled and limited.
      3) Separate pre- and post-PCR areas.
      4) In pre-PCR area, separate areas for extraction and PCR set up.
      5) Dedicated equipment (marked) for amplification room and dedicated storage for PCR product.
      6) Air handling appropriate for prevention of sample contamination.
      7) **A** minimum of 4 linear feet of bench space per analyst during processing in both pre- and post-PCR areas or sufficient space to prevent sample contamination when more than one analyst is working.

   B. Personnel:
      1) DNA Analysts (caseworkers) have primary responsibility for analyses performed and

must be capable of defending their work in court ; they may be assisted by other analysts, molecular biologists, technicians and technologists in the performance of their work.

2) DNA Analysts must have a Baccalaureate degree or higher; a Masters degree in forensic science, biochemistry, or molecular bioiogy or certification in medical technology is preferred.

3) DNA Analysts must have documentation of training and at least six months experience in DNA analysis, as well as experience in mtDNA sequencing of degraded DNA from aged bone specimens, to include demonstrated competency in performing such analysis.

4) Technical Director of the laboratory must:
   a) hold a doctorate-level graduate degree.
   b) have at least one year postgraduate experience in molecular biology.
   c) a member in good standing of the American Academy of Forensic Sciences.

5) Technical Director and DNA Analysts must participate in continuing education in-house and out-of-house yearly. Training and education must be documented.

C. External proficiency testing: Every analyst must undergo proficiency testing twice a year, at least one of which must be an external proficiency test and every lab must undergo blind proficiency testing twice a year. Every deficiency must be explained and corrective action must be demonstrated; approval withdrawn until demonstrated competency after incorrect determinations and reapproved after documentation of further applicable training, reasonable investigation of cause, documentation of corrective action, and demonstration of proficiency to the Quality Assurance Oversight Committee.

1) Open: College of American Pathologists, Parentage Survey (PI).

2) Blind: Duplicate samples will be sent twice a year by CILHI to the laboratory as a blind proficiency test. Exact sequence match is anticipated, although the amount of sequence may differ.

D. Written internal quality assurance program:

1) Documentation of deficiencies and corrective action.

2) Use of reagent blanks during extraction procedure.
3) Use of negative control, positive control, and reagent blank in each amplification reaction.
4) Evaluation of negative control, positive control, and reagent blank in product gels.
5) Written guidelines for acting on positive and negative control results out of specification.
6) Use of National Institute of Standards and Technology (NIST)-specified positive human control.
7) All results must be read independently by a second qualified individual.
8) All reported sequences must be confirmed by duplicate testing or preferably by sequencing of the reverse strand.

E. Chain of custody:
1) Use of DA Form 4137 form for external chain of custody.
2) Must maintain internal and external chain of custody in accordance with Army Regulation AR190-45.
3) Each sample must be labelled with a unique identifier.
4) Casework specimens must be stored under proper seal.

F. Case documentation in single case file and available to the government to include:
1) transportation slips.
2) telephone conversation notes.
3) chain of custody forms.
4) photographs of evidence and appropriate data.
5) case notes sufficient to explain and support analysis.
6) raw data.
7) quality control results.
8) reagent and control lot numbers.

G. Standard Operating Protocols:
1) Protocols must be written and followed for specimen handling, testing procedures, and interpretation.
2) Analytical protocols must be validated.
3) Amplification cycles limited to 40 or less per amplification, second round of amplifications acceptable.
4) Quality control and assurance must be documented and incorporated into standard operating protocols.

5) Written protocols must be submitted, approved, and filed by the Quality Assurance Oversight Committee before use in casework.

H. Reagents:
1) Documented quality control of production, acquisition, and use of all critical reagents.
2) Water:
a) NCCLS Type I water.
b) < 0.1 ppm metal ions (including zinc, iron, lead, and magnesium), monitored weekly.
3) PCR Primers:
a) 95% purity
b) tested for function
c) tested for human mtDNA contaminants
4) Polymerase:
a) licensed for PCR.
b) tested for function.

I. Approved report format:
1) Signed by analyst and certifying official, if not the analyst (i.e. technical director, program manager, corporate official, or other designated person).
2) CILHI and laboratory case number must be incorporated into the final report.
3) AFIP directed format/rules.
4) Terminology:
a) reference to Anderson sequence. [Nature Vol. 290, pp. 457-465, 9 April 1981]
b) use of common nomenclature. [Wilson, et al, Crime Laboratory Digest, Vol. 20, pp. 68-77, Oct 1993]
5) Sequence information must be in format compatible with AFDIL computer system.

J. Analysis:
1) Unknown bone samples must be routinely sequenced before putative reference samples. (except in the creation of a database)
2) Sequenced results must be independently analyzed by a second individual.
3) Statistical analysis by 'counting' method [frequency of observations/population].

K. Database for use in statistical analysis:
1) Database sequences must be confirmed by duplicate testing or preferably by sequencing of the reverse strand.
2) Database sequences must be made public.

3) database sequence must include at a minimum:
   a) Hypervariable Region I: 16024 - 16365.
   b) Hypervariable Region II: 73 - 340.

III. Quality Monitoring

1. Inspections: AFDIL, Civilian Expert Consultant, in the case of a contract lab, the Contract Officer's Representative, if available. Performed semi-annually for the first two years and annually thereafter. Reports to be filed with the Quality Assurance Committee.
2. Copies of all accreditation materials and correspondence with the accrediting organizations filed with the Quality Assurance Oversight Committee.

IV. Quality Assurance Oversight Committee

1. Function: Programmatic and technical oversight of quality assurance program, monitors execution and compliance, grants DoD approval, reviews accreditation materials, monitors proficiency testing results, oversees inspection reports, approves protocols, resolves technical disputes, assures satisfaction of military concerns; does not take action but reviews records and recommends corrective action.
2. Structure: The committee should be a subcommittee of the AFIP Scientific Advisory Board (SAB), composed of five (5) committee members appointed by the AFIP Director: a member of the SAB (chair), a DoD DNA Registry Representative, and three Technical Consultants.
3. Meeting Schedule: Annually and on ad hoc basis when necessary to resolve issues.
4. This meeting will be funded by the AFIP.

The Assistant Secretary of Defense for Health Affairs has ultimate responsibility for quality assurance of DNA testing performed for identification.

Annex D

"X-6" Case Synopsis and Report of Findings

ANNEX D

"X-6" Case Synopsis And Report Of Findings

2 Sep 1958: A US Air Force C-130 was shot down by Soviet MIG Fighters over Soviet Armenia. There were 17 American crew members on board.

16 Oct 1958: Soviet authorities released the remains of six crew members from the plane. Identification of four of the six remains was completed by 1 November. Two of the remains that could not be identified were interred at Arlington National Cemetery (X-3 and X-6).

6 Jan 1993: At the request of a family member, the USAF Mortuary Affairs had the two remains exhumed for evaluation and possible mitochondrial DNA testing.

24 Feb 1993: Skeletal specimens received and DNA testing initiated on the skeletal remains by the Armed Forces DNA Identification Laboratory (AFDIL).

12 Apr 1993: Based upon forensic anthropology evaluations of the remains, made by the Armed Forces Medical Examiner, and successful testing of DNA material from the skeletal remains, family reference specimens were requested.

16 Apr 1993: Family blood reference specimens received by AFDIL.

21 Jun 1993: Testing initiated on the family reference specimens.

23 Jun 1993: Testing completed on family reference specimens.

25 Jul 1993: AFDIL issued a report on DNA testing of both the skeletal remains and the family reference specimens. The report indicated the presence of two individuals, consistent with the anthropological findings. In addition, the DNA type of the X-6 remains was identical to the family reference specimens.

21 Oct 1993: The family requested through the USAF Mortuary Affairs, that a second laboratory perform independent DNA testing of the remains.

8 Nov 1993: Remaining skeletal specimens from AFDIL sent via Federal Express to a research laboratory of the University of California, Berkeley for DNA testing.

ANNEX D

"X-6" Case Synopsis And Report of Findings

25 Jan 1994: Second laboratory reported different DNA testing results for the skeletal remains (see Table). The reported DNA type from the skeletal material did not match the family references.

3 Feb 1994: A moratorium placed on use of DNA as a primary means of identification by the Assistant Secretary of the Army, DoD Executive Agent for Mortuary Affairs.

4 Mar 1994: Meetings held to establish guidelines for a DoD Quality Assurance Program for Military and Contract Laboratories performing mitochondrial DNA testing.

24 Mar 1994: At the request of the Army's Central Identification Laboratory, HI (CILHI), third party testing was initiated by the British Forensic Science Service (FSS), a forensic laboratory. A portion of the "X-6" skeletal remains was sent to the FSS.

7 Apr 1994: "X-6" remains transferred from Dover Air Force Base to CILHI.

19 Apr 1994: Family blood reference specimens sent to the FSS.

18 Jul 1994: A blind test submitted by CILHI, received as a routine skeletal remains case, was received by AFDIL and assigned to two separate DNA teams for duplicate analysis. The teams generated results independently, and came up with the same DNA type.

29 Sep 1994: AFDIL issued a preliminary report of DNA analysis to CILHI on the case received on 18 Jul 94.

3 Oct 1994: AFDIL informed by CILHI that the specimens received 18 Jul 94 were those from the "X-6" remains. The DNA results were identical to the first testing performed, and matched the family references. This process constituted a blind test of the AFDIL laboratory procedures.

9 Dec 1994: Preliminary report from the British FSS indicated that the DNA type of the skeletal remains, "X-6", is the same as the family references and the same type reported by AFDIL.

2 Feb 1995: Final report from the British FSS confirmed that the DNA type generated from the 'X-6' skeletal remains was the same as that reported by AFDIL, and different from the type reported by the UC Berkeley laboratory (see Table).

ANNEXD

"X-6" Case Synopsis And Report of Findings

TABLE: DNA Testing Results Indicating Location of Polymorphisms in the Mitochondrial DNA Control Region

Testing Laboratory	Skeletal Remains DNA Type	Family References DNA Type
AFDIL	16129 A	16129 A
British FSS	16129 A	16129 A
UC Berkeley Laboratory	16223 T, 16291 T	16129 A

Annex E

Memorandum Lifting DoD Moratorium

OFFICE OF THE ASSISTANT SECRETARY OF DEFENSE
WASHINGTON, DC 20301-1200

HEALTH AFFAIRS

06 JAN 1995

MEMORANDUM FOR DIRECTOR, ARMED FORCES INSTITUTE OF PATHOLOGY V-P)

SUBJECT: Mitochrondrial DNA Remains Identification

During a December 22, 1994, meeting with COL. David Suttle, COL Jeanne Hamilton and COL Michael Spinello, it was reported that the British Home Office (BHO) had submitted a preliminary report-concerning the discrepancy noted between the Armed Forces DNA Identification Laboratory (AFDIL) and the Hary-Claire King Laboratory. It was stated that the preliminary report found a sequence which matched the Bourg reference sample. The preliminary report noted that this confirmed the AFDIL results. During further conversation, the Amy representatives expressed confidence in the quality assurance process used by AFDIL.

Since the AFDIL quality assurance procedure has been evaluated as acceptable, the previous verbal requirement to submit all request6 to this office for approval prior to performing DNA analysis is rescinded. Please respond to all Army requests for analysis in an expeditious manner, and consider that Amy request have had a waiver at Army level to the Army imposed moratorium.

Edward D. Martin

Edward D. Martin M.D.
Principal Deputy Assistant Secretary

cc:
COL Suttle
COL Hamilton
COL Spinello

HA Control #a
Due Dated

22 DEC 1994

ASSISTANT SECRETARY OF DEFENSE
(HEALTH AFFAIRS)
EXECUTIVE SUMMARY/COVER BRIEF

MEMORANDUM FOR THE PRINCIPAL DEPUTY ASSISTANT SECRETARY OF DEFENSE (HEALTH AFFAIRS)

THROUGH: Dr. Sue Bailey, DASD(CS)

FROM: COL 8. M Cirone, VC, USA

SUBJECT: Mitochondrial DNA Remains Identification

PURPOSE:

SIGNATURE sign a letter: reinstating routine mitochondrial DNA identification of remain at AFDIL.

DISCUSSION:

Became of discrepancies noted in the identification of associated remains and because of the question concerning use of DNA technology for the primary identification of Korean War remains, a moratorium has been placed on the use of DNA technology has the primary means of remains identification until the Defense Science board (DSB) reviews the technology. A final report is not expected prior to *February 1995.*

As a corollary action, samples involved in the associated remains discrepancies were forwarded to the British Home Office (BHO) for: resolution. Current information is that a resolution of this problem may not come from the BHO However a preliminary report war submitted but has not been seen by this office.

In the interim the Armed Forces DNA Identification Laboratory (AFDIL) was told verbally to perform preliminary work in the remains identification process without actually collecting reference l mpleo and performing associated remains identification. AFDIL was told to contact this office and raquest approval prior to the confirming

identification of remains using the mtDNA technology.

Although the AFDIL quality assurance procedures are currently being peer reviewed, the initial reports indicate an approval of the technology with some recommended modifications of the overall quality assurance program. This is in progress The final report is expected in January.

At a meeting yesterday, 21 Dec 94, Army noted that the premliminary report from the British Home Office was received two days ago, and it verifies the AFDIL results . The Army noted that it Was satisfied with the AFDIL QA. All individuals at the meeting agreed that it would be appropriate to resume routine mtDNA testing without further delay.

This memorandum instructs AFDIL to perform routine DNA testing without the need for formal approval by this office.

RECOMMENDATION:
Sign the memorandum.

COORDINATION:
COL Armbrustmacher _____
Mr. **Wold, DASD POW/MIA** _____

AASD (HA) DECISION

APPROVED: _____

DISAPPROVED: _____

OTHER: _____

Annex F

Mutations

MUTATIONS

Dr. Douglas C. Wallace
Department of Genetics & Molecular Medicine
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At birth, the mtDNA genomes appear to be relatively homogeneous throughout the body. Multiple investigations have demonstrated that mtDNA rearrangement mutations, generally assessed using the common 5 kilobase (kb) [4977 nucleotide pair (np)] deletion, are rare or absent in the newborn. This low level of somatic mtDNA mutations appear to be sustained in rapidly dividing tissues such as white blood cells (Cortopassi et al., 1992a, Monnat and Ray, 1986). However, post-mitotic tissues of individuals over 30 years have been found to accumulate a wide variety of deleterious mtDNA mutations including both rearrangement and nucleotide substitution mutations. Studies on the accumulation of the common 5 kb deletion have revealed that the highest levels of somatic mtDNA rearrangements occur in brain, followed by muscle, heart, and kidney, with deletion levels increasing more that 10,000 fold from young to old age (Cortopassi and Amheim, 1990; Cortopassi et al., 1992b: Corral-Debrinski et al., 1991,1992a and b; Soong et al., 1992).

In the brain, the 5 kb deletion can increase to 10% of the total mtDNAs in the basal ganglia, by age 80,0. 1 to 2% in the cerebral cortex, by age 75. By contrast, the level of this deletion remains low, about 0.OOl%, in the cerebellum throughout life (Corral-Debrinski et al., 1992; Soong et al., 1992). Significant levels of mtDNA deletions have also been observed in extraocular muscle (Munscher et al., 1993a and b; Munscher, et al., 1993a and b;) skeletal muscle (Simonetti et al., 1992); heart (Corral-Debrinski et al., 1991 and 1992); and other tissues (Cortopassi et al., 1992; Zhang et al., 1992), though at lower levels then found in the basal ganglia and cortex.

A number of nucleotide substitution mutations have also been found to accumulate with age. All three pathogenic tRNA mutations examined were found to accumulate with age. These included the np 8344 (MERRF) mutation in $tRNA^{Lys}$, the np 3243 (MELAS) mutation in $tRNA^{Leu(UUR)}$, and the NP 10006 $tRNA^{Gly}$ mutation. By contrast, the neutral polymorphism at np 12308 in $tRNA^{Arg}$ did not increase (Munscher et al, 1993 a and b; Reigers et al., 1993; Zhange et al., 1993). The reason for this selective increase in deleterious mtDNA mutations in post-mitotic cells is unknown. One possibility, however, is that cell nuclei in the presence of defective mitochondria sense the localized bioenergetic defect through the concentration of some mitochondrial metabolite. This signal then stimulates the nuclei to increase the synthesis of the surrounding mitochondria in an effort to compensate for the defect. However, this creates a distinctive cycle, for instead of increasing the levels of normal mitochondria, the nucleus stimulates the replication of the adjacent defective mtDNAs.

New mtDNA mutations have also been shown to arise in the female germline. A portion of the oocytes from normal female donors have recently been to harbor the 5 kb deletion (Suganima et al., 1993); a neutral, heterplasmic mutation has been reported in the ND6 gene at np 14560, in a family for three generations (Howell et'al., 1992); and the AFDIL has observed two

cases of the fixation of mtDNA sequence differences between a mother and child. The most dramatic demonstration of the frequent appearance of mutations has come from the rapid identification of new mtDNA mutations in human degenerative diseases (Wallace, 1992a and b; Wallace et al., 1994).

All of these data demonstrate that post-mitotic tissues such as bone may harbor heteroplasmic, mutations, of both somatic and germline origin. Hence, caution should be exercised when drawing conclusions based on a single genetic characteristic state.

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Annex G

Outreach Program

OUTREACH PROGRAM

PURPOSE

The purpose of the outreach program is to contact persons authorized to direct the disposition of remains and acquire reference blood specimens from the families of Americans whose remains were not recovered from the Vietnam, Korean and Cold Wars.

ASSUMPTIONS

Every family of an American service member whose remains were not recovered from Southeast Asia, Korea, or the Cold Wars that can be contacted will be offered an opportunity to contribute a blood sample from two different maternal relatives.

The government will take maximum advantage of having family donor blood samples drawn at government facilities proximate to the location of the donor at no additional direct cost to the government.

The organization dedicated to performing the outreach function will be quartered in government owned facilities or premises already under government contract.

DISCUSSION

Background.

There are approximately 8 100 American Korean War casualties whose remains were not recovered or identified. Fewer than 500 Korean War families from this group are immediately contactable by their respective Service. Additionally, a significant portion of the personnel records covering this period were destroyed in a 1973 fire at the National Personnel Records Center in St. Louis, Missouri. Even when these personnel records are available, there is a dearth of medical and dental records that can be used to identify remains.

There are approximately 2200 Americans listed as unaccounted for from the war in Southeast Asia. Approximately ninety percent of the families of the servicemen and civilians in this group are contactable by their respective Service.

There are approximately 132 Cold War losses. Most of these losses are Navy and Air Force servicemen. About 95 percent of the family members of these Cold War losses can be contacted by the Navy/Air Force.

The poor condition of remains recovered from Korea, the paucity of records necessary to make an identification, and the increasing lack of identifiable portions from Southeast Asia, have reduced the effectiveness of the traditional anthropological and dental techniques to identify these fallen Americans. DNA technology is the best available technology to offer any prospect of identifying these remains.

Using DNA techniques on remains from the wars in Southeast Asia, Korea, and the Cold War will require identifying and contacting prospective DNA donors from relatrves of the service member. A substantial effort will be needed to identify eligible DNA donors and concurrently contact persons authorized to direct the disposition of remains that may be recovered. The Outreach

Program is intended to support this effort.

The consensus within the forensic community is that two blood samples from maternal relatives of the person to be identified are adequate to conduct mtDNA analysis for the purpose of making a comparison for identification. Although nuclear DNA technology is not currently a viable technology to identify old remains, this technology may become feasible in the future. Should nuclear DNA technology prove to be a feasible future technology, identifications could be made using samples from relatives other than maternal kin, in particular, siblings. An outreach sampling strategy of collecting not more than two MtDNA and two nuclear DNA samples per family would allow samples for current and future technology to be collected as a part of the same program.

Of the 8 100 servicemen not recovered or identified from the Korean War, an early assessment was that 5000 remains might be recovered from North Korea. Subsequent analysis opined that up to 3000 American remains may be forthcoming should the North Koreans **allow** optimum access to recover and repatriate American remains. (See enclosure 1). Korean War losses (bodies not recovered) represent the bulk of the population toward which the outreach effort will be focused.

It is unlikely that the government will be able to contact and acquire DNA samples from all of the families of service members not recovered from Vietnam, Korea, and the Cold War. The Defense Nuclear Agency has had experience in attempting to contact persons exposed to radiation during the Korean-era. Their effort, although not identical to the prospective DNA outreach effort, has many of the same impediments: client unawareness, poor records availability, aging population, etc.

The Defense Nuclear Agency's experts best guess estimate is that a concerted DNA outreach effort may net contacting 40-70 percent of the target population (DNA donors/person authorized to make disposition decisions). A working rate of 70 percent contactability was used for costing purposes.

Concept of the Outreach Program.

An outreach program for Vietnam War', Korean War, and Cold War families of Americans whose remains were not recovered would have three principal purposes:

- to identify and contact persons authorized to direct the disposition of remains;
- to identify and contact prospective DNA donors;
- to raise public awareness of government efforts to repatriate, identify, and bury with honor the remains of Americans lost in Southeast Asia, Korea, and the Cold War.

An organization will be needed to accomplish the goals of the outreach program. An organization specifically designed for and dedicated to the outreach mission has the best prospect for successfully accomplishing this mission. With Department of Defense downsizing, military personnel resources are at a premium. As a result, contractual resources may be more available than military personnel to address the outreach effort. Military presence in the effort to contact the family members of servicemen killed during war is critical to reinforcing the Services' continuing commitment to its fallen comrades and their families. Given the military personnel constraints, a military-supervised, contractor-operated operation would be the most effective approach to address a program like DNA outreach.

The program would be accomplished by a dedicated cell. The contractor will be co-located with the military supervisors. The contractor will proactively contact persons authorized to direct the disposition of remains as well as appropriate DNA donors for a period of 2 years or when 70% of the donors/persons authorized to direct the disposition of remains are contacted, whichever occurs first. This latter condition will ensure that the outreach effort does not inordinately continue when diminishing returns would indicate reduced effectiveness.

There are five cost components to the outreach program:

- Contractor development, automated database operation, and client information input cost (enclosure 2).

- DNA blood sample collection cost (enclosure 3):
  - DNA blood sample collection kit cost.
  - DNA blood sample collection kit mailing cost.
  - -- DNA blood sample draw cost.

- Travel cost to participate in various veteran and advocacy group activities, conventions, media events, and advertising (enclosure 4).

- Staffing:

  Civilian: Temporary government civilian word processor cost:
  1 - GS/4 Wordprocessor (enclosure 5).

  Military:

  3 - Officers (Army) 4 - enlisted (l/Service)

  Contractor: See enclosure 2.

- Facilities and space will be provided at no additional expense by using currently available space.

The aggregate cost of each of the outreach program components is summarized in enclosure 6.

INFORMATION PAPER

TAPC-PED-P
19 January 1995

SUBJECT: Potential for Remains Recovery from North Korea

1. Purpose. To provide an estimate of the potential number of remains that may be recovered through joint United States/North Korean Peoples Army recovery operations and investigations in North Korea.

2. Facts.

a. Based on the available records and information, the U.S. Army Central Identification Laboratory, Hawaii (CILHI), Casualty Data Section, established preliminary figures for the number of recoveries as indicated below.

b. The available information can be categorized into two areas:

(1) Incidents or areas where there is sufficient information to establish a known location where the recovery of remains is possible, i.e. known prisoner of war (POW) camps, temporary cemeteries, and known aircraft loss sites.

Reported burials in former POW camps with known locations	1612
Known burials in cemeteries north of the Demilitarized Zone (DMZ)	181
Known aircraft general loss sites north of the DMZ	633
Total number losses with documented loss sites	2426

(2) Incidents or losses which may lead to remains recovery:

Reported POW camp burials with no known location	535
Reported interments in cemeteries with no location	1
Aircraft losses without fixed or general location	32
Total number of individuals without a fixed or known loss site	568

c. The sum of the two categories (2994) above represents the upper limit of the reasonable prospect for the number of individuals that may be recovered through joint investigations and recoveries.

Mr. Webb/@OS) 44843903
Approved by COL M. T. Spinello

Enclosure 1

OUTREACH PROGRAM

CONTRACTOR DEVELOPMENT AND OPERATIONS COST ESTIMATE

Contractor development and automated database operation for a DNA outreach program was designed based on the following assumptions:

a. The system will contain up to 33,000 records associated with Americans whose remains were not recovered from the Korean and Cold Wars or who remain unaccounted for from Southeast Asia including information on persons authorized to direct the disposition of remains and DNA blood donors.

b. The Outreach database will be compatible with and capable of transmitting data to the Army Casualty Information Processing System (ACIPS).

The specifications of the contract would provide for the following terms of work:

a. The contractor will provide all computer hardware and software.

b. The contractor will provide outreach data operators.

c. An interactive voice response (IVR) system will answer initial calls and record the pertinent information (i.e., caller name, the unrecovered Americans' name and service number, relationship to the unrecovered soldier, etc.).

d. The contractor's operators will return valid calls and manually key pertinent information into the database.

e. The contractor's operators will validate the information captured from the initial calls against the database.

f. The contractor will develqp a custom database package that wiIl allow for storage and retrieval of outreach information and enhance ACIPS by providing a subsystem that directly accesses outreach information.

g. The contractor will preload the database with the names and social security numbers and/or service numbers of unrecovered Americans from the Vietnam Korean and Cold Wars.

h. The contractor will develop up to ten management reports to Army specifications.

i. The contractor will provide training on the use of the system to military staff.

j. The contractor will verify familial relationships.

System Configuration

- Staff: Five operators

- Equipment:

  Five IBM compatible 486 PC workstations (40 megabyte of RAM and 80 megabytes of disk storage and an ethernet local network card).

  One 486 IBM compatible PC complete with the IVR hardware and software.

  Local Area Network software and hardware capable of sustaining 5 workstations.

  Multi-user database management system.

- **Cost**

System setup	**$214,000**
Staffing cost/year	**$250,000**
Total cost for 2 years	**$714,000**

MtDNA BLOOD SAMPLE COLLECTION COST

In 1994, the Defense Nuclear Agency was tasked to locate servicemen exposed to nuclear radiation as a result of experiments conducted during the Cold War. Their empirical results in locating Korean-era Department of Defense affiliated personnel were used to estimate the number of families that may be contacted as a result of an outreach effort. They were able to locate about sixty percent of the estimated population.

Collection Cost:

- DNA blood sample collection kit cost: $10
- Mailing the DNA blood sample collection kit cost: $14
- Drawing DNA blood sample cost: $40

Total Collection Cost: $64 each

Sample Population:

- Number of Southeast Asia Unrecovered servicemen: 2,211
- Number of Korea War Unrecovered servicemen: 8,100
- Number of Cold **War** Unrecovered: 132

Approximate Sample Population: 11,000

Maximum Number of Kits at an average Three per Family: 11,000 x 3 = **33,000**

Maximum Total Collection Cost: **$64 x 33,000 x .7 = 985,600**

Enclosure 3

OUTREACH PROGRAM PUBLICITY COST

TEMPORARY DUTY TRAVEL COST - Estimated cost to attend veterans conventions, media events, and advertising.

1st year	$20,000
2nd year	$20,000

ADVERTISING - Estimated cost to propagate the outreach message in various media.

1st year	$10,000
2nd year	$10,000
Total estimated publicity cost for two years	$60,000

TEMPORARY GOVERNMENT CIVILIAN WORDPROCESSOR COST

GRADE	JOB TITLE	ANNUAL COST
GS 4/5	Office Automation Operator	$21,000
Total cost for two years operation:		$42,000

OUTREACH COST SUMMARY

(TWO YEARS)

CONTRACTOR DEVELOPMENT AND OPERATIONS COST ESTIMATE	**$ 714K**
DNA BLOOD SAMPLE COLLECTION COST	**$986K**
OUTREACH PROGRAM PUBLICITY COST	$ 60K
TEMPORARY GOVERNMENT CIVILIAN WORDPROCESSOR COST	$ 42K
TOTAL COST	$1802K

Annex H

Projected Resource Requirements for Korean Remains
Phase I

KOREAN WAR REMAINS IDENTIFICATIONS PROJECTIONS

PHASE I

ASSUMPTIONS:

1) @ 40% OF FAMILIES, 6,480/3520 = Approximately 1.8 years
2) @ 70% OF FAMILIES, 11,340/3520 = Approximately 3.2 years

MARGINAL COSTS:

1) LABOR:

10 ADDITIONAL PERSONNEL = $498,000 PER YEAR

2) SUPPLIES & REAGENTS

Equipment Requirements:

$220,000	2 ABI 373A DNA Sequencers (220K)
$24,000	2 Per-kin-Elmer Thermal Cyclers ($5OK)
$50,000	Misc. Equipment ($50K)
$294,000	TOTAL COST of additional **equip**

Capacity with new equipment and additional personnel:

8 cases/shift/day = 16 cases/day x 220 days/year = 3520 cases/year

'In the course of one work day, one run in the morning and one in the afternoon will be run.

Timeframe:

DURATION IN YEARS	POSSIBL NUMBER SAMPLE	OUR ANNUAL OUTPUT	ANNUAL COSTOF SUPPLIES	TOTAL COSTOF SUPPLIES	TOTAL COSTOF LABOR	TOTAL COST OF PROJECT
1.8	6480	3520	$211,200	$388.800	$896,400	$1,579,200
3.2	11340	3520	$211,200	$680,400	$1,593,600	$2,568,000

Additional Personnel:

1 Supervisory DNA Technologist @ $65,000 = $65,000
6 DNA technicians @ $49,000 = $294,000
2 Administrative support person @ $45,000 = $90,000
1 Computer support person @ 49,000 = $49,000

TOTAL COST OF ANNUAL LABOR = $498,000

Annex I

Projected Resource Requirements for Korean Remains
Phase II

KOREAN WAR REMAINS IDENTIFICATIONS PROJECTIONS

PHASE II

ASSUMPTIONS:

1) 3000 SETS OF REMAINS
2) 4 BONE SAMPLES PER SET OF REMAINS
3) DNA TESTING PERSONNEL @ 50% INCREASED EFFICIENCY

MARGINAL COSTS:

1) LABOR COSTS
2) SUPPLIES & REAGENTS

FIXED COSTS:

$1,050,000 FOR LEASE & UTILITIES
$140,868 FOR 3 ADMINISTRATIVE PERSONNEL
$102,874 FOR 5 YEAR AMORTIZED INSTRUMENTS & EQUIPMENT COSTS
$50,000 FOR MAINTENANCE AGREEMENTS
$1,343,742 PER YEAR

	DURATION IN YEARS	NUMBER OF CASES YEARLY	TOTAL LABORATORY PERSONNEL NEEDED	FIRST YEAR TOTAL COST	** TOTAL LABOR COST	*** TOTAL MARGINAL COST	**** TOTAL PROJECT COST
OPTION 1	8	375	42	$5,855,942	$26,143,974	$39,943,974	$50,693,910
OPTION 2	10	300	33	$4,961,542	$27,498,555	$38,538,555	$51,975,975
OPTION 3	12	250	28	$4,356,342	$28,803,306	$38,003,306	$54,128,210

NOTES:
** SUBSEQUENT YEARS MUST ACCOUNT FOR INFLATIONARY INCREASE IN LABOR
— INCLUDES 4.5?kANNUAL INFLATIONARY INCREASE
— MARGINAL + FIXED COSTS

Figure 1

Human Mitochondrial Genome

Figure 1.

Human Mitochodrial Genome

Human mtDNA is a circular loop of double-stranded DNA, 16,569 basepairs in length. One strand is termed the heavy strand and the other the light strand, based on the ability to separate the two strands by ultracentrifugation. Position 1 is arbitrarily designated in the middle of the Control Region, also known as the Displacement Loop.

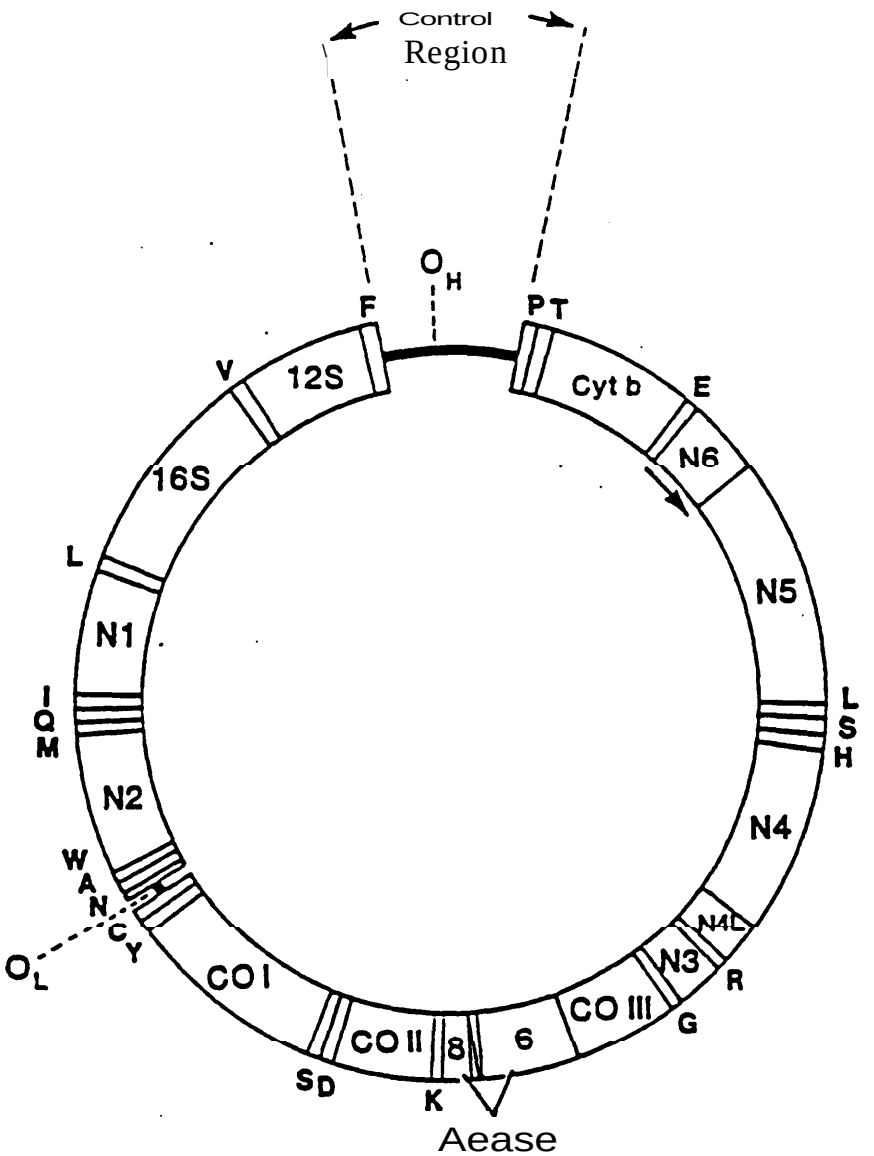

Figure 1. The circular human mitochondrial genome, with fknctional regions indicated as follows: two ribosomal RNA genes (l2S and 16S); 13 protein-coding genes [three cytochrome oxidase subunits (COI-COIII), seven NADH-dehydrogenase subunits (Nl-N6, N4L), two ATPase subunits (6 and 8), and cytochrome b (Cyt b)]; and 22 transfer RNA genes (designated by single letter code). The thin solid bars indicate the major noncoding regions, containing the origin of replication of the heavy (OH) and light (OL) strands.

Figure 2

Human Mitochondrial Genome Regions

Figure 2.

Human Mitochondrial Genome Regions
The circular mtDNA is primarily composed of functional coding regions, as: two ribosomal RNA genes (12s and 16s); 13 protein-coding genes [three cytochrome oxidase subunits (COI-III), seven NADH-dehydrogenase subunits (Nl-N6, N4L), two ATPase subunits (6 and 8), and cytochrome b (Cyt b)]; and 22 transfer RNA genes (designated by single letter code). The thin solid bars indicate the major noncoding regions, containing the origin of replication of the heavy (OH) and OL) strands.

Figure I: Human Mitochondrial Genome

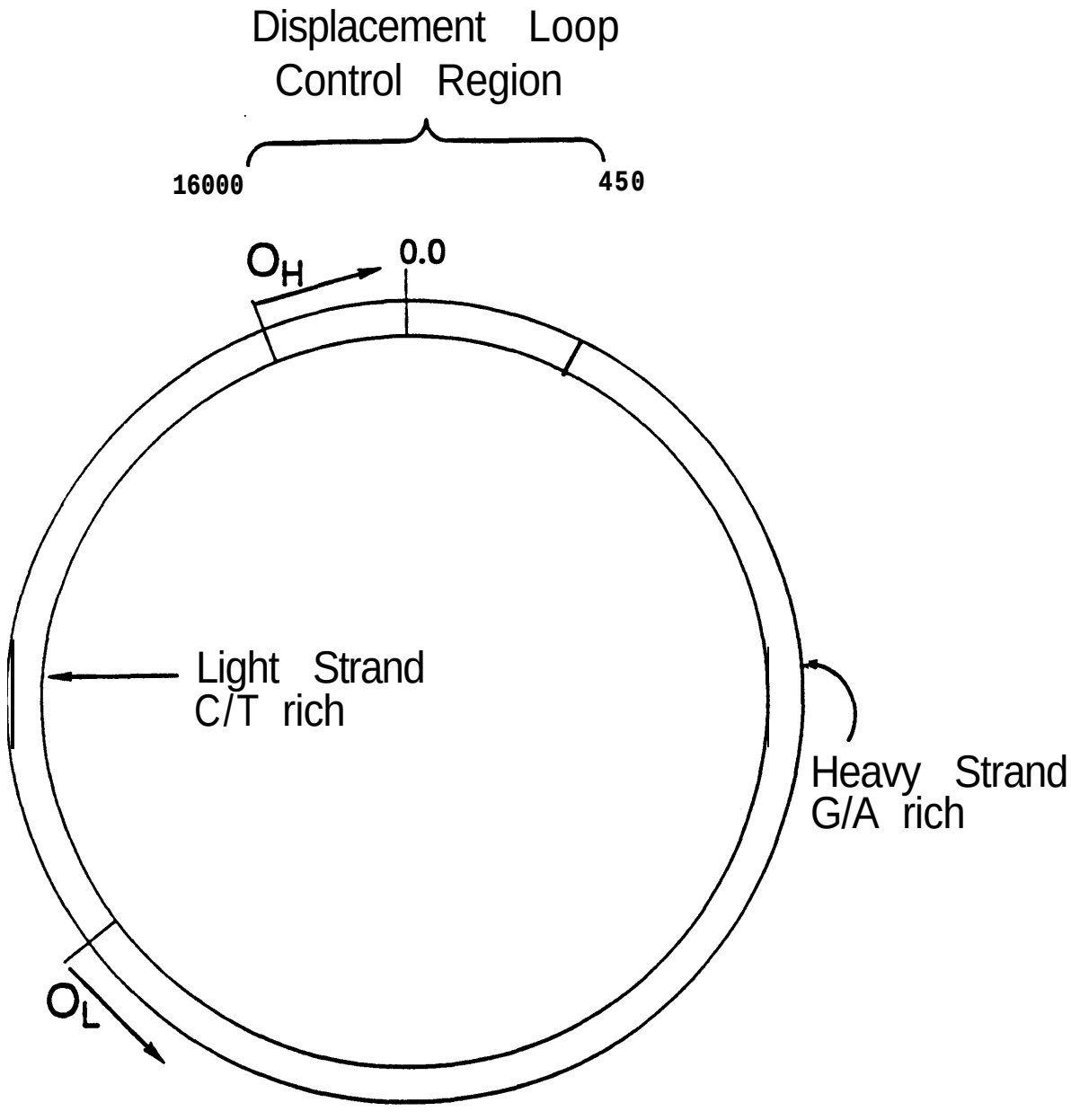

Figure 3

Human Mitochondrial Control Regions

Figure 3.

Human Mitochondrial Control Regions
Diagram of the human mtDNA control region (displacement loop). The control region is flanked by proline and phenylalanine tRNA genes. Shown are the location of the two hypervariable segments, the nine SSO-defined regions, and the primers initially used for PCR reactions. PCR primer sequences are given by Vigilant et al. (1989).

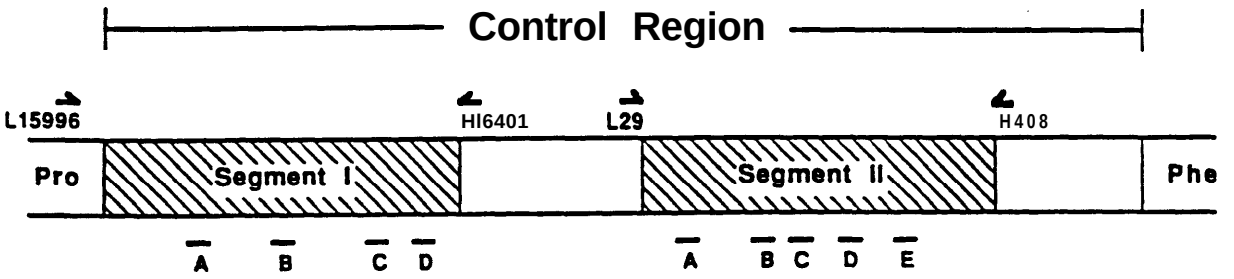

Figure 2. Diagram of the human mtDNA control region. The control region is flanked by proline and phenylalanine tRNA genes. Shown are the location of the **two** hypervariable segments, the nine SSO-defined regions and the primers used in PCR reactions. PCR primer sequences are given by Vigilant et al. (1989).

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Figure 4

Bequence of the Control Region Eypervariable Segments

Figure 4.

Sequence of the Control Region Hypervariable Segments
The actual DNA sequence of the hypervariable segments of the human mtDNA control region. The sequence is that of the "Anderson" sequence, the first reported sequence of the mitochondrial genome in Nature (1981). The numbers represent base number designations.

Hypervariable Region One

16030	16040	16050	16060	16070	
TTCTTTC	ATGGGGAAGC	AGATTTGGGT	ACCACCCAAG	TATTGACTCA	**Anderson**
16080	**16090**	**16100**	**16110**	**16120**	
CCCATCAACA	ACCGCTATGT	ATTTCGTACA	TTACTGCCAG	CCACCATGAA	**Anderson**
16130	**16140**	**16150**	**16160**	**16170**	
TATTGTACGG	**TACCATAAAT**	**ACTTGACCAC**	**CTGTAGTACA**	TAAAAACCCA	**Anderson**
16180	**16190**	**16200**	**16210**	**16220**	
ATCCACATCA	**AAAccccctc**	**CCCATGCTTA**	**CAAGCAAGTA**	**CAGCAATCAA**	**Anderson**
16230	**16240**	**16250**	**16260**	**16270**	
CCCTCAACTA	**TCACACATCA**	**ACTGCAACTC**	CAAAGCCACC	**cctcacccac**	**Anderson**
16280	**16290**	**16300**	**16310**	**16320**	
TAGGATACCA	**ACAAACCTAC**	**ccacccttaa**	**CAGTACATAG**	**TACATAAAGC**	**Anderson**
16330	**16340**	**16350**	**16360**		
CATTTACCGT	**.ACATAGCACA**	**T'I'ACAGTCAA**	**ATCCCTTCTC**	**GTCCC**	**Anderson**

Hypervariable Region Two

80	90	100	110	120	
ATGCACGC	**GATAGCATTG**	CGAGACGCTG	**GAGCCGGAGC**	**ACCCTATGTC**	**Anderson**
130	**140**	**150**	**160**	**170**	
GCAGTATCTG	**TCTTTGATTC**	**CTGCCTCATC**	**CTATTATTTA**	**TCGCACCTAC**	**Anderson**
180	**190**	**200**	**210**	**220**	
GTTCAATATT	**ACAGGCGAAC**	**ATACTTACTA**	**AAGTGTGTTA**	**ATTAATTAAT**	**Anderson**
230	**240**	**250**	**260**	**270**	
GCTTGTAGGA	**CATAATAATA**	**ACAATTGAAT**	**GTCTGCACAG**	**CCACTTTCCA**	**Anderson**
280	**290**	**300**	**310**	**320**	
CACAGACATC	**ATAACAAAAA**	**ATTTCCACCA**	**AACCCCCCCT**	CCCCCGCTTC	**Anderson**
330	**340**				
TGGCCACAGC	**ACTTAAACAC**				**Anderson**

Figure 5

Polymorphism Distribution of the Mitochondrial DNA Control Region

Figure 5.

Polymorphism Distribution of the Mitochoadrial DNA Control Region
Distribution of polymorphic nucleotide positions across the mtDNA control region derived from the control region of 22 individuals of African, Asian, and European ancestry. The figure indicates the number of polymorphic sites occurring in 50 bp intervals across the control region. The majority of the polymorphisms occur in two hypervariable regions.

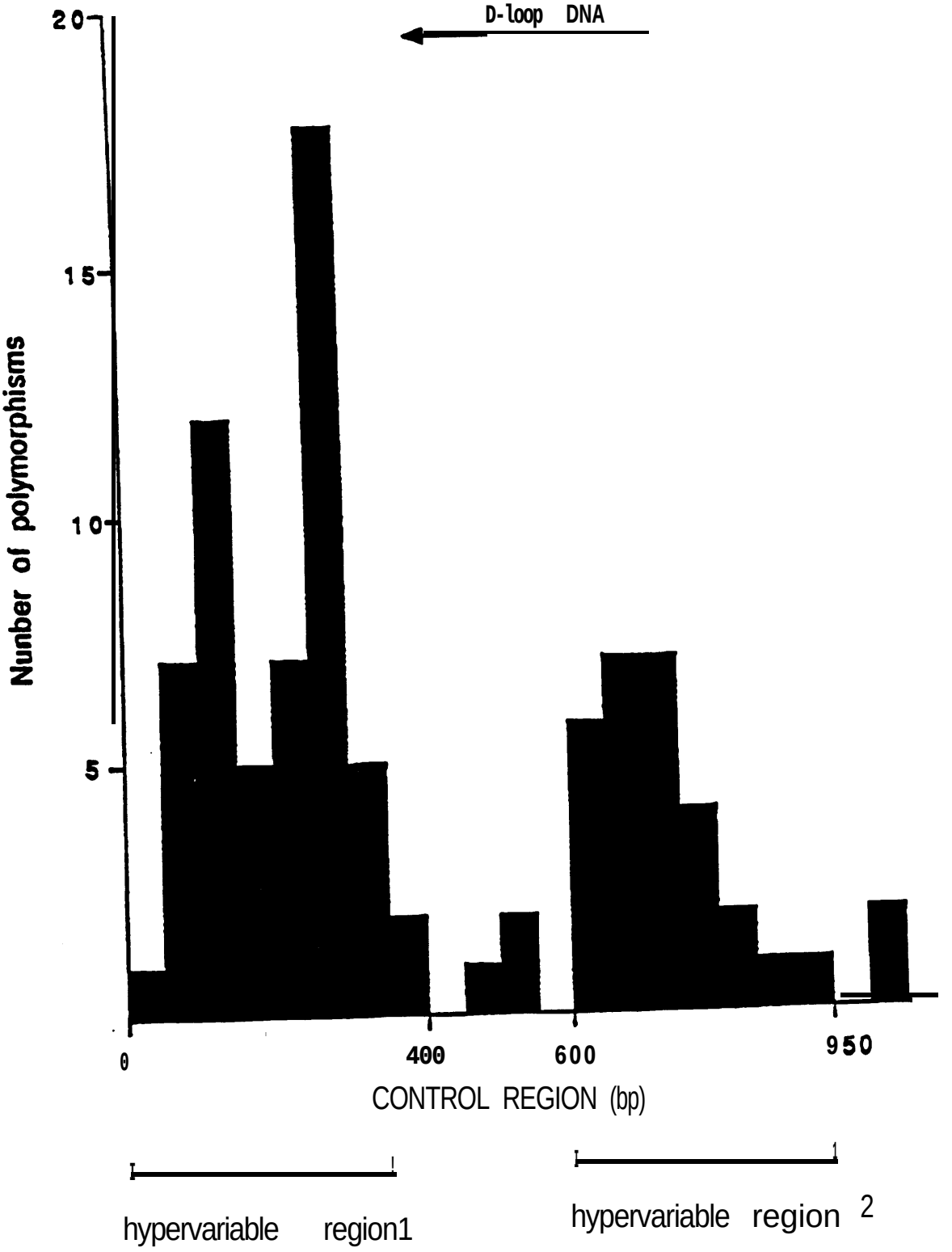

D-loop DNA
20
15
10
5
Number of polymorphisms
0
400
600
950
CONTROL REGION (bp)
hypervariable region1
hypervariable region 2

Figure 6

Constraints on Polymorphism of the Mitochondrial DNA Control Region

Figure 6.

Constraints on Polymorphism of the Mitochondrial DNA Control Region

Variation within eight functional segments of the control region. TAS is the termination associated sequence, CSB-1 through 3 are the conserved sequence blocks, LSP and HSP are the light and heavy strand promoters, and mtTF-L and mttf-H are the binding sites for the mtDNA transcription factor. The large nucleotides are polymorphic in the 189 humans surveyed. The observed and expected number of polymorphic nucleotides for each segments are significantly different only in the case of the TAS. Expected values were calculated by assuming a random distribution of variability across the entire control region and for some segments included only information from the complete control region sequences.

		Number of changes	
		Observed	Expected
TAS	ACATAAAAACCCAAT	9	3.8
CSB-1	TTAATGCTTG TA G GACAT A A	5	5.1
CSB-2	CAAACCCCC C C T CCCC	4	4.0
CSB-3	TGCCAAACCCCA AAAACA	1	4.6
LSP	TTCAAATTTTATCTTTTGGCGGTATGCACTTTTAACAGTCACCC	0	3.6
mtTF-L	ACTTTTAACAGTCACCCCCCAACT	0	1.9
mlTF-H	GCTGCTAACCCCATACCCCGAACC	0	1.9
HSP	GACCAACCAAACCCCAAAGACA	0	1.8

Figure 7

AFDIL Mitochondrial DNA Amplification and Sequencing Scheme

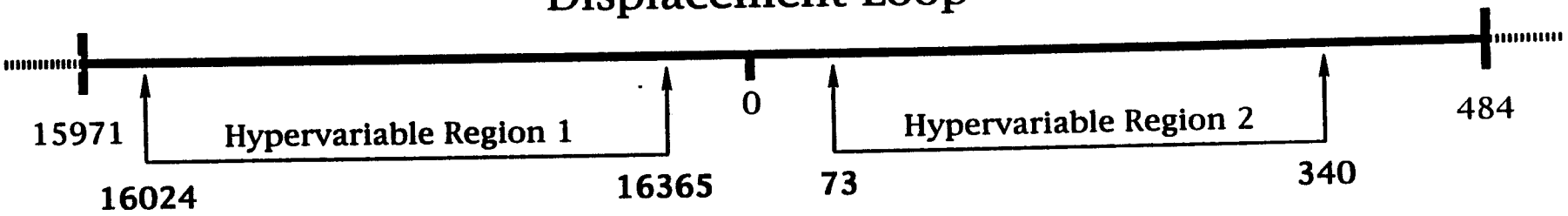
Figure 7.

AFDIL Mitochondrial DNA Amplification and Sequencing Scheme
AFDIL performs mtDNA analysis by amplification and sequencing of two sets of overlapping segments. The sequence 15971-16410 includes Hypervariable region 1 and the sequence 15-389 includes Hypervariable region 2.

AFDIL
Mitochondrial DNA Amplification and Sequencing Scheme

*All Primers are 20-mers, Numbers Reflect The 5'-end

Displacement Loop



15971 Amplification 16258

15971 Sequencing 16258

15 Amplification 274

15 Sequencing 274

16144 Amplification 16410

16144 Sequencing 16410

155 Amplification 389

155 Sequencing 389

(or 484)

Figure 8

Division of Labor for Current AFDIL Casework

Figure 8.

Division of Labor for Current AFDIL **Casework**

Approximately half of **the** effort spent in current casework is expended in analysis.

BREAKOUT BY TASK (TIME)

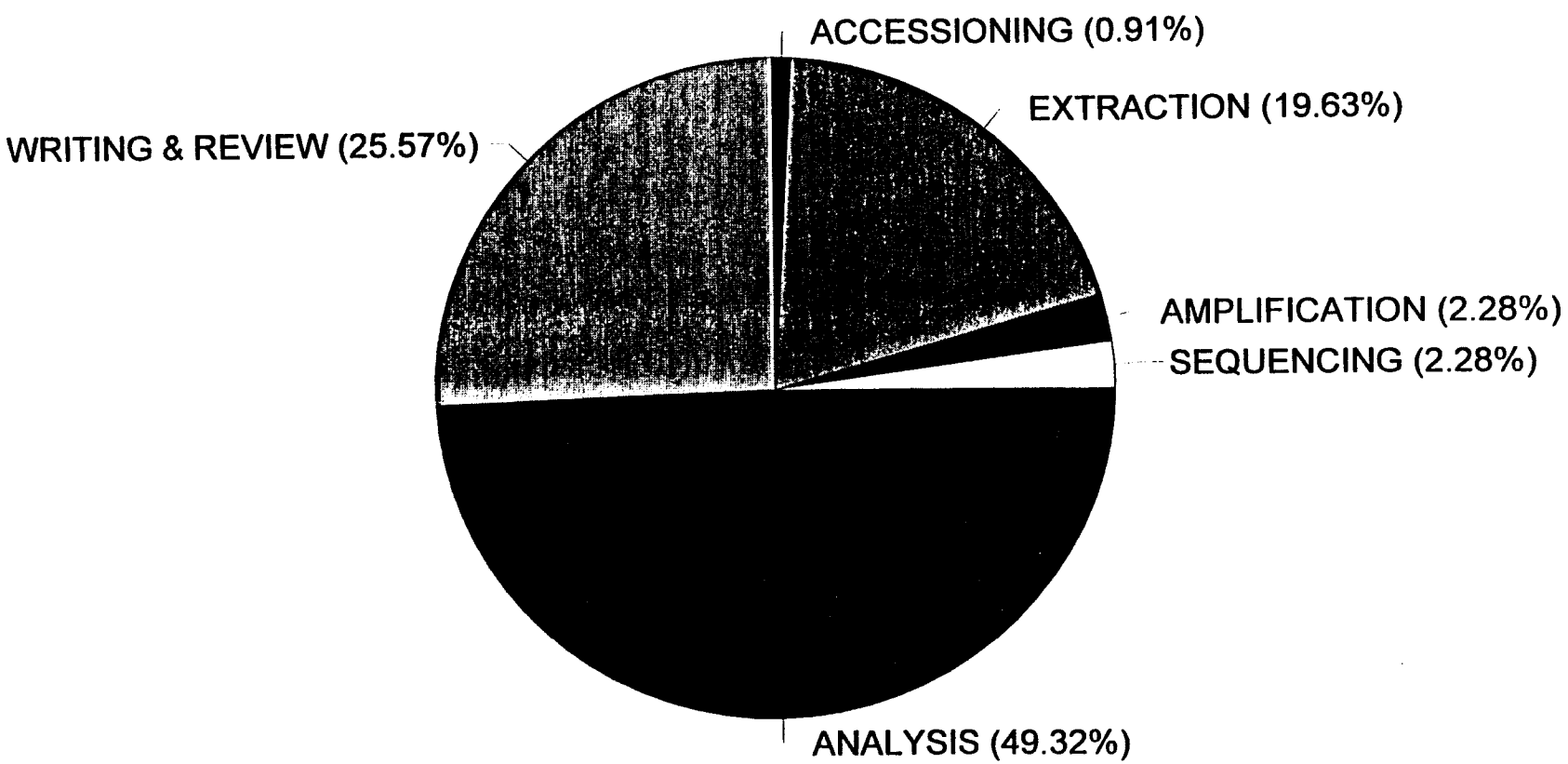


Figure 9

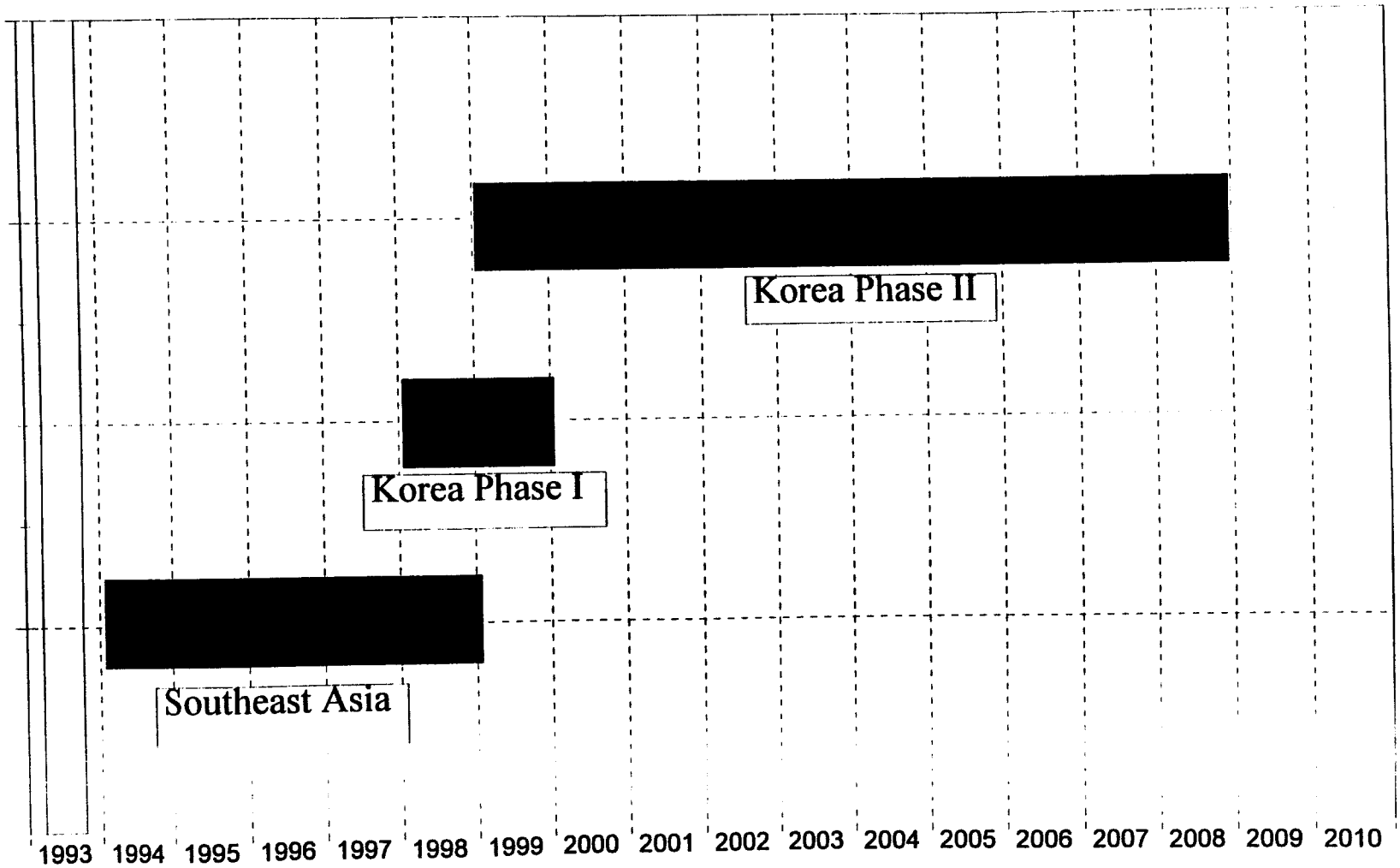
Projected Time-Line for Korean Remains Identification

Figure 9.

Projected Time-Line for Korean Remains Identification

Korean remains testing is anticipated to be phased in as Southeast Asia casework is phased out. A database of family reference specimens should be generated during the initial phase of a Korean remains testing program.

PROJECT DURATIONS



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