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Large scale DNA identification: The ICMP experience

Thomas J. Parsons*, Rene M.L. Huel, Zlatan Bajunović, Adnan Rizvić

International Commission on Missing Persons, Koninginnegracht 12a, The Hague, Netherlands

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ABSTRACT

The International Commission on Missing Persons (ICMP) is a treaty-based international organization with a global mandate to address the issue of missing persons. It works with governments, civil society organizations, and others, and utilizes data systems and technical assistance in forensic science. ICMP's initial work focused on the ~40,000 people missing in the Western Balkans from the conflicts of the 1990s. A "DNA-led" approach to large-scale DNA identification of the missing was developed, based on high-throughput autosomal STR testing of skeletal remains from mass graves and other sites, and the establishment of a regional database of DNA profiles from family members of the missing. Database pairwise and pedigree kinship searching is conducted using in-house DNA matching software, the Identification Data Management System (iDMS), providing high-certainty DNA matches that are integrated in a multi-disciplinary identification process. Anthropological guidelines for sampling skeletal remains for DNA testing are based on tens of thousands of tests from a wide range of skeletal elements, allowing for prioritization based on DNA preservation. Large-scale collection of family reference samples has been conducted, resulting in a database of more than 100,000 family reference DNA profiles across all projects and delivering family DNA match reports for more than 20,000 individuals. From the 1995 Srebrenica event, ICMP provided DNA matches for 6887 of the ~8000 missing from that event. In assistance to justice, ICMP has provided extensive evidence and expert testimony in multiple war crimes trials, including those conducted at the ICTY. This article provides an overview of ICMP's technical involvement over the last 17 years in areas of DNA testing and database matching, and training and capacity building projects with partners. It also touches on the development of massively parallel sequencing (MPS) strategies specifically tailored to missing persons applications.

1. Introduction

The International Commission on Missing Persons (ICMP) was established in 1996 at a G7 Conference, as a mechanism to help deal with the legacy of some 40,000 persons missing as a result of the conflict of the early to mid- 1990's that resulted from the breakup of Yugoslavia. The mandate of the ICMP is to secure the co-operation of governments and other authorities in locating and identifying persons missing as a result of conflicts, human rights abuses, disasters, organized violence and other causes and to assist them in doing so. ICMP also supports the work of other organizations in their efforts, encourages public involvement in its activities and contributes to the development of appropriate expressions of commemoration and tribute to the missing. ICMP's activities include provisions to build institutional capacity of governments and other partners, and to encourage public involvement and address the needs of justice. It also involves provision of technical assistance in locating, recovering and identifying missing persons.

In 2004, the mandate of the ICMP was formally extended to a global reach that has come to encompass activities relating to missing persons around the world, regardless of what caused them to go missing, and whether the missing are alive or dead. This encompasses historical or recent armed conflict, terrorism or other crimes, human trafficking and child separation, and disaster victim identification (DVI) due to transportation incidents and/or natural disasters. In 2014, the Foreign Ministers of the Netherlands, the United Kingdom, Sweden, Belgium and Luxembourg signed a treaty-level agreement constituting ICMP as an international organization with privileges and immunities in its headquarters in The Hague, Netherlands. This status allows protection of data and premises from any government or other seizure. At the time of writing, the ICMP treaty has been signed by ten countries, and the ICMP DNA laboratory moved to The Hague in January 2018, from its previous location in Sarajevo, Bosnia and Herzegovina.

The ICMP acts at many levels, including institution and legal development, policy, and civil society. This paper will not review these activities in detail, but in focusing on the DNA identification aspects will note instances of intersection with these other realms, as well as

* Corresponding author.
E-mail address: Thomas.parsons@icmp.int (T.J. Parsons).

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with the interrelation with other disciplines such as forensic archaeology, forensic anthropology, pathology, aerial and satellite imagery, and data coordination.

2. A pressing need for large scale DNA

In 1996, the International Criminal Tribunal for Yugoslavia (ICTY) was established to investigate and prosecute war crimes that occurred during the conflict, and international teams of investigators were deployed under ICTY auspices to, among other things, exhume mass graves and document evidence relating to war crimes and other violations under their mandate. Over the years, this resulted in many thousands of cases of human remains having been exhumed. Already traumatized families, desperate to know the fate of their missing, were further stressed by the discovery and exhumation of these remains with no organized or effective provision for their identification and appropriate return to families for legal closure and commemoration. A detailed, non-technical exposition on the history, context and efforts of ICMP and other actors to address the issue the missing in Bosnia and Herzegovina, and Kosovo, may be found in Sarkin et al. [1] and ICMP, 2017 [2].

Pioneering work in Latin America relating to enforced disappearances by dictatorial regimes in South and Central America had established a then prevailing model for identification that had an emphasis on the role of forensic anthropology, due to the fact that the human remains in question were predominantly skeletonized [3]. In what may be considered the "traditional" identification approach, family members provided information on the missing regarding physical characteristics, personal effects and circumstances of disappearance, and this information was compared to information from the post mortem cases under examination [4]. In the absence of access to today's recognized primary scientific identifiers of fingerprints, odontology or DNA [5], consistency in such areas as age, sex and stature, generally taken in the context of circumstances and number of people missing could be used as grounds for an identification and handover of the remains to families. Such identifications are often referred to as "presumptive" identifications. With the advent of newly developing DNA methods, in the 1990's usually involving mitochondrial DNA (mtDNA) [6], presumptive identifications were sometimes then tested by DNA to confirm or refute a particular hypothesis of identity. With a positive result, a scientifically based identification could be made, but in the not uncommon instance of a DNA exclusion, the case would be left without a defined path toward resolution.

In the Western Balkans, the vast scale of the event, the displacement of surviving family members, and the dearth of distinctive medical or dental records, made it difficult to establish investigation-driven hypotheses of identity that could be tested by DNA. In the early years after the conflict, local authorities and sometimes families themselves performed visual or other traditional identifications, which is recognized by present day standards to run a significant risk of error. Moreover, many recovered human remains were fragmented and commingled, a situation resulting in some instances from inexpert recovery by various actors, or in a vast number of cases, the commingling originated from the creation of secondary graves.

In July 1995, the UN Safe Area Srebrenica, where thousands of almost exclusively Bosnian Muslim refugees from ethnic cleansing had congregated, fell to Bosnian Serb forces. We now know that approximately 8000 men (almost exclusively) were systematically killed [1,7] and initially most were buried in five very large primary mass graves. Shortly after reports of this atrocity attracted international attention, the perpetrators excavated the primary graves with heavy equipment and distributed the then fragmented remains to over 90 clandestine secondary mass graves. Upon excavation by investigators over the years (ICTY and others initially, ICMP and local authorities later), the skeletonized remains were found to be fragmented and commingled within and between graves, posing a vast challenge of not only individual identification, but re-association of remains on a scale that rendered solely anthropological approaches to re-association for the most part unreliable and impractical. Extensive efforts by multiple actors to use traditional methods to establish presumptive identifications proved ineffective, and instances where samples were sent to various labs for DNA confirmation resulted in a high rate of exclusion.

In 2000, ICMP set out to attempt DNA identifications on a massive scale, independent from pre-existing hypotheses of identity, by conducting high- throughput autosomal STR testing from skeletal remains and comparing the profiles to large regional-scale databases of STR profiles of family members of the missing [8]. This effort proved to be an unprecedented success and established what has become referred to as a "DNA-led" approach to DNA identification of the missing on a very large scale.

3. DNA extraction from degraded skeletal remains

By the time ICMP undertook its large-scale DNA experiment, the human remains were mostly skeletonized, or in any case bone or tooth samples presented the best prospects for DNA recovery. However, at the time, DNA testing from degraded skeletal remains was primarily the realm of mtDNA testing [9,10], due to its high copy number and relative ease of successful PCR amplification. In the context, there was no option for obtaining direct Ante Mortem (AM) reference samples from the missing (such as biopsy samples, or known personal effects), so identification would have to be performed by genetic kinship analysis of family members. Also, mtDNA in its traditional usage offers advantages in that even distant maternal relatives can be used as references, but it was recognized that, given the scale of the event and the lack of distinctive non-DNA identification evidence in almost all cases, the resolving power of mtDNA would be insufficient for the task at hand. Thus, nuclear autosomal DNA testing was the only route. This presented three major requirements: 1) DNA extraction and amplification methods that would provide a sufficient success rate on degraded skeletal material, 2) DNA reference samples from multiple close references would have to be obtained for each missing person, and 3) effective software for large scale kinship matching.

Initial work in some forensic and ancient DNA labs showed some encouraging prospects for DNA recovery from skeletal remains [11–13]. However, whether it could work on a sufficient rate and scale was quite unknown. The remains in question at the outset of the DNA program were from 5 to 9 years postmortem, but it was clear that the identification effort would last for years, with increasingly aged material. Moreover, remains were recovered from a very wide range of environmental conditions, ranging from surface remains exposed to the elements, to caves, wells, lakes and rivers, and single and mass graves in many types of soil and moisture, making it a certainty that any generally successful approach would have to work on samples with very little surviving DNA. The microbial environment of mass graves has not been well studied, but could be considered to be sites of extreme microbial activity that in some cases might accelerate degradation.

An initial emphasis was on optimization of DNA extraction methods, which had to contend not only with DNA degradation, but the serious problem of co-purification of PCR inhibitors that characterizes skeletal remains that have been in contact with soil [14]. At the time, the most widely used method for DNA extraction from bone was digestion with protease K and detergent, followed by phenol chloroform extraction [10,13]. While successful in many instances using large quantities of bone, the organic extraction was suboptimal for inhibitor removal, so a large-scale extraction based on silica purification was devised and optimized that provided good yields and superior performance with inhibition [15]. This method was applied to tens of thousands of cases over the years, with a success rate of around 85% when applied to preferred samples such as femur and teeth, but ranging to lower success with less preferred elements, and a weak but significant negative correlation with the postmortem interval dating from 1992 to 1995 to

1999 [16].

In 2007, the method for DNA extraction from bones and teeth was substantially improved by adapting full demineralization extraction methods involving digestion of bone powder with a large volume ratio of 0.5 M EDTA, and full retention of the lysate [17]. The optimized method resulted in an improvement in success rate, a decreased cost per sample, and the use of significantly less starting bone material [18]. The method was adapted to a semi-automated format that employs robotic liquid handling but still requires manual steps in the concentration of large extract volumes via centrifugation in molecular exclusion columns [19].

4. DNA amplification and genetic systems

The success rates referred to above reflect criteria set forth in standard operating procedures (SOPs), which rely on internally validated profile/allele submission criteria, where partial profiles may be submitted to the ICMP database if they comprise a minimum of 11 loci plus Amelogenin [18]. Rare exceptions to the minimum submission criteria are made on a case-by-case basis. In order to obtain a high success rate, enhanced PCR parameters are employed with increased cycles of amplification (31 or 32 cycles depending on the amplification kit being used).

In 2001, the primary multiplex PCR kit that was chosen for testing was Promega PowerPlex16 (PP16), and this is the kit used for the vast majority of the 93,030 family reference profiles from the Western Balkans in the ICMP database. However, more recently, additional kits have been validated to obtain more genetic information as needed for a particular case, to increase the number of loci so as to meet the threshold for a match report. To obtain more loci from highly degraded samples, the short-amplicon multiplex Applied Biosystems (ABI) Minifiler is sometimes used. Today, newly collected reference samples and Post Mortem (PM) samples are tested at the outset with Promega PowerPlex21 (PP21) or ABI Globalfiler (depending on project and its partners), as the additional loci they afford are often useful for determining a kinship match. In many instances, candidate family matches were found with PP16 that did not meet the 99.95% surety threshold (described below), so the Promega PowerPlex ESX17 (ESX17) kit was used to provide eight additional loci to increase the kinship statistics. In large scale database matching, adventitious hits to families at below-threshold statistics are not rarely encountered, and the addition of ESX17 loci often had the effect of providing for exclusions of adventitious (false candidate) matches, or, in cases where the candidate match was correct, increased the kinship likelihood ratio (LR) by a factor of 10,000 on average (data not shown). The results of testing low-threshold cases with additional loci, between 2011 and 2017, are listed in Table 1.

Lineage markers such as Y-chromosome testing with ABI YFiler are also employed in particular cases, depending on the reference samples available for a case, or to resolve discrepancies in reported relationships. Given the low power of discrimination of mtDNA testing, but, more significantly, its requirement for much higher effort and cost, mtDNA has been used only rarely in the ICMP laboratory. For a period of time, an updated version of the time-efficient Linear Array hybridization assay (Roche Applied Science) was used in cases where mtDNA was desired [20].

Table 1
Statistics for below-threshold candidate match cases selected for additional testing with Promega EXS17. A majority of these candidate matches proved correct, allowing for DNA match reports to be issued. Cases where statistics increased to values still below-threshold would be candidates for additional reference sample collection and/or issuing a statistical comparison report. Instances where statistics decrease with additional data probably represent false, adventitious matches. See the section on DNA Matching below for additional detail on match reporting.

# Below-threshold cases, 2011–2017	632	
Threshold reached	344	54.43%
Threshold NOT reached-stat. increased	90	14.24%
Threshold NOT reached-stat. decreased	104	16.30%
Threshold NOT reached-exclusion	95	15.03%

5. DNA in multidisciplinary identification

The great advantage of the DNA-led approach to identification of the missing is that a homogenous and highly quality-controlled work flow can be applied on a large scale at the outset, in a manner that is independent of time-consuming investigation. The use of DNA has often been criticized as being too costly and time-consuming in comparison to other modalities, however in some instances this may be due in part to a lack of a well-developed and efficient DNA identification system, and a lack of full accounting for the costs associated with largescale collection of other antemortem identifiers such as fingerprints or dental records. In any case, the ICMP DNA-led approach should not be understood as a DNA-only approach.

The ICMP provides DNA match reports to various official partners in various projects, most often court appointed forensic pathologists who are the identification authorities. In many instances, particularly in Bosnia and Herzegovina, ICMP anthropologists work directly in association with these pathologists, assisting in case examination and sampling for DNA. Anthropological examination assesses whether there is comingling in the case, and develops a PM biological profile with regard to age, sex, stature, distinctive characteristics including skeletal abnormalities and medical protheses or history (e.g., bone breakage during life), and indications of trauma associated with death [21]. Upon receipt of a DNA match report, pathologists and anthropologists cross check all this PM information with the AM information on the missing person listed on the match report to ensure consistency, including comparison of place of recovery to reported place and circumstances of disappearance and information on personal effects. One key role for anthropology in the identification process is to distinguish between siblings, when DNA cannot (in cases where neither sibling has children as references). If an identification is warranted, case files are presented to families that explain the basis of the identification, including DNA. If there is an inconsistency between the case and the DNA report, a formal review is conducted, and if necessary the case is sampled again for confirmation.

In events such as Srebrenica, with very large numbers of fragmented and commingled cases, DNA plays a critical role not only in identification, but in re-association. Mortuaries were faced with the need to perform thousands of body part re-associations, and the scale and complexity of the event provided little prospect for re-association apart from DNA testing. For re-associations, direct DNA matching between cases is performed, in principle requiring fewer loci than needed for kinship matching. With this in mind, ICMP developed novel in-house mini-STR kits for less expensive and more sensitive testing specifically for re-association [22]. After mini-STR testing and re-association, the best sample was then “pushed” for PP16 and matching to the family reference database. Over 5000 re-associations were processed in this manner, with fast information exchange between the DNA laboratory and the anthropologists to expedite merging and management of cases. This effort was not without complications, however, most notably relating to occurrence of many related missing persons in the event. If a mini-STR kit with 6 loci experienced locus drop out, the resulting direct match statistics were sometimes insufficient to tell definitively if matches were due to same-source samples, or to samples from close relatives. This gave rise to the need to resolve the re-association cases by re-testing with PP16, which occurred often enough that it was deemed more process-efficient to revert to PP16 at the outset for re-association purposes.

The ICMP has tested over 65,000 degraded skeletal samples. The

very large number and wide variety of skeletal remains tested has provided insight into the relative preservation of DNA in different skeletal elements [16,23]. This data was gathered over the years, and feedback between the DNA laboratory and anthropologists and pathologists progressively informed case sampling and management in the mortuaries. The outcome is ICMP's current skeletal remains sampling Standard Operating Procedure (SOP) that indicates preferred sampling locations on a wide range of complete or partial skeletal cases that might be encountered, ranked by success rates [24]. Our findings mirror and extend observations by others [10,25,26], where intact teeth and dense weight bearing bones such as femur or tibia are preferred sources, while arm bones such as humerus, radius and ulna are poor sources despite their density. ICMP's historical sample sizes are lower for some elements we now consider to be among the better sources, such as the petrous portion of the temporal bone (an excellent source, but comparatively difficult and destructive to sample), and bones of the wrist, hands, ankles and feet.

6. Family reference DNA testing, consent and data security

At the time of writing, in its various projects, ICMP has issued DNA match reports on over 20,000 individuals. While the DNA-led approach does not require extensive investigatory information for high certainty DNA matches to lead the identification process, it does require highly effective systems for reaching out to family members of the missing, obtaining genetic reference samples, and accurately registering information. The key points of information needed for DNA matching are: a unique name designation of the missing person (or other unique identifier), the biological relationship between the reference donor and the missing person(s), biological relationships between reference donors, and the existence and relationships of related missing persons. Starting in the Western Balkans, extensive multi-media family outreach was conducted, including the organization and education of family organizations to foster fact-based awareness and transparency. At the time of writing, the ICMP family reference DNA database contains over 100,000 profiles from family members, representing over 34,000 missing persons. Samples are mostly collected as finger-prick blood stains on treated paper, or presently, FTA (GE Healthcare, Chicago, Ill) cards, although collection kits also accommodate buccal swab collection onto FTA.

In addition to the information listed above, ICMP's Missing Persons forms document additional AM data on the missing, as well as contact information for family members. The forms and associated "Information Sheet on DNA Testing" also provide the means for documenting informed consent from donors. ICMP considers family information and genetic profiles as sensitive personal data, and views data protection as a fundamental component of its institutional identity and structure.

In its programs, ICMP accepts reference samples from any family member that wishes to provide a sample, but in cases where multiple close relatives are available, not all the samples will necessarily be tested. For example, an aunt or uncle adds no value to an identification if references are available for both parents. However, during family contact and sample collection, it is generally unknown how many other references will be available, and moreover, it is not comfortable for family members who have come forward to be informed that their samples are not needed. In the Western Balkans, ICMP has collected on average 3.2 family reference samples from each missing person. Closer family members, of course, are preferred, and spouses are valuable if children are also available as reference donors. These guidelines reflect testing done with traditional Capillary Electrophoresis (CE) autosomal STR analysis. With advances in Massively Parallel Sequencing (MPS), the number and degree of reference samples required for identification will likely change in the future.

ICMP maintains an in-house team of software developers who have worked closely with technical staff and other departments over the years to develop a comprehensive database system that in its latest iteration is called iDMS (Identification Data Management System). iDMS comprehensively covers ICMP's operations, including archaeological field activities, mortuary activities, missing persons and relatives information, and DNA Matching. The ICMP database and the information it holds is protected both physically and legally by a treaty-level agreement that affords ICMP with privileges and immunities that protect the data, premises and communications from any government or other intrusion or seizure. The iDMS system is designed so that only select staff members, or outside partners under specific agreement, have access to only those areas of information to allow for the processing or daily work.

7. DNA matching

DNA kinship matching at the ICMP through large scale database comparisons has evolved over the years. Initially, scripts were devised to search DNA profiles within and between sub-databases in a pairwise manner, for direct matches, and allele sharing such as half-band matching for parental references. This was quickly refined to include several kinship indices. In pairwise matching, when a bone profile was screened against reference profile databases, a ranked list of the paternity, maternity and sibling indices was produced, indicating potential associations to family members. When association to a family was detected through pairwise comparison to individual donor(s), the potential match was checked by bringing all the reference profiles for the missing person into a full kinship analysis, performed for many years using DNA-View [27], driven by in-house automated scripts that easily pulled the appropriate profiles into an export for analysis.

In the last year, ICMP has additionally implemented within iDMS an in-house database matching program that performs full pedigree searches. For large database searches such as those needed in ICMP's work, efficient algorithms are required to conduct millions of kinship calculations in a reasonable time. In a recent project, iDMS matching compared 90 bone profiles against 35 families in 6 minutes, based on full kinship calculations. However, computation time increases not only with the number of profiles, but especially with the complexity of family pedigrees that are searched. Upon initial roll out of iDMS pedigree matching, 5200 unidentified bone profiles were run against ~8000 family pedigrees which often had complex constellations of close and distant relatives. In that massive screen, the computation time was 7.5 days. A manuscript describing the iDMS DNA Matching module is in preparation.

It should be noted that neither full pedigree nor pair-wise matching alone is sufficient to ensure finding matches in a large database. There are cases where full pedigree searching may not find the missing (i.e., issues within the stated relationships), and therefore pair-wise matching should also be utilized. Likewise, the need for experienced individuals performing the matching should not be underestimated. In many instances, cases need to be further investigated or evaluated to ensure the proper hypotheses are tested.

The ICMP typically issues a DNA match report when the posterior probability of an identification is 99.95% or greater, and a great majority of reports have been issued at much greater levels of certainty. This Bayesian approach requires the use of prior probabilities. ICMP uses a very general and conservative approach to prior probabilities based on the total number of missing persons from a particular event, region and/or time period that relate to the case in question. For example, at the outset of DNA-led matching associated with Srebrenica graves, the prior probability was taken at 1/8000. The prior probability was increased over time as identifications were made, and currently Srebrenica associated cases are calculated with a prior probability of 1/875. If prior probabilities are not available, as in some partnerships where ICMP works, reports can be issued presenting only likelihood ratios (LRs). In other programs of operation ICMP coordinates closely with partners to specify prior probabilities and reporting thresholds

based on their context and requirements. In the absence of a well-defined prior probability, the ICMP sometimes reports a "requisite prior" together with the LR. The requisite prior is the prior probability that would be needed to achieve a posterior threshold (usually 99.95%). Conceptually this can be quite helpful in case evaluation; for example, it may be difficult to specify a prior probability, but easy to evaluate that it would be more than one in a million.

ICMP also issues "statistical comparison reports" that permit objective reporting of kinship statistics, even when the statistics fall short of the 99.95% threshold. Usually this is done in cases where exchange with anthropologists or pathologists indicate a presumptive identification, or when near-threshold matches are communicated to determine if probable identifications could be well supported by non-DNA information. If this is determined to be the case, a statistical comparison report will be issued. This again emphasizes that DNA can and should work in combination with other types of evidence, where possible. ICMP does not consider DNA Match Reports to be identification reports—they are intended be used by identification authorities who consider all aspects of the case and make an official determination of identity. If cases are submitted as presumptive identifications, naming a particular family comparison to be made to the case, ICMP will issue a DNA Match Report (if $\geq$ 99.95% in favor), a Statistical Comparison Report (between 99.95% and 0.05%), or an Exclusion Report ($<$ 0.05%).

The occurrence of multiple related missing persons is a serious complication in most large-scale identification efforts, and particularly so with Srebrenica, where it was not uncommon for many males in an extended family to have been killed. With related missing persons it is very useful to use the DNA profile of an identified missing person as a reference sample for other missing relatives. For example, if one brother is identified using his wife and children as references, his DNA profile can be used as a reference for his missing sibling. However, it is essential to avoid a circular argument in solving cases of related missing persons, where assuming 100% identity of one profile allows you to match a related missing person, and assuming 100% identity of that person allows you to match the first.

Indeed, it is possible to identify groups of related missing persons when none could be identified individually based only on the available references, but to do so requires that multiple hypotheses of all reasonably possible relationships be considered simultaneously, and their relative likelihoods evaluated. An ICMP case exemplifies this: a woman, her husband and child were reported missing. One set of remains was identified as the missing woman based on genetic family references for her, and her case was officially ruled as an identification based on all evidence. Later, skeletal samples from this event/region of 700 missing persons gave DNA profiles from two individuals, consistent with a father:son relationship (paternity index of 82,180). One of those profiles was also consistent with being the son of the identified woman (maternity index of 11,290— a 94% probability of identity). If we consider profiles M (known mother), S (profile consistent with maternal relationship to M) and F (profile consistent with paternal relationship to S), both the father and son were able to be matched with certainty by simultaneously evaluating the relative probabilities of the following scenarios: Hyp 1) M, F, S related as mother, father, son; Hyp 2) M and S related as mother and child, F unrelated; Hyp 3) F and S related as father and son, M unrelated; and Hyp 4) all unrelated. This was calculated using the Bayesian Calculator function of DNA-View, with prior probabilities for each scenario based on 700 missing from the event, with an estimated 50 father:son pairs among them (Table 2). With the hypotheses spanning all relevant possibilities, hypothesis 1 is favored at 99.9996% over all other hypotheses, meeting the threshold for issuing DNA match reports on both the missing father/husband and son.

Once a DNA database is established and properly configured informatically to link to all necessary and accurate information (a huge hurdle in its own right), powerful kinship searching and reporting software makes it relatively easy to find straightforward matches.

Table 2
Multi-hypothesis approach to case resolution for known mother with profile M, and missing father and son, with bone profiles designated F and S which are under matching evaluation. The event has 700 missing persons and an estimated number of 50 father:son pairs among the missing. Prior probabilities are listed; for example, the prior probability for hypothesis 3 is based on 100 individuals involved in 50 father:son pairs which is a chance of 1 in 7 of bone F coming from one of those pairs, and 698/699 being the chance bone S is related to bone F as father:son. Posterior Probabilities do not sum to 100% as listed, due to roundoff.

Hypothesis	Prior Probabilities	Relative Likelihoods	Posterior Probability
1. M,F,S related	(1/700) x (1/699)	3.43×10^{11}	99.9996%
2. M,S related, F not	(1/700) x (698/699)	10020	0.0002%
3. F,S related, M not	(1/7) x (698/699)	78930	0.0002%
4. All unrelated	(699/700) x (698/699)	1	0.0001%

However, in large, complex events, the experience base of a DNA matching unit remains extremely important, including a high degree of knowledge of the event context.

One case exemplifies the type of the steps that often go into resolution of a DNA match. A male PP16 profile from a skeletal remains case was run for pairwise kinship index comparisons across the family reference database. Often, highly elevated statistics (such as a maternity index in the thousands), quickly provide clues of family associations. In this instance, no single pairwise kinship index stood out. When pedigree searching was applied, however, a candidate family match at 97.6% posterior probability was quickly obtained for a particular family. The pairwise kinship indices for the references in this case were indeed unimpressive: mother, maternity index 142; sister, sibling index 46; sister, sibling index $<$ 1. As there were no additional family references to collect and test, all samples were analyzed in ESX17, providing enough additional loci to confirm the match at $>$ 99.999% posterior probability.

The above example shows the great benefit of software that allows full pedigree searching, as the initial candidate match would have been evident immediately if pedigree searching had been used. However, there are important instances where pedigree matching, if used alone, could miss matches. If there are problems in reported relationships or recorded data, pedigree matching may indicate an exclusion, while pairwise matches nonetheless indicate a family association that should be investigated. There have been a number of cases where ICMP has discovered family matches to individuals who were not reported missing. In one case, pairwise matching showed a clear association to a family for a set of male remains, but not consistent with the individual reported missing by the family. Communication with the family indicated that the father of the person reported missing from the 1990′s was missing from World War II, and the identification was confirmed for that family. This case is indicative of the fact that locations selected for body disposal tend to have a constellation of characteristics, which can lead them to being used repeatedly.

Numerous other cases of identification of individuals not reported missing have to do with cases that were incorrectly traditionally identified prior to the use of DNA. An example of such an instance would be a family with a missing son and daughter who prior to the DNA-led process received a traditionally-identified body as the son, and after the advent of the DNA-led process provided reference samples to find their still-missing daughter. DNA matching of bone profiles, however, indicated a new set of skeletal remains coming from the family's actual son, indicating the previous traditional identification had been incorrect. In such instances, testing of the incorrectly identified remains can in turn result in a correct identification, and return of the remains to a family who has been waiting for years after having provided reference samples.

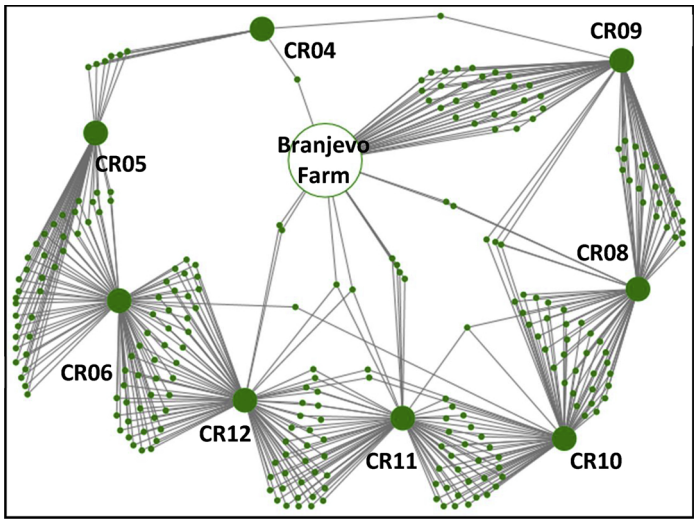

Fig. 1. Branjevo Farm was one of the largest primary mass grave of Srebrenica, and several months after it was originally created, it was exhumed by perpetrators with heavy equipment, and the contents distributed to a series of secondary graves along Cancari Road (CR). When excavated by forensic investigators, Branjevo Farm yielded a relatively small number of fragmentary remains that were left behind, but these were shown by DNA to have body-part re-associations to the CR graves. The secondary graves in turn showed numerous linkages between them, indicative of the pattern of creation of the graves and distribution of body parts, which accounted for over 1700 individuals, all but 22 of whom were identified by DNA. Lines and small dots indicate a DNA match between body parts among the graves.

Pairwise matching is essential for resolving a certain class of case, where inconsistency in family relationships are involved, relating to "incidental findings" [28], usually reflecting false paternity. Such instances are to be expected in any sizeable missing persons event, including disaster victim identification (DVI), and should be planned for in policy and practice. Pedigree searching software often has a "validation" function that checks if relationships among reference profiles are as stated, allowing problems to be identified in advance of matching to unknown profiles. However, non-paternity may become evident only once the victim/missing person is introduced into consideration, and in our large projects we have had multiple such instances. One example case was when two parents and two of their children provided reference samples for a third child who was missing. There were no problems with the reference profiles, and pedigree matching showed no match to unidentified remains profiles. However, pairwise matching indicated a high maternity index between a bone profile and the mother, but exclusion of full sibling status to the sibling references; when additional loci were added through confirmatory testing with ESX17, the posterior probability with the mother alone as reference exceeded 99.99%, permitting an identification when all identification information was evaluated.

Clearly, policies and practices are needed so that incidental findings about family relationships do not cause harm to families or individuals, that is: these incidental findings are not made known to families. Measures to avoid imparting harmful or distressing information to families should not, however, be detrimental to the ability to identify and return the missing to their families. One element in navigating this ethical and practical challenge is to establish in the first instance effective lines of communication with families, including at the time of reporting a missing person and collection of reference samples. What appears to be a possible incidental finding could be a result of misreported or mis-recorded information, so resolution begins with a review of all collected information and knowledge of the context. Depending on case-by-case considerations, families may be contacted for follow-up information confirmation, and/or asked if additional reference individuals might be available as a means to assist with the case. For ICMP match reports in most contexts, information on family relationships is listed in the report, although genetic profiles are not. A common solution to resolving cases and issuing match reports would be to conduct additional tests to obtain sufficient certainty on non-problematic references, sometimes investigating with additional systems such as Y-chromosomal STRs to fully establish the validity of the match. Then problematic references can be dropped from the final calculations and report. ICMP has not received pointed inquiries from families in such circumstances, but is able to respond with the general information that not all reference samples are necessarily needed for match and therefore some may not be included.

8. Assistance to Justice

DNA identification techniques to resolve the fate of missing persons can become involved in the criminal trials of the perpetrators. ICMP evidence has played a role in numerous trials conducted by the ICTY in The Hague, and in domestic trials in the Bosnia and Herzegovina State Court. These include five major indictments (Haradinaj et al, Popović et al, Tolimir, Mladić, and Karadžić, see www.icty.org) where ICMP provided scientific methodology reports, archaeology and anthropology reports, DNA match report lists, selected DNA case files (see below) and expert witness testimony. The most recent trials were those of former Bosnian Serb President Radovan Karadžić, and his former General Ratko Mladić who was in command at Srebrenica, with indictments and guilty verdicts for both including charges of genocide, crimes against humanity, and violations of the laws and customs of war. DNA evidence presented at these trials related not only to thousands of individual identifications, but to hundreds of DNA re-association linkages established between linked primary and secondary graves. Prosecutors presented these linkages as evidence of the systematic criminal enterprise associated with the killing and creation of clandestine secondary graves of Srebrenica (Fig. 1).

Within a perspective of support for human rights with its DNA work, ICMP works not only to provide answers and the return of the missing to families, but to afford their right to justice through effective investigations that are official, transparent and capable of establishing facts. In cooperating with justice mechanisms, however, the ICMP nonetheless safeguards sensitive personal data that individuals have entrusted with informed consent. Prior to 2007, this consent extended to use of data and samples only for the purposes of identification. Since 2007, reference donors additionally have the option to indicate their willingness for their data to be used in justice mechanisms. This goes to the issue of provision of evidence to Defense for counter-examination. In the case of Karadžić, agreement was reached to provide to Defense DNA case files for 295 randomly selected representative cases, constituting over 9000 pages. In so doing, over 1000 family members were contacted to provide consent, with nearly 100% consenting to participate.

Ratko Mladić and Radovan Karadžić were found guilty, among numerous other charges, of genocide with regard to Srebrenica. At the time of writing, ICMP's work on Srebrenica is summarized in Table 3.

In its assistance to justice, the ICMP does not act as part of the prosecution, but recognizing the relevance of its work to justice

Table 3
Summary of ICMP DNA testing results from Srebrenica, associated with the event beginning in July 1995.

Number of Individuals Reported Missing to ICMP	7751
Number of Family Reference Samples Collected/Profiled	22,320
Number of Unique DNA Profiles from Srebrenica Graves	6971
Number of Individuals Identified with DNA Match	6887
Number of Reassociation Match Reports	10,580

mechanisms, conducts its operations according to rigorous principles of forensic science. In the DNA laboratory, this rigor has been defined by a Quality Management System (QMS) that is the basis for ICMP's ISO 17025 accreditation, first acquired in 2007. Other aspects of the QMS that have led to the acceptance of ICMP's science in international courts are interlaboratory exchange testing (on samples not part of ICMP's casework), the German DNA Profiling Group (GEDNAP) external proficiency test, the International Society of Forensic Genetics (ISFG) bone proficiency test, and publication of methods in peer reviewed journals.

9. Center for excellence and training

As a forensic DNA laboratory, ICMP is unique in having a sole focus on missing persons applications, and for years has worked to refine methods, work flow and informatics to increase effectiveness in practical application. Part of ICMP's mandate also is to disseminate expertise to generally bolster global capabilities. This relates not only to optimized methods and a range of validated DNA systems, but even more so to how all elements must work together in order for cost- and time-effective resolution of a large number of cases: DNA laboratory methods, informatics, the interface with anthropology and pathology, outreach to and trust of families, and institutional roles and responsibilities in the context of medical-legal death investigation.

Over the years, ICMP has provided in-depth training and development consultation to partners in many contexts, with larger efforts in Iraq, Libya, Colombia and the countries of the former Yugoslavia (Western Balkans), and additional formal training in specialized techniques to officials from Philippines and Vietnam. These efforts at global capacity building have recently been brought under the mantle of a Center for Excellence and Training (CET), to emphasize this fundamental element of the mandate. Under the CET, ICMP organizes subject-matter specific training packages, most often involving testing of highly degraded skeletal remains. In its work on application of massively parallel sequencing (MPS) methods, the CET will be structured to serve as a training and demonstration laboratory of newly optimized MPS methods, in an effort that at the time of this writing is supported by QIAGEN (Hilden, Germany) and Verogen (La Jolla, CA) with regard to adaptations and applications of their MPS systems.

10. Global reach, diverse applications

In post-conflict or human rights violation contexts, ICMP has provided DNA testing and/or matching assistance to Bosnia and Herzegovina, Brazil, Canada, Chile, Colombia, Croatia, Cyprus, El Salvador, Iraq, Kosovo, Libya, Montenegro, Serbia, South Africa, USA and Vietnam (see Table 4). Different projects varied in scale, and often this work has been coupled with capacity building and training. In some of these instances, countries are faced with numbers of missing that are staggering in scale, but do not have internal identification systems developed that are even nearly sufficient in scale or efficiency, as indeed these are immensely difficult to put quickly in place, and in some cases the challenge is unprecedently large.

As a matter of principle, ICMP promotes establishment of domestic capacity in countries or regions that are conducting missing persons projects, but additionally provides a model where early success can be

Table 4
Overview of ICMP's global assistance to DNA-led programs, to include post-conflict, disaster and routine cases of missing persons. A: Overview of AM samples processed. B: Overview of PM samples processed. C: Overview of DNA reports based mostly on kinship matching. All figures accurate as of 06-09-2018.

Reference Samples	Western Balkans	Norway	Chile	South Africa	Iraq	Kuwait	South Africa	Libya	Thailand	Iraq	Cyprus	Libya	Typhon Frank	Kenya Flight 507	Other
Number of Reference Samples Analyzed	93,030	45	2,570	23					1,413			1,325	2,440	114	229
Number of Missing Persons Represented by Reference Samples	30,153	42	1,094	11					694			493	931	108	91

PM Samples	Western Balkans	Norway	Chile	South Africa	Iraq	Kuwait	Libya	Thailand	Cyprus	Typhon Frank	Kenya Flight 507	Hurricane Katrina	Other
Number of PM Samples Received	60,831	150	286	47	1,555	10	249	1,819	2,301	559	316	268	113
Number of PM DNA Profiles Obtained	43,737	32	194	39	911	7	249	1,739	1,632	544	265	245	44
Estimated Number of Unique Profiles	21,925	57	135	38	626	7	247	1,558	419	516	107	239	28

DNA Matching Reports	Western Balkans	Norway	Chile	South Africa	Iraq	Libya	Thailand	Typhon Frank	Kenya Flight 507	Other
Total Number of DNA Reports Submitted	39,485	26	85	6	5	457	802	483	218	70
Number of Different Individuals Represented	18,440	14	46	6	5	137	802	456	89	39
Number of Re-association of Separated Skeletal Elements	19,859	12	24	0	0	1	0	27	129	28
Number of Exclusion of Presumptive Cases	1,186	0	15	0	0	319	0	0	0	3

demonstrated through application of ICMP's DNA testing and matching and information systems, concurrently with development of internal capabilities based on the integrated systems and rigorous laboratory standards that are required. In provision of DNA assistance, ICMP's role has varied with respect to different partners, sometimes providing DNA testing and/or partial assistance with matching or case review, and in other instances providing full project-wide DNA and matching capacity.

In Kosovo, for example, the ICMP conducted all family reference sample collection (14,831 family reference samples related to 4427 missing persons), and post-mortem sample DNA testing and matching, to result in the identification, under the auspices of partners, of 2550 individuals missing from the conflict in 1999 [2]. In 2012, the ICMP was contracted to perform DNA testing and matching for Cyprus, in this instance with coded (anonymous) family reference profiles provided through the Cyprus Committee on Missing Persons (CMP), that was in charge of the project and managed the overall identification effort. ICMP's work for Cyprus resulted in the effective application of a blind DNA matching program that provided 1632 bone DNA profiles, and resulted in 1517 DNA family match and/or re-association reports. An analysis by the International Committee of the Red Cross [29] examines the identification efforts in Kosovo and Cyprus and claims to have applied "close observation" to discern "the factors that may influence the development of forensic programs" in such situations. However, this paper, which purports to analyze in detail the time course of actors, activities and outcomes, glaringly omits any reference to when, how and by whom identifications were achieved by large scale DNA matching in either context. The paper therefore overlooks the beneficial role that engagement of a highly efficient external DNA identification system can play in abetting the development and success of local forensic systems.

The DNA identification system honed at the ICMP has proven to be directly applicable to assistance in Disaster Victim Identification (DVI). This was first demonstrated in the response to the devastating tsunami that occurred off the coasts of Sumatra in on December 26, 2004. International assistance with DVI centered on Thailand where many international teams arrived in response to the many foreign nationals that were killed there. The incident came to be managed by Thailand authorities in cooperation with INTERPOL, and was a defining event in international DVI response [30,31]. As a mode of identification, DNA lagged far behind other disciplines such as odontology and fingerprints due to a lacking of standing, centralized capability in dealing with environmentally degraded DNA and kinship matching [see 31,32]. In June, 2005, ICMP was asked to apply its capabilities to degraded samples and matching, and came to provide DNA profiles from 1819 bone samples, reflecting a 99% success rate of typing, and DNA matches on 802 individuals.

The experience in Thailand stimulated the need for enhanced international preparedness, and in 2007 resulted in a standing cooperation agreement between ICMP and INTERPOL. This partnership was activated in 2008 in response to Typhoon Frank in the Philippines, where within 2 weeks effective family reference sample collection was conducted through training and cooperation with local authorities. Body recovery from the sunken *Princess of the Stars* ferry was protracted, but ICMP testing of 559 bone samples provided DNA matches for 456 individuals [32].

Other DVI incidents that ICMP has been involved in, with various roles, are: 2005 Hurricane Katrina (DNA profiling from bones only, in assistance to the state of Louisiana), 2007 Kenya Air flight 507 crash in Cameroon (DNA profiling of reference and PM samples, DNA matching), the 2013 rail disaster in Lac-Megantic, Canada (DNA profiling from bone samples only), and assisting the Dutch Authorities in the processing of 1002 cases from flight MH-17, which was shot down over Ukraine.

Finally, in other areas of operation, the ICMP has been involved in historical casework dating to World War II [33], and also assisting other countries with the resolution of decades-old cold casework of missing persons [see, for example, 34–36].

11. Future development

One prominent area of future development of methods specifically for missing persons will focus on Massively Parallel Sequencing (MPS). In general, the biggest limitations to resolution of missing persons cases relate to: 1) advanced degradation of DNA in post mortem samples, where recovery of STR data is problematic or impossible, and, 2) the requirement of CE-based STR systems for multiple close relatives in order to establish conclusive kinship matches. For example, among the still missing from the Western Balkans conflict, more than 2000 cases are represented by family reference samples that are deemed as "insufficient," by virtue of the number and/or closeness of relatives, to be sure of finding a match. This is compounded by partial profiles that may be obtained from bone samples, making it even less likely that a match to, say, a single sibling acting as a reference sample might be found.

For samples where DNA preservation permits STR loci to be amplified, MPS methods offer the means for obtaining many loci simultaneously. For example the Verogen DNA Signature Prep kit targets 27 autosomal STRs and 29 Y-chromosomal STRs in a single assay [37], while the ThermoFisher Precision ID Globalfiler NGS STR Panel v2 targets 32 STR markers [38]. This amount of additional genetic information, including increased discrimination based on sequence rather than length variation [39], offers substantial advantage in the power of kinship matching, especially if complemented by autosomal SNP data also afforded by those systems.

MPS appears to hold great promise for increasing the ease and power of mtDNA sequence analysis generally, and for missing persons applications in particular. The ability to obtain large amounts of sequence data in a single run opens the door for routine access to sequences from the entire mtDNA genome [40,41], adding greatly to the resolution power of the system [42]. The ability to sequence multiple small amplicons simultaneously permits effective targeting of degraded DNA, typical of forensic case samples [43,44]. We predict these characteristics, taken together, will bring mtDNA testing closer to the utility of nuclear DNA in resolving missing persons cases, when applied to even more highly degraded trace quantities of DNA.

Ultimately, we consider that maximum utility of MPS methods for missing persons will be realized through large multiplex panels of single nucleotide polymorphism (SNP) markers. SNPs have the advantage of permitting assays that target very small fragments of DNA. Additionally, SNP data are simpler to handle from the standpoint of nomenclature and bioinformatics, and in this way may contribute to the efficiency of largescale identification. In work not yet ready for publication, ICMP is working with support from QIAGEN corporation, the University of Santiago de Compostela, and Linkoping University & Swedish National Board of Forensic Medicine to develop a ~1200 SNP multiplex assay designed specifically for missing persons applications. This panel targets many tri-allelic SNPs with elevated heterozygosities compared to usual bi-allelic SNPs, as well as a number of selected microhaplotype loci adapted from Kidd et al. [45]. In simulations, this panel has extreme power in kinship analysis, with kinship simulations indicating the ability to produce high-certainty matches with even a single first cousin as a reference sample (A. Tillmar, personal communication).

With MPS testing as well as traditional systems, one of the central aims of the ICMP DNA laboratory is the continued refinement of DNA methods, work flow, and integrated practices to push forward global capabilities in missing persons identification. These capabilities are intended to be applied as a global standing capacity that can be turned to in response to episodic events, various longer-term theatres of operation or specialized projects, or as augmentation of capacity for developing partners. Through the Center for Excellence and Training, ICMP seeks to disseminate expertise and new developments.

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