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Identification of Skeletal Remains of Communist Armed Forces Victims During and After World War II: Combined Y-chromosome Short Tandem Repeat (STR) and MiniSTR Approach

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Aim To report on the use of STR, Y-STRs, and miniSTRs typing methods in the identification of victims of revolutionary violence and crimes against humanity committed by the Communist Armed Forces during and after World War II in which bodies were exhumed from mass and individual graves in Slovenia.

Methods Bone fragments and teeth were removed from human remains found in several small and closely located hidden mass graves in the Škofja Loka area (Lovrenska Grapa and Žolšče) and 2 individual graves in the Ljubljana area (Podlipoglav), Slovenia. DNA was isolated using the Qiagen DNA extraction procedure optimized for bone and teeth. Some DNA extracts required additional purification, such as N-buthanol treatment. The Quantifiler™ Human DNA Quantification Kit was used for DNA quantification. Initially, PowerPlex 16 kit was used to simultaneously analyze 15 short tandem repeat (STR) loci. The PowerPlex S5 miniSTR kit and AmpF*ℓSTR*® *MiniFiler* PCR Amplification Kit was used for additional analysis if preliminary analysis yielded weak partial or no profiles at all. In 2 cases, when the PowerPlex 16 profiles indicated possible relatedness of the remains with reference samples, but there were insufficient probabilities to call the match to possible male paternal relatives, we resorted to an additional analysis of Y-STR markers. PowerPlex® Y System was used to simultaneously amplify 12 Y-STR loci. Fragment analysis was performed on an ABI PRISM 310 genetic analyzer. Matching probabilities were estimated using the DNA-View software.

Results Following the Y-STR analysis, 1 of the "weak matches" previously obtained based on autosomal loci, was confirmed while the other 1 was not. Combined standard STR and miniSTR approach applied to bone samples from 2 individual graves resulted in positive identifications. Finally, using the same approach on 11 bone samples from hidden mass grave Žološče, we were able to obtain 6 useful DNA profiles.

Conclusion The results of this study, in combination with previously obtained results, demonstrate that Y-chromosome testing and miniSTR methodology can contribute to the identification of human remains of victims of revolutionary violence from World War II.

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Y-chromosome short tandem repeat (Y-STR) markers are highly polymorphic. In addition, Y-chromosomes are passed down from generation to generation with little or no change. Therefore, they may be quite useful in following the paternal lineages in population studies, as well as in forensic DNA analysis, including the identification of human skeletal remains. Y-STRs show sufficient variability among individuals in a population and a high degree of geographical differentiation (1). Recent identification of numerous, informative biallelic markers in the non-recombining region of Y-chromosome has already significantly contributed to the understanding of European pre-history and history (2-4). Y-chromosome testing may provide very valuable results in forensic analysis where the nature of evidence limits the effectiveness of autosomal tests. Typical examples are sexual assault cases with the evidence in the form of mixtures with predominant female fraction (5). Also, Y-linked markers usually significantly increase the possibility of identification of male missing persons when the only available reference is a male paternal relative.

MiniSTR assays can help recover information from degraded DNA samples that typically result in partial profiles and total loss of information from regular STR amplicons (6). This approach has already been used in the analysis of highly degraded samples like those processed within the identification of victims from the World Trade Center terrorist attacks (7). Also, miniSTR primer sets for all CODIS STR loci have already been developed (6), but many other loci were taken in consideration (8). Finally, several miniSTR commercial kits have been released in the last 2 years. Considering all relevant information, the most recent concept of miniSTR kits should certainly upgrade the analysis of DNA from old bones and teeth, including those originating from World War II (WWII) skeletal remains.

Identification of human remains relies on various procedures, such as identification by direct facial recognition, fingerprint and dental analysis, identification of special features, recognition of clothing and belongings, autopsy findings, forensic anthropologist's findings, reconstruction of facial features from skulls, hair comparisons, and DNA analysis (9). Unfortunately, with significant number of remains buried in one mass grave or reburied in secondary graves in order to hide the location, identification of such remains is much more difficult (10). Considering a temporal gap of 60 years, DNA analysis seems to be the only viable approach to the identifying the remains dating from the period during and immediately after the WWII (11). Similar studies have already been performed in different parts of Europe (11-13). Different DNA markers were employed for the same purpose, from autosomal STR markers in Slovenia (11) to mtDNA markers in Finland (12) and Y-STR markers in western Herzegovina (13). The differences in molecular approach were mostly dictated by the quantity and quality of DNA isolated from the remains. Regional differences in the level of DNA preservation are likely to exist due to the climatic conditions and chemical properties of the soil (12).

There is no precise official data about the number of missing persons in Slovenia but rough estimates suggest that the approximate number could amount to tens of thousands. Almost 600 scaffold and hidden mass graves are verified in Slovenia (14) and the latest mass grave was discovered in Barbarin rov (Huda jama, Laško) in March 2009 by Slovenian Government Commission Regarding the Question of Hidden Mass Graves. In this grave, skeletal remains of 300 victims killed by Communist Armed Forces after WWII were found (15). In 2006, the Commission for Recording and Managing Hidden Graves of the Major of the Municipality of Škofja Loka (a city located around 40 km to the northwest from the Slovenian capital Ljubljana) put in a significant effort to identify victims of the Communist Armed Forces discovered in 2 hidden mass graves uncovered at the location Lovrenska Grapa (Figure 1). Due to the advanced state of the remains decomposition, application of conventional methods of human identification was unfeasible and DNA identification was requested. We have reported the first results of these analyses earlier (11). In our previous study (11), comparison of victims' profile against a reference sample database resulted in 4 statistically significant matches. In addition, another 5 profiles were associ-

Figure 1.

Mass grave at Lovrenska Grapa site.

ated to certain reference samples with insufficiently high probability. Two of those victims were associated with possible male paternal relatives, thus the samples were further subjected to Y-STR typing. At this point, we present the results of additional analysis based on Y-STR markers.

Encouraged by these results, the same authorities requested DNA analysis for the victims found in newly uncovered hidden mass grave situated near Škofja Loka, location Žološča (Figure 2). Interestingly, after the public announcements of previously achieved results in the local media, numerous persons requested DNA analysis for the skeletal remains found in several individual graves, presumed to belong to their beloved. These samples turned to be extremely challenging for processing and at this point we present some of the obtained results.

Figure 2.

Mass grave at Žolšče site.

MATERIALS AND METHODS

Analysis of skeletal remains from Lovrenska Grapa mass grave

Previous statistical comparison, based on the obtained autosomal STR profiles, suggested possible relation between 2 bone samples (laboratory-codes BS0016 and BS0017) and 2 male DNA profiles bearing laboratory-codes RS0031 and RS0059 previously deposited into the reference sample database (11). The bone samples were harvested from mortal remains recovered from the larger of the 2 small collective hidden graves, which were located in the woods, close to a spring (11). Pre-amplification handling and processing of reference samples (RS0031 and RS0059) from presumed living male paternal relatives and the procedures leading from the process of skeletal remains recovery to DNA amplification were described in detail in our previous article (11). PowerPlex Y kit (Promega Corp., Madison, WI, USA) was used to simultaneously amplify 12 Y-STR loci. Amplification reactions were prepared according to the Promega technical manual (16). The polymerase chain reaction (PCR) amplification was carried out in the PE GeneAmp PCR System Thermal Cycler (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations with 32 amplification cycles. Fragment analysis was preformed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The raw data was compiled and analyzed using accessory software: 310 Data Collection Software and GeneMapper™ 3.2 (Applied Biosystems). Numerical allele designations of the profiles were obtained by processing with PowertyperY Macro (Promega Corp.). Previous comparative analysis of autosomal DNA profiles obtained from skeletal remains and reference samples, estimation of potential familial relationships, calculation of paternity and sibling indices, and calculation of matching probability were performed in DNA-View program (17). Calculation of Y-STR profiles matching was based on the counting method with an upper bound confidence limit (5) and the data from the YHRD database (18).

Analysis of skeletal remains from Žolšče mass grave

Mortal remains of 11 victims were recovered from a small hidden mass grave uncovered in the woods at the location Žolšče, close to the Slovenian town of Škofja Loka. The excavation of skeletal remains was performed under the supervision of archeologist Draško Josipovič. Anthropologist Petra Seljak-Leben performed the anthropological analyses of victims. Unfortunately, no precise information as to the identity of the victims was available. However, local accounts suggest that the remains may belong to local people who were executed by the communist forces in the summer of 1945. The remains were recovered and processed by local archeologists and anthropologists. Samples for DNA analysis (femoral fragments and teeth) were collected and labeled. After that, the samples were transported to the Laboratory of Forensic Genetics at the Institute for Genetic Engineering and Biotechnology, University of Sarajevo, Bosnia and Herzegovina. Upon arrival at the Institute, the samples were assigned case numbers and the relevant information was entered into Chain of Custody forms. Each bone sample was cleaned and powdered following previously described procedure (11). Double extractions were performed for each sample following previously described optimized Qiagen procedures

(19). Centricon-100® centrifugal filter units (Millipore, Billerica, MA, USA) were used for additional DNA purification and concentration. Some samples required an additional purification step with ***n***-butanol. The concentrates were transferred to 1.5-mL microcentrifuge tubes and diluted with DNA-free ddH_2O to a final volume of 50μL. DNA concentration was determined using Quantifiler Human DNA Quantification Kit (Applied Biosystems) (20). The reaction was carried out in AB 7300 Real-Time PCR System (ABI, Foster City, CA, USA) according to the manufacturer's recommendations. Preliminary analysis was performed using PowerPlex 16 kit that contains 15 STR loci, but some samples yielded weak partial profiles or no profiles at all, due to extremely small amounts of isolated DNA. Therefore, additional analyses employing the PowerPlex S5 miniSTR kit and AmpF*ℓSTR® MiniFiler* PCR Amplification Kit were performed (21,22). The PCR amplification was carried out in PE Gene Amp PCR System Thermal Cycler (Applied Biosystems) according to the manufacturer's recommendations. Fragment analysis was preformed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Reference samples (18 buccal swabs) from potential living relatives were collected, recorded, labeled, and processed according to the previously described procedure (11). All statistical analyses were performed in the DNA-View program (17).

Analysis of skeletal remains from 2 hidden individual graves (Podlipoglav site)

Two brothers were looking for their mother, missing from the Fall of 1942. According to the testimonies, she was kidnapped in front of her children on August 2, 1942 from a train by territorial workers of Communist Security-Intelligence Organization VOS (Varnostno-Obveščevalno Službo [in Slovenian]). She was in an advanced stage of pregnancy. According to the testimony, she was murdered soon after kidnapping. Within the past 10 years, her children intensified the search for information about her burial place. In more than 60 years they managed to identify 2 gravesites that could possibly contain her remains. The excavation of skeletal remains was performed under the supervision of archeologist Draško Josipovič. Anthropologist Petra Seljak-Leben performed the anthropological analyses of skeletal remains. Samples for DNA analysis (femoral fragments from skeletal remains and buccal swabs from her 2 sons) were collected, labeled and transported to our laboratory. Pre-amplification handling and processing procedures of bones (BS0033 and BS0034) and reference samples (RS0074 and RS0075) were detailed in a previous paper (11). Initially, PowerPlex 16 kit was used to simultaneously amplify 15 STR loci, but in the case of bone BS0034, the procedure was unsuccessful. Therefore, additional analyses employing PowerPlex S5 miniSTR kit and AmpF*ℓSTR® MiniFiler* PCR Amplification Kit (21,22) were performed on that sample. Fragment analysis was preformed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). All statistical analyses were performed in DNA-View program (17).

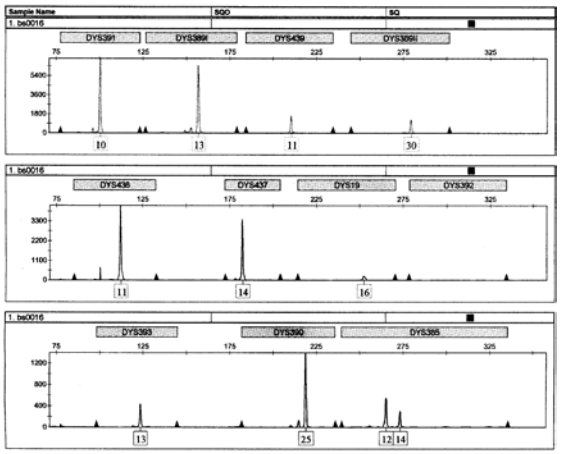
RESULTS

Additional information for Lovrenska Grapa hidden mass grave

Bone samples (femoral fragments) from 2 skeletal remains previously analyzed at 15 autosomal STR loci were subjected to additional Y-STR analysis. The number of previously detected loci per PP16 profile was 13 for BS0016 and 15 for BS0017 (11). Estimates based on autosomal STR profiles indicated that sample BS0017 was 2000 times more likely to be a relative (presumed parent) of reference RS0031 than an unrelated individual within the population. The same analysis indicated that the sample BS0016 was 1000 times more likely to be a relative (presumed parent) of reference RS0059 than an unrelated individual within the population. Since both sample pairs (BS0017-RS0031 and BS0016-RS0059) were male-male combinations we performed Y-STR analysis.

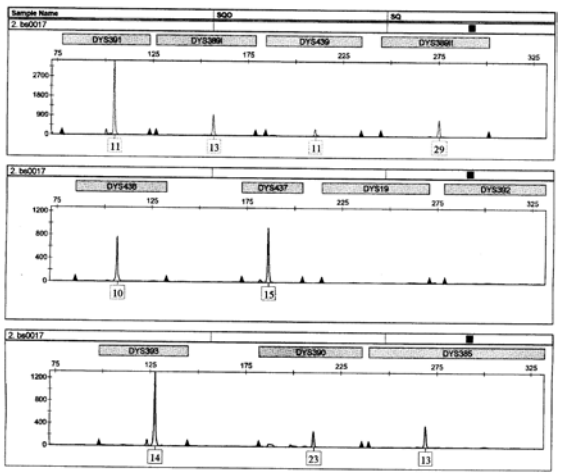
Consequently, we obtained 2 partial PPY-STR DNA profiles. The number of detected loci per profile was 11 for BS0016 (Figure 3) and 9 for BS0017 (Figure 4). Simultaneously, we

Figure 3.



Y-STR profile for BS0016.

Figure 4.



Y-STR profile for BS0017.

generated complete PPY STR profiles for both reference samples.

Comparative analysis suggested that sample BS0017 and reference RS0031 shared the same Y-STR profile across the detected loci (Table 1), with (calculated using data from the YHRD database and counting method with an upper bound confidence limit) an expected frequency in the European population of 1.6580752×10^{-3}. When these results were combined with the previous results of autosomal STR DNA testing, it was shown that BS0017 was approximately 1 206 000 times more likely to be the father of reference RS0031 than an unrelated individual in the population. Since the rough estimate of the number of victims reported missing from this area is 400 persons, in accordance with the ISFG Recommendation #11 (23), the threshold for the direct match was set to 1 in 400 000. Consequently, posterior probability was sufficient and higher than the recommended 99.9%.

On the other hand, Y-STR DNA testing showed discordance at 8 of 11 detected loci between the bone sample BS0016 and reference RS0059 (Table 2). Therefore, Y-STR analysis did not support father-son relation between samples BS0016 and RS0059.

Žolšče hidden mass grave

Samples collected from 11 skeletal remains found in a small mass grave in Škofja Loka area were processed.

TABLE 1. Comparison of obtained Y-chromosome short tandem repeat (Y-STR) profiles for bone sample BS0017 and reference sample RS0031 that share the same Y STR profile across the detected loci

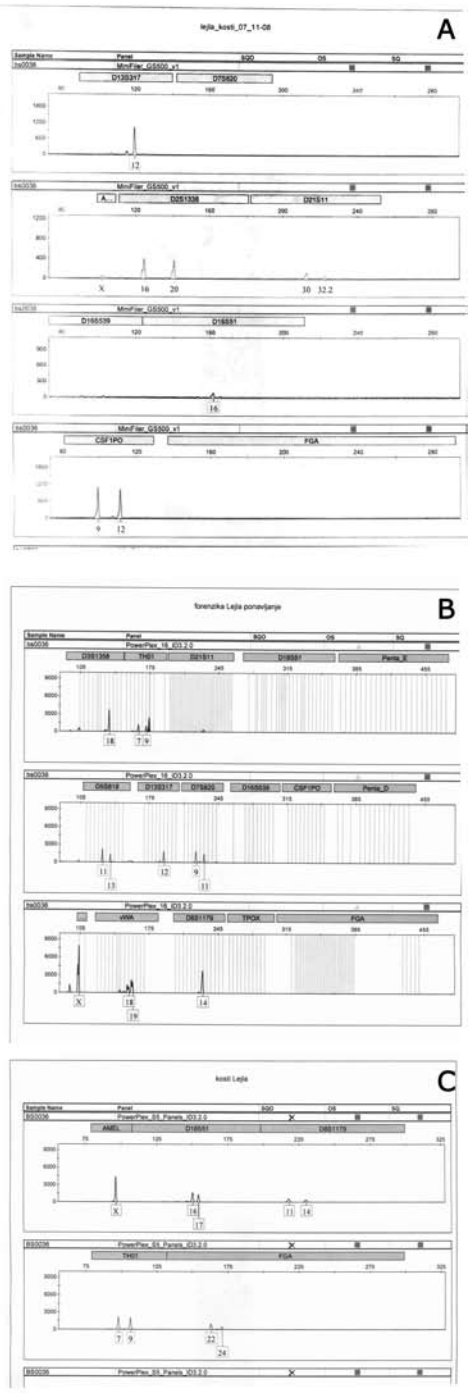
Sample	Y-STR allele profile of	
Y-STR LOCI	BS0017	RS0031
DYS391	11	11
DYS389I	13	13
DYS439	11	11
DYS389II	29	29
DYS438	10	10
DYS437	15	15
DYS19	–	17
DYS392	–	11
DYS393	14	14
DYS390	23	23
DYS385	13	12
DYS385	–	13

TABLE 2. Comparison of obtained Y-chromosome short tandem repeat (Y-STR) profiles for bone sample BS0016 and reference sample RS0059 that do not share the same Y STR profile across the detected loci

Sample	Y-STR allele profile of	
Y-STR LOCI	BS0016	RS0059
DYS391	10	12
DYS389I	13	13
DYS439	11	14
DYS389II	30	31
DYS438	11	10
DYS437	14	15
DYS19	16	16
DYS392	–	11
DYS393	13	13
DYS390	25	24
DYS385	12	14
DYS385	14	15

Based on quantification results, an extended PCR procedure (32 cycles with elongation time extended to 90 seconds) was used for all samples, but provided poor results (from 2-4 loci) in 6 cases, and no results in 5 cases due to extremely low (undetectable) amount of isolated DNA. Therefore, 2 miniSTR kits (PowerPlex S5 and AB Minifiler) were used in order to obtain additional information. Finally, useful DNA profiles were obtained for 6 bone samples (Figure 5A, Figure 5B, Figure 5C, Table 3). The number of detected loci varied between 12 and 16 per profile. The remaining 5 samples provided either poor results or no results at all.

Figure 5.



Obtained profiles for the sample BS0036. (**A**) Obtained MiniSTR profile for the sample BS0036 (AB Minifiler). (**B**) Obtained PP16 profile for the sample BS0036. (**C**) Obtained MiniSTR profile for the sample BS0036 (Promega S5).

TABLE 3. Obtained "cumulative" short tandem repeat (STR) profile (employing PowerPlex 16, PowerPlex S5, and AB Minifiler) for the bone sample BS0036, one of the samples from the skeletal remains exhumed from Žolšće site

Sample	BS0036	
STR LOCI	Allele 1	Allele 2
D3S1358	18	–
TH01	7	9
D21S11	30	32.2
D18S51	16	17
PENTA E	–	–
D5S818	11	13
D13S317	12	–
D7S820	9	11
D16S539	–	–
CSF1P0	9	12
PENTA D	–	–
VWA	18	19
D8S1179	11	14
TPOX	–	–
FGA	22	24
D2S1338	16	20
Amelogenin	x	x

Also, 18 reference buccal swabs collected from relatives of persons missing from that area were successfully profiled. Comparison of victims' profile against the reference sample database resulted in no positive matches. In addition, all DNA profiles were included in the existent database, which contains all the remains' and reference profiles that we have processed, but in which no positive identifications were detected.

Podlipoglav individual hidden graves

First, 2 brothers were successfully typed using PowerPlex®16 kit. Also, the bone sample (BS0033) of the presumed missing mother was profiled at 12 STR loci. The comparison of the profiles showed discordance at more than 3 clearly detected loci between the bone sample BS0033 and references RS0074 and RS0075. Consequently, the presumption that remains labeled BS0033 belonged to the mother of the analyzed references was rejected.

Unfortunately, we were not able to generate a useful PowerPlex 16 profile for the other bone sample (BS0034). DNA was unquantifiable using Quantifiler Human DNA Quantification Kit. Anyway, we performed additional typing using PowerPlex S5 system. We obtained a complete female profile (over all 5 loci) that was matched to

TABLE 4. Summary of performed DNA analysis for the World War II skeletal remains from Slovenia

Exhumed remains	48
Obtained profiles	41
Ratio of obtained profiles (%)	85.4
References samples	90
Positive identification	6
Ratio of positive identification (%)	12.5

both brothers with likelihood ratio (LR) = 295. These results were further supported using AB Minifiler kit. Combined analysis of all loci from both kits (total number of detected loci 9) significantly increased the LR to 2.21×10^9. This "strong match" led us to the positive identification and confirmation of presumption that skeletal remains labeled BS0034 belong to the references' mother.

Cumulative results of DNA identification of skeletal remains of missing persons during and after of the WWII

Compiled results of all the performed analysis are presented in Table 4. Total number of processed skeletal remains was 48. For 41 of them, the analysis led to making useful DNA profiles, which represents 5/6 of all processed skeletal remains. The total number of processed reference samples is 90. Finally, 6 remains were matched to living relatives, which represents 1/6 of all the exhumed skeletal remains.

DISCUSSION

During the last few years, we have employed an approach based on using Y-STRs and miniSTR for different purposes, such as basic population data announcements (24), examination of settlement process in South-Eastern Europe (4), processing of complex forensic cases (25), and identification of mass disaster victims (7). In this study, these markers were used for the first time to provide genetic information sufficient for the identification of victims killed in the period during and after WWII from uncovered hidden graves. More than 60 years after the crimes, DNA analysis has emerged as the ultimate tool for identification of the victims from that period. This study once again confirmed the conclusions from a previous report (11) that the experience gathered through the process of identification of missing persons in the former Yugoslavia may provide vital insight necessary for the identification of skeletal remains of the victims killed in the period during and after WWII. Y-STRs and miniSTR approach, combined with standard autosomal STR analysis, helped ensure that uncovered skeletal remains could be assigned to a missing person after more than half a century and offered hope to the families that their loved ones will be given a dignified resting place and provides closure to a lifelong quest. Therefore, the introduction of the most recent methods such as miniSTR and Y-STR testing allows the forensic science community to achieve results that were inconceivable just a few years ago.

Of course, another DNA lineage marker (hypervariable regions of mtDNA) may also be a very valuable source of information for the analysis of WWII skeletal remains (12) and offer very interesting insight in archeological skeletal samples (26).

The findings presented in this study reinstate that the experience gathered through almost two decades of identification of missing persons in this region, reinforced by the latest methodology, can successfully provide information necessary for the identification of victims from and after the WWII period.

Therefore, we propose this approach as a model for further DNA identification of human remains exhumed from WWII mass graves that are, unfortunately, scattered throughout Europe. As stated before (11), these 6 positive matching reports are not only 6 individuals whose identity was restored, but also represent families who have received the final answer as to the fate of their loved ones. These 6 people reinstate hope for other families, especially from Croatia and Bosnia and Herzegovina, that they could also find their missing relatives and get answers to the long-standing questions.

It is apparent that 35 profiles remain unidentified as they await suitable reference. One of the ways forward is to enlarge reference samples database. Obviously, more than 60 years that have elapsed since the end of WWII is close to an average human life-span and for many bodies, living reference samples may not be available. It is also enough time for families to lose hope. However, a wider media campaign may help prompt living relatives to come forward, donate samples, and provide additional information.

Also, there is the remaining issue of bodies that could not be profiled. We envisage that the solution lies in the refinement of DNA extraction procedure rather than in the application of additional typing protocols.

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