Genetic Kinship Research

Determination of biological relationships within the medieval and post-medieval cemetery of the St. Plechelmus church in Oldenzaal using ancient DNA

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Genetic Kinship Research: Determination of biological relationships within the medieval and post-medieval cemetery of the St. Plechelmus church in Oldenzaal using ancient DNA

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1. Introduction

1.1 The Oldenzaal research project

1.1.1 The Archaeological excavation at Oldenzaal

Between September 2011 and February 2013 an archaeological investigation was performed by ADC ArchaeoProjecten in cooperation with RAAP prior to the renovation of the Plechelmusplein and the Kerkstraat around the Plechelmus church in Oldenzaal. The town Oldenzaal is located approximately 10 kilometers north of Enschede in the province of Overijssel, the Netherlands (figure 1).This project was performed with the financial support of the Rijksdienst voor het Cultureel Erfgoed (RCE). The disturbance of the archaeological features and material was due to the construction of a tree planting slot with a depth of 1 meter and 2,5 meters wide, a new sewer slot around the church with a depth of 2 meters and 300 meters in length, and the renovation of the square.

Figure 1. Map of Holland from the Middle Ages with the present-day boundaries. The red dot indicates the location of Oldenzaal (modified from van Bavel 2010, 50).

The excavation area consisted of almost 5000 m² with 111 trenches. The excavation exposed approximately 2750 graves, with the oldest dating from the 8th or 9th century A.D. The depth of the graves varied between +44,58 m and +46,04 m NAP. Most of the skeletons were in supine position with an east-west orientation. Specific areas within the cemetery have deviating burial orientations, possibly related to a temporary shift in religious policy (Williams 2013, 27). Occasionally wood from the coffins was found around the skeletons. However, in most cases the wood was already decayed and only color changes in the soil were visible. Grave goods were very scarce from the excavated burials. The uncovered human remains were carefully documented, excavated and stored.

Prior to the excavation, it was decided that population research would take place. Therefore, 1005 (37%) excavated skeletons were sampled during fieldwork of which a selection of 200

samples was made for further analysis, due to the large number of burials encountered at the excavation. The data obtained from physical anthropological research of the human remains and DNA and isotope samples will be used to provide more insight in the population structure. This thesis is part of a population research project based on different bioarchaeological techniques, namely physical anthropology, DNA research and research on strontium, oxygen, carbon and nitrogen isotopes. The aims of the research project are reconstructing family relationships and to demonstrate if genetic continuity occurred between different time periodes in order to answer archaeological research questions and provide more insight into biological selection processes that occurred in the past, due to various diseases. The human bioarchaeological material from the Oldenzaal project was first tested to provide a clear indication about whether the material was suitable for further research. A small examination of the quality and quantity of the ancient human DNA took place in order to examine the success rate of the technique to analyze the DNA of the collected samples. This preliminary DNA analysis was performed by Eveline Altena and Risha Smeding from the Forensic Laboratory for DNA-research (FLDO) of the Leiden University Medical Center (LUMC) on dental samples from 27 skeletons. The results were promising and it was decided to continue the DNA research.

1.1.2 The historical context of the St. Plechelmus church and its cemetery

The current Plechelmus church was built around 1150 AD in a late-Roman style and was placed on the location where missionary Plechelmus erected the Silvester church around 765 AD. The cemetery of the Plechelmus church was used for at least ten decades, until 1829 AD. In medieval times the church was one of the seven most important churches of the diocese of Utrecht (Boon 2006). It is assumed that surrounding villages each had their own separate area within the cemetery. However, there are no known written documents of this possible division of the cemetery. Based on dated skeletal remains, the area located northwest of the church was interpreted as the first phase of the cemetery. The excavated skeletal remains do not necessarily provide a representative sample of the original cemetery population, because certain areas within the excavation area were excavated only to a limited depth.

It is estimated that the cemetery around the Plechelmus church contained at least 14.000 primary graves at the time of the excavation. However, the total amount of burials that took place in this cemetery probably exceeds this number. Unfortunately, an unknown part of the original graves has been lost with the construction of surrounding buildings and clearing of

burials. Investigating the burial complexes can provide information regarding taphonomic processes (Nawrocki 1995), death rituals, social structure and familial ties, and the relative dating of the burials (Pearson 1999, 12).

1.1.3 Harris Matrices and the family grave

Within the excavation terrain, five areas (A to E) with high burial density have been selected for extended research (figure 2). Sequence diagrams of the burials and Harris matrices were created for these specific areas. These Harris matrices provide an overview of the chronological succession of the burials. The sequence of burials mapped by the Harris matrices also enables the indirect dating of burials, based on their locational relationship with each other and the Law of Superposition.

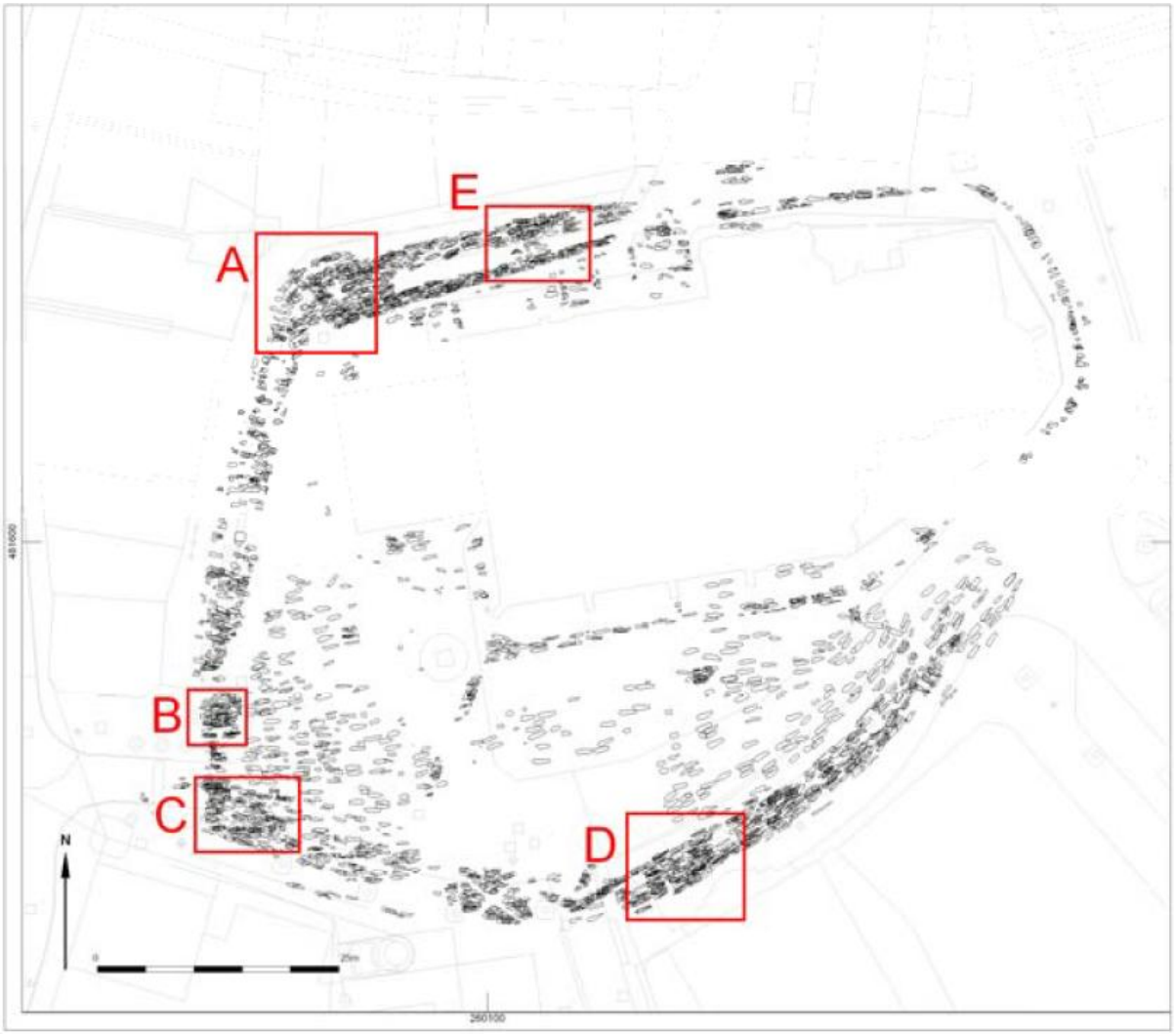

Figure 2. Overview of the five selected areas with a high burial density, within the excavation terrain of the cemetery (Williams 2013, 34).

In the north-west part of the excavation terrain, within matrix A, a cluster containing six individuals was found and is interpreted as a possible ‘family grave’ because the individuals were laying on top of each other in a single burial context (figure 3). The exact identity of the individuals is unknown due to the absence of gravemarkers or written documents.

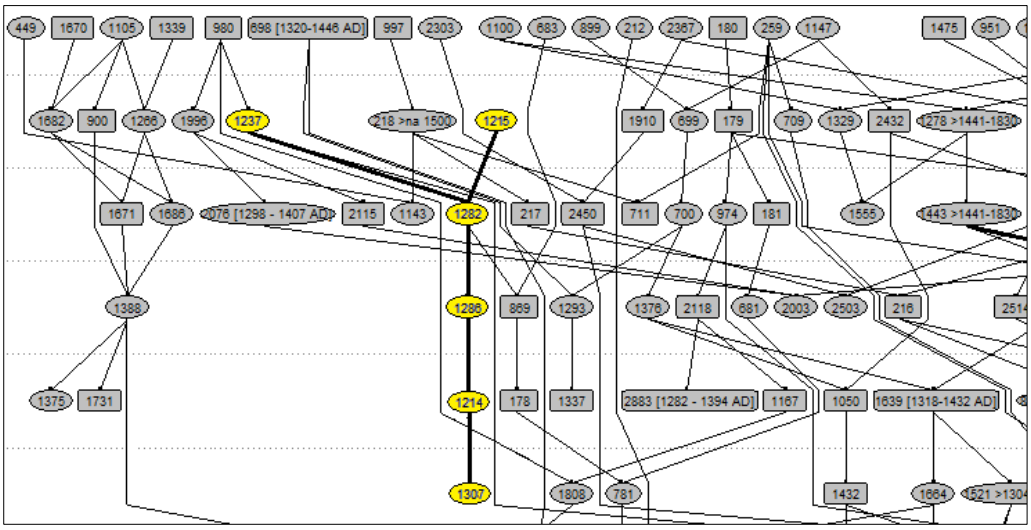

Figure 3. The ‘family grave’ burials within a section of the Harris matrix of matrix A.

Besides the six skeletons of the ‘family grave’, 81 other skeletons were selected from the total 275 within Matrix A for further research. This selection was based on the following criteria: the skeletons had to be sampled for DNA and isotope research, and were most efficient for dating purposes; a dated skeleton could enable the indirect dating of many surrounding skeletons. All the individuals from matrices B, C, D, and E which were in accordance with the criteria were selected and 81 from matrix A were added to come up with a total of 200 individuals.

Figure 4. The ‘family grave’ during the excavation (Williams 2013, 33)

In this thesis the possible kinship relations between individuals from the ‘family grave’ as well as the selected individuals from matrix A are investigated and discussed. Due to the

absence of gravemarkers and written grave registers with names of the buried individuals, the best method for determining possible kinship relations within Matrix A and the 'family grave' is DNA analysis.

1.2 Kinship Analysis

1.2.2 Physical anthropological research

Since DNA analysis cannot provide information regarding the age-at-death of an individual, physical anthropological research has to be incorporated for age estimation. The age of an individual is important for genetic kinship research to get more insight into the likelihood of an individual being the parent or child within a possible parent-offspring relationship. The excavated skeletons were described in the field by a junior physical anthropologist. Based on a quick first impression at the archaeological excavation, a total of 2229 skeletons were defined as adults, 189 as juveniles and 291 as children. From 41 skeletons it was not possible to estimate the age. Besides age-at-death, physical anthropological research can provide information on sex, stature, ethnicity, and health and disease and possibly the cause of death of an individual. As the extended laboratory-based physical anthropological research is still being conducted at the time of writing, this information is not available at the moment.

Genetic components can determine the morphological outcome of a skeletal trait. This means that familial characteristics can sometimes be expressed in specific morphological traits. Various studies have focused on specific skeletal traits in an attempt to demonstrate relationships within and between populations (Alt and Vach 1995; Spence 1974; Velemínský and Dobisíková 2005). Because no singular morphological trait exists that can conclusively indicate a biological relationship amongst individuals, the presence and appearance of many morphological traits needs to be assessed and analyzed, while also taking into consideration the nature of the traits in the population or origin. However, it is rarely possible to suggest anything more than levels of similarity. Skeletal traits can also be converted by environmental factors during and after growth, this influences the reliability of possible morphological similarities. Therefore, metric and non-metric morphological skeletal analysis does not provide the same level of accuracy or precision as DNA analysis.

1.2.1 Relevance genetic kinship research

This study is important because the determination of family relationships within a population based on ancient DNA obtained from skeletons is a relatively new research approach which

can provide additional information, other archaeological research techniques are not capable of. This research can contribute to studies focusing on the social structures in past populations. Genetic kinship analysis can provide interesting information within an archaeological investigation, for instance regarding the possible family relations of individuals which are buried together. What is interpreted in the field as a 'family grave' might after genetic kinship analysis appear something different. Furthermore, kinship analysis can also give an indication for certain family areas within the cemetery, confirm historical documents or gravemarkers, and contribute to the construction of an archaeological genetic database. This database can be used to provide future ancient DNA research with allele frequencies.

The importance of a better understanding regarding the genetic composition of a population lies in the level of relatedness which provide information about various aspects of the population, such as migration. Changes in the genetic composition of a population in the course of time can be attributed to migrations. This is important information for all kinds of research, including medical-genetic research towards past populations.

1.3 Research Questions

Multiple buried individuals in a single grave context leads to the question of whether the individuals belonged to the same family. The potential family relations between the individuals of the 'family grave' will be explored in this thesis by means of DNA research. In addition to the 'family grave', it is also the aim of this research to reconstruct kinship relations between the selected individuals within matrix A of the Plechelmus church cemetery.

Because this research deals with the reconstruction of kinship relationships in the family grave as well as in matrix A, the main research question can be divided in two and is defined as follows:

- *Are there genetic indications for kinship relations between the individuals of the 'family grave' at the Plechelmus cemetery which can confirm that this cluster of burials actually represents a family grave?*

- *Are there genetic indications for kinship relations between the individuals within matrix A of the Plechelmus cemetery?*

2. Methods and Techniques

2.1 Introduction DNA analysis

2.1.1 Structure of DNA

Almost all organisms, except for a few viruses, have deoxyribonucleic acid (DNA) molecules as their genetic components. DNA is a polymer, and consists of monomeric molecules named nucleotides. The four different variants of nucleotides are the bases adenine, guanine, cytosine and thymine. The bases are attached to each other by a phosphate group and the sugar deoxyribose forming a strand; the "sugar-phosphate backbone" (Jobling et al. 2013, 21). Adenine of one strand will always pair with thymine of another strand and this also goes for guanine and cytosine. Therefore, these two bonded strands are complementary by the base pairing rules and form a double stranded helix (Prescott *et al.* 1996, 214). The sequence of these nucleotide molecules carries the genetic information.

2.1.2 The principle of inheritance

The present-day understanding of the mechanism of inheritance comes from the theory proposed by Gregor Mendel in 1865 (Miko 2008). Before the discovery of genes, Mendel designed a model in which the transmission of genetic traits from parents to offspring was explained. The heritable elements, units of DNA now known as genes, are made up of two alleles one derived from each parent. This process, through which a new combination of alleles is formed, is called genetic recombination. Individuals are homozygous for a particular gene when they possess two identical alleles, and heterozygous if both alleles are different from each other.

Most DNA is located in the nucleus, and a relative small amount in the mitochondria of almost every human cell. Within the nucleus the genetic material is divided into 23 pairs of chromosomes: twenty-two autosomal chromosome pairs which are identical between the sexes and one pair of sex chromosomes which differ between the sexes. Females have two copies of the same X chromosome, while males have one X chromosome and one Y chromosome. The Y-chromosome is a non-recombining region and can therefore be used to study patrilineal inheritance patterns.

2.1.3 Genetic kinship analysis

The genetic kinship analysis performed on the Oldenzaal samples makes use of the inheritance approach by examining the degree of resemblance of the obtained genetic data from different individuals. Generally, based on the principle of inheritance we are genetically more similar to our close relatives compared to non-relatives. However, the only reliable kinship relation that can be reconstructed is the parent-offspring relationship. Offspring can statistically inherit the exact opposite half of both parents genetic material, and therefore have the possibility of showing no resemblances to each other.

The DNA analysis performed at the Forensic Laboratory for DNA-research (FLDO) of the Leiden University Medical Center (LUMC) is based on genetic information in the form of alleles on non-coding DNA sequences, which are not affected by selective pressure. The inheritance of these selectively neutral markers through generations enables the demonstration of kinship relations through comparisons of the genetic information. Mutations of the loci contribute to the genetic variation between individuals. The genetic variation can be studied in small regions with known locations on the autosomal and Y-chromosomal DNA. The high variability between individuals within these short DNA sequences leads to a high potential of discrimination. The level of discrimination depends on the number of used genetic markers and the frequency of the alleles within a specific population. A selection of short autosomal and Y-chromosomal DNA sequences is analyzed in the Oldenzaal project and is described in section 2.5.

Mitochondrial DNA (mtDNA) is also valuable for kinship analysis because of the high mutation rate and mtDNA remains unaltered through inheritance, because it is inherited from the mother with no recombination. However, mtDNA yields less variety compared to autosomal DNA and it is not possible to perform Short Tandem Repeat analysis on mtDNA (see section 2.6 for more information regarding STRs). The relatively limited amount of variation in mtDNA makes phylogenetic inferences questionable since unrelated individuals can potentially share the same mtDNA. Nevertheless, mtDNA analysis remains useful for this kinship relation research and will be performed at a later stage of the Oldenzaal research project. Therefore, the mtDNA analysis will not be described in this thesis.

Besides demonstrating kinship relations, ancient DNA research can also contribute to archaeology by determining the sex of individuals. In the case of an incomplete skeleton at an archaeological excavation, determining the sex of a skeleton can be difficult. Subadult skeletons are not possible to sex, because the morphological characteristics which are used to distinguish male and female individuals are not (fully) developed at this stage. The presence

or absence of the Y-chromosome can be used to confirm uncertain sex determinations based on morphological characteristics of the skeletal material.

2.2 Technical implications in ancient DNA studies

Analyzing archaeological samples in the DNA laboratory is generally a problematic application due to the poor preservation of DNA. The biological material from the Oldenzaal excavation has been exposed to a variety of environmental conditions for many centuries. Therefore, DNA molecules are possibly degraded or contaminated to some extent. The ancient DNA is often highly fragmented into sequences with a length of only several hundred nucleotides.

2.2.1 Degradation of ancient DNA

After the death of an organism the enzymatic systems which monitor and repair DNA stop functioning. As a result the degradation process starts in which endonucleases will break down the DNA molecules into smaller pieces. This results in fragmented DNA, loss of bases, deamination (the chemical alternation of ancient DNA in which the hydrolysis reaction turns cytosine into uracil) and cross-linked sites (which can block the polymerase). Temperature, pH level, humidity and salinity are conditions that affect the degree to which DNA decays (Jobling et al. 2013, 111). Degraded DNA samples may result in unsuccessful PCR amplification, because primers will only extend as far as the break in a template (Butler 2005, 146).

2.2.2 DNA contamination

In conjunction with the degradation process, the sample can be contaminated with microbial or modern human DNA. Contamination can be defined as the accidental transfer of DNA into a sample (Butler 2005, 152). This can occur prior to the research process with genomic DNA from the environment and during the research process by contaminated consumables, employees who come into close contact with the samples, or between samples.

Modern DNA has high concentrations and a relatively good condition compared to ancient DNA. Therefore, modern DNA will more easily be amplified during the PCR. Particularly studies of ancient human DNA are problematic, because the PCR primers will amplify the ancient human DNA as well as the modern human DNA. Because PCR reactions have the ability to amplify low quantities of DNA, laboratory protocols were designed in order to avoid contamination.

2.2.3 Precautions

The FLDO, as part of the Human Genetics Department of the LUMC, has strict guidelines to prevent contamination and further degradation of the samples. Archaeological samples are generally degraded as mentioned in section 2.2.1. To prevent them from degrading even further samples are stored in a freezer at -20°C. Under these conditions the degradation of DNA and bacterial growth will be minimalized.

In order to prevent contamination during DNA research it is important that all the samples are packed separately, clean plastic gloves and protective clothes are used, and the working environment is kept sterile. The FLDO laboratory for ancient DNA research is separated from the main genetics laboratory. The air pressure within the laboratory is higher than the surrounding areas to prevent contaminated air from entering. Therefore, the laboratory has a ventilation system which regulates the pressure. UV-C light and bleach are used for the sterilization of the consumables.

Negative controls have to be included in the extraction and PCR process to determine if contamination had occurred. Furthermore, the genotypes of the entire staff of the DNA laboratory are stored in an elimination database to be able to indicate the potential source of contamination.

2.3 Research material

2.3.1 Sample selection

The first step in the process of the DNA analysis took place in the field. During the archaeological excavation, samples were carefully collected under forensic circumstances to avoid contamination. The biological material used in this research was dental elements. In the archaeological context teeth are the preferred human tissue to extract DNA from. The dental hard tissue produces a layer which protects the DNA from environmental conditions such as humidity, extreme temperatures, and fungus and bacteria activity (Malaver 2003).

The anatomical position of the DNA in a tooth is primarily in the soft tissue within the coronal and radicular pulp chamber that is physically enclosed by the mineralized tissue of enamel and cementum, which covers the tooth root and provides an anatomical composition of great sustainability. Therefore, DNA extracted from teeth is generally of higher quality and is less often contaminated compared to DNA extracted from bones (Adler 2011; Girish *et al*. 2010; Higgins and Austin 2013). Teeth from archaeological excavations do not contain pulp

as it is decomposed, which leaves dentine as the targeted material within the teeth for DNA extraction.

From each of the selected individuals at the archaeological excavation of Oldenzaal, three teeth were removed; one for isotope research, one for DNA research, and the last one for future purposes. Once teeth were selected for isotope research, a selection was made out of the two remaining teeth for each individual for DNA research. This selection was based on preservation condition, tooth size, the presence of caries, and with a preference for closed roots (see Appendix I for all selected samples).

2.4 DNA extraction

The extraction of DNA from tooth material is a destructive process. Hence the teeth were photographed before the extraction in order to assess the biochemical preservation of the teeth in later phases of the research project. Prior to the pulverizing process the samples were placed under an UV-C lamp on two sides for 45 minutes each to remove possible contamination. DNA molecules have to be isolated from the biological sample before they can be analyzed. Therefore, DNA extraction methods were performed to separate the DNA molecules from other cellular material. The first step in the isolation process is pulverizing the teeth with the use of the Mixer Mill 400. Pulverizing teeth results in a large amount of heat exposure. The samples were put into small zirconium oxide containers containing a zirconium oxide bead, which were placed in liquid nitrogen for 20 minutes to counteract the heat exposure. The pulverizing interval was exactly 10 seconds, a longer duration of the intervals could have resulted in damaging the DNA due to the impact of heat exposure. The number of pulverizing intervals was determined based upon the size and the condition of the teeth, but normally 2x three intervals. Once the samples were pulverized for three intervals of 10 seconds they were placed in liquid nitrogen for an additional 10 minutes. The total amount of obtained powder was weighted (Appendix I.).

A lysis buffer containing 1 ml 0.5 M EDTA pH 8.0 and 5 % sarkosyl, and 90 µl proteinase K was added 0.4 gram of powder from every sample. EDTA is a salt which regulates the acidity of the lysate. Proteinase K hydrolyzes histone proteins and can be used over a wide pH range (4-12.5) and is not affected by EDTA. The detergents are added to break up membrane structures and separate histone proteins from DNA. The samples were incubated overnight at 56 ºC in a thermomixer at 1000 rpm and thereafter centrifuged for 3 minutes at

13000 rpm. The supernatant was purified with the QIAquick PCR Purification Kit from Qiagen in accordance to the company's manual, eluted in 80 µl sterile water and finally centrifuged for 2 minutes at 8000 rpm in the soft mode.

During the extraction process one negative control was added that had been treated exactly the same way as the other archaeological samples. This negative control contains no cellular material and serves to detect possible contamination during the extraction process.
Finally, the extracted DNA was stored in a freezer at -20°C in the laboratory.

2.5 The polymerase chain reaction

Between 1983 and 1985 Kary Mullis developed the PCR technique that enabled the replication of large quantities of DNA sequences with small amounts of template DNA. Therefore, this technique is very useful for studying ancient DNA. Prior to the PCR process specific fragments, called primers, with sequences identical to the flanking unit of the targeted DNA fragment have to be selected in order to amplify .

The first step in the PCR cycle is the denaturing phase in which the two complementary strands of the DNA separate by heating the sample. The second step is the annealing phase where the primers are added and by lowering the temperature will bind to both single stranded DNA sequences. Finally, an enzyme (most often the Taq polymerase) extends the primers with the use of nucleoside triphosphates and amplifies the targeted DNA sequence. At the end of each cycle the previous amount of DNA fragments have been doubled, making it a chain reaction (figure 5). A PCR with 30 cycles can provide around a billion

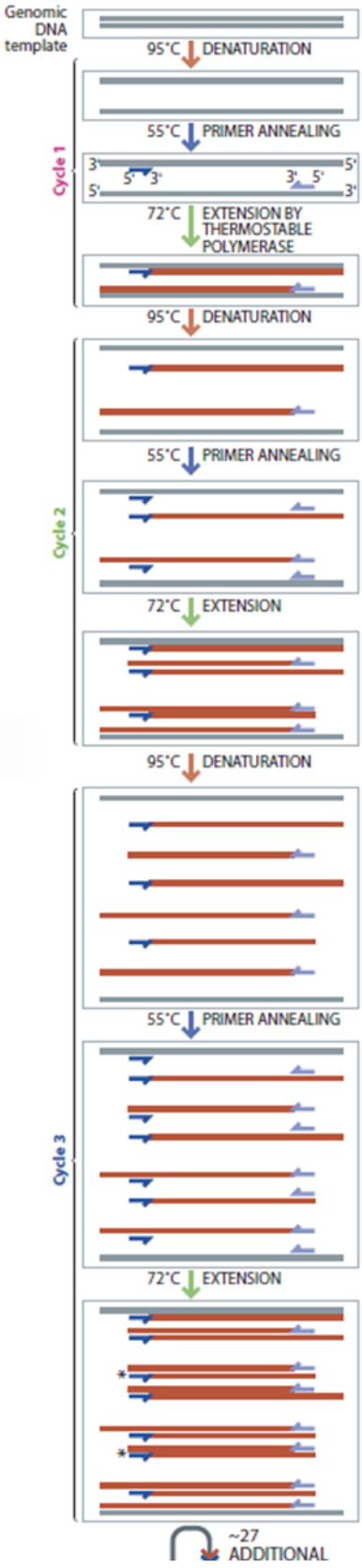

Figure 5. A schematic illustration of the different phases of the polymerase chain reaction (Jobling et al. 2013, 100).

copies of a particular fragment (Prescott et al. 1996, 312). In this research the Applied Biosystems GeneAmp® PCR System 9700 was used.

2.5.1 Quantification

The quantity and quality of DNA needs to be measured before different PCRs are performed. The determination of the amount of DNA that is present in the samples is important because the PCR works best within a small concentration range. A shortage of DNA can lead to allele drop-out due to failing amplification of DNA in the PCR reaction. Too much DNA can cause artifacts in the DNA profiles. In addition, highly degraded DNA and the presence of co-extracted inhibitors can cause a failure of DNA amplification (Butler 2005, 50/53). The overall benefit of a quantification test is a more efficient use of analyst time and sample material because of a decrease in reagent consumption on samples that contain high DNA concentrations.

Highly concentrated DNA samples will need less DNA extraction volume for following PCR reactions than low concentrated DNA samples. The DuoHuman Concentration outcome of the quantification has to be at least 0.02 ng/µl to consider a sample highly concentrated.

After the DNA in the samples has been isolated the quantity and quality were assessed with the use of the Quantifiler® Duo DNA Quantification kit from Applied Biosystems. The Quantifiler® Duo kit contains two markers: Humane Ribonuclease P RNA component H1 (RPPH1) for human DNA and the sex-determining region Y (SRY) for male DNA. Each PCR reaction contained 2 µL of DNA template, 5.25 µL Quantifiler Duo Primer Mix and 6.25 µL Quantifiler Duo PCR-Reaction Mix providing a total reaction volume of 13.5 µL.

The thermal cycling process for the Quantifiler® Duo kit was performed with the Biosystems 7500 Real Time PCR System from Applied Biosystems. The Applied Biosystems 7500 Real Time PCR System is a PCR instrument with a specific additional application. The PCR System measures the cycle-to-cycle change of a fluorescence signal, which is the result of the displacement of a dual dye-labeled TaqMan probe from a sequence during the PCR (Butler 2005, 76). The amount of light emission provides an indication for the quantity of the PCR product by comparing it with a standard curve obtained from samples containing an already known DNA concentration. The thermal cycling process for the quantification contained the following cycling parameters: incubation for 10 minutes at 95 °C which was followed by 40 cycles of 15 seconds at 95 °C, and 1 minute at 60 °C and a final hold at 4 °C.

2.5.2 PCR inhibition

Besides degradation and contamination, amplifying DNA from archaeological remains can also be influenced by inhibitors within the samples. Possible inhibitors are melanin from tissue and hair (Eckhart *et al.* 2000), humic compounds in the soil (Tsai and Olson 1992) and urea in urine (Mahony *et al.* 1998). These substances can remain in the sample even after the extraction process and cause PCR amplification errors by disrupting polymerase activity, disrupting the cell lysis in the process of DNA extraction, and cause nucleic acid degradation or capture (Wilson 1997). The presence of inhibitors often results in incomplete DNA profiles due to the loss of alleles from larger STR loci.

2.6 Short Tandem Repeat Analysis

Approximately three percent of the human genome consists of repeated DNA sequences. These repeated sequences are described as satellite DNA and are characterized by the number of successive repeated units and the number of base-pairs within a core unit. Core repeat units which are composed of two to six base-pairs are defined as micro-satellites or short tandem repeats (STRs). Since STR regions are non-coding, no selective pressure will occur against mutations, which results in a high variation between individuals. The STRs are useful for the identification of different individuals due to the high variability of the number of repeats in STR markers among people (Butler 2005, 85). STR alleles are relatively small in size which makes them suitable for archaeological research where DNA is generally degraded. Longer repeat sequences will not amplify when highly fragmented. Shorter repeat sequences tend to be disturbed by artifacts such as PCR stutter, this is caused by miscopying or slippage of DNA during the PCR process, which results in a by-product that is one repeat unit smaller than the primary allele.

Kinship relations can be determined by comparing the obtained STR profiles of the individuals of the family grave, as well as individuals of matrix A to each other. The DNA typing makes use of a standardized set of markers in order to amplify the targeted STRs.

2.6.1 Autosomal STR analysis

In this research various tetranucleotide repeat core loci were analyzed with the use of the PowerPlex® ESX and ESI 16 kits from Promega, including the 13 CODIS STR loci. With these widely used STRs it is possible to compare the results with other research results. The PowerPlex® ESX 16 and PowerPlex® ESI 16 were applied to the Oldenzaal samples in order

to demonstrate family relationships and were run following the manufacturer's recommendations (except half the total reaction volume was used). The PowerPlex® ESX 16 and ESI 16 Systems utilize different PCR primer combinations with different size range and dye label configurations to co-amplify the following 16 loci: D3S1358, TH01, D21S11, D18S51, D10S1248, D1S1656, D2S1338, D16S539, D22S1045, vWA, D8S1179, FGA, D2S441, D12S391, D19S433 and Amelogenine (figure 6).

The PowerPlex® ESX and ESI 16 kits amplify the same set of loci, however, they differ from each other by means of the different locations at which the primers will bind. The advantage

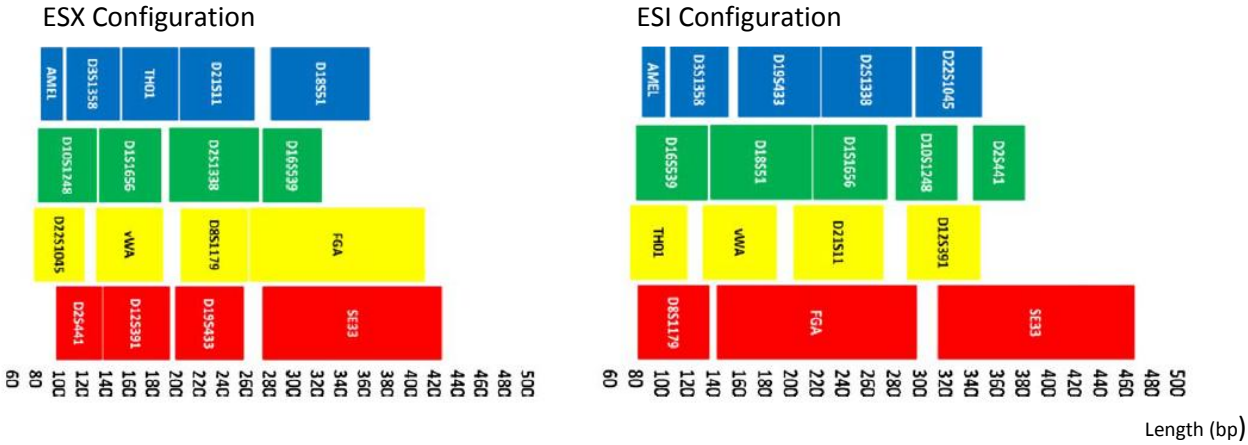

Figure 6. The configuration of the PowerPlex® ESX 16 and ESI 16 Systems (Sprecher *et al.* 2009,2)

of utilizing both kits is based on the differences in the size of the amplified fragments. The absence of long fragments in archaeological samples due to degradation of the DNA can result in failing amplification because primers require too long fragments for one particular kit. However, the other kit might amplify shorter fragments for the same loci and therefore eventually give results. The use of both kits also enables the detection of allelic dropout or "null alleles" within the data, which occur due to primer binding site mutations. Because primers bind at different sites for both kits, these mutations preclude the annealing process of one particular primer, where the primer of the other kit might possibly not be affected by the mutation.

The ESX and ESI were both applied in twofold in order to confirm the results of the previous and to provide sufficient concordance between the typing results for the two kits. Depending on the quality of the samples a second ESX was performed.

Based on the quantification results the samples were divided in 2 and 5 µL volumes. Each PCR reaction for low concentrated samples contained 5 µL of DNA template, 2.5 µL PowerPlex® ESX or ESI 5X Master Mix, 1.25 PowerPlex® ESX or ESI 10X Primerpair

Mix, and 3.75 µL diH_2O providing a total reaction volume of 12.5 µL. Each PCR reaction for high concentrated samples contained 2 µL of DNA template and 3 µL extra diH_2O compared to the PCR reaction with low DNA concentration samples in order to provide the same reaction volume of 12.5 µL.

The thermal cycling process for the PowerPlex® ESX and ESI 16 kits was performed with a GeneAmp® PCR System 9700 from Applied Biosystems with the following cycling parameters: incubation for 2 minutes at 96 °C which was followed by 30 cycles of 30 seconds at 94 °C, 2 minutes at 59 °C, and 90 seconds at 72 °C; and concluded with 45 minute incubation at 60 °C. A final hold at 4 °C was added until samples were removed.

2.6.2 Sex determination

The method for sex determination used in this research relied on a Y-specific PCR product. The presence or absence of a small fragment of the amelogenine gene can give an indication for sex. The amelogenine gene is involved in the development of enamel in teeth and is found on both the X and Y chromosomes. However, a difference between the X chromosome and Y chromosome versions of the amelogenine gene is present. The amelogenine X gene is six base pairs shorter in comparison with the amelogenine Y gene. This difference in size and sequence enables it to be used for sex determination. The primers that have been used for the amplification of the specific regions on the gene target fragments with a length of 106 bp and 112 bp for the X and Y chromosomes respectively (Sullivan *et al.* 1993). The presence of the Y allele after PCR amplification strongly suggests that an individual is male. However, the absence of the allele does not unquestionably mean that the individual is female. The absence can also be the result of the low quantity or quality of DNA within the sample or due to the presence of inhibition factors (Stoneking 1996). When auSTR profiles contained less than five typed markers in every single ESX or ESI analysis, the absence of the Y allele for the amelogenine marker could not give a reliable indication the individual was female. Therefore, the sex of these individuals will be referred to as ‘indeterminate’. Based on the Quantifiler® Duo DNA Quantification and amelogenine results a selection was made for additional STR analysis on specific Y-chromosomal loci.

2.6.3 Powerplex Y23

After the PowerPlex® ESX 16 analysis and PowerPlex® ESI 16 analysis were performed on all the samples, a good indication was provided which samples were from male individuals. Thereafter, the PowerPlex® Y23 was applied to all individuals of the Oldenzaal samples which yielded an Y chromosoom according to the Y quantification and amelogenine results,

in order to demonstrate possible patrilineal relations. The PowerPlex® Y23 was applied in twofold in order to confirm the results of the previous, following the manufacturer's recommendations (except half the total reaction volume was used). The PowerPlex® Y23 kit contains the following 22 loci: DYS576, DYS389I, DYS448, DYS389II, DYS19, DYS391, DYS481, DYS549, DYS533, DYS438, DYS437, DYS570, DYS635, DYS390, DYS439, DYS392, DYS643, DYS393, DYS458, DYS385 a/b, DYS456 and Y- GATA- H4. The ySTRs are located on the non-recombining part of the Y chromosome and therefore occur only as single alleles. However, the STR locus DYS385 consists of two linked STR subloci and reveals two peaks in the DNA profile. The two fragments of this polymorphic marker can be considered as a single allele (Schneider *et al.* 1998).

Each PCR reaction contained 5 µL of DNA template, 2.5 µL PPY-23 5X Master Mix, 1.25 PowerPlex Y-23 10X Primerpair Mix, and 3.75 µL diH_2O providing a total reaction volume of 12.5 µL. The thermal cycling process for the PowerPlex® Y23 kit was also performed with a GeneAmp® PCR System 9700 from Applied Biosystems with the following cycling parameters: incubation for 2 minutes at 96 °C which was followed by 30 cycles of 10 seconds at 94 °C, 1 minute at 61 °C, and 30 seconds at 72 °C; and concluded with 20 minute incubation at 60 °C. A final hold at 4 °C was added until samples were removed.

2.6.4 Sequencing STR alleles

Following the amplification of the targeted STRs, the PCR products were separated by fragment length in order to make a clear differentiation between the various alleles. From each sample 1 µL was diluted in 11 µL diH_2O at which 1 µL CCS ILS-500 Orange internal size standard was added for the Powerplex® ESX and ESI kits, whereas the Powerplex® Y23 kit made use of the ILS600 Y23. Prior to the separation process, rapid heating to 95°C and snapcooling on ice was performed to denature the DNA. An allelic ladder containing all the STR alleles that can be amplified with the specific kit, was added to enable the confirmation of PCR products with the ladder and for monitoring the system resolution. Finally, positive and negative controls were added to examine the automatic genotyping steps performed by the software (see 2.6.4) and to detect possible contamination. The samples were analyzed with an ABI PRISM® 3100 Genetic Analyzer from Applied Biosystems using POP-4 polymer and a 36-cm capillary array (see Appendix II for an extended description of the ABI 3100 Genetic Analyzer). The PowerPlex® ESX, ESI 16 and Y23 kits enable the amplification of various loci in a five-color detection platform using four channels for the PCR products and the fifth channel for the size standard (Hill *et al.* 2011).

2.6.5 Data analysis

The length variants of the amplified STRs were visualized and genotyped using the GeneMarker 2.4.0 software (SoftGenetics LLC, State College, Pennsylvania, USA). Prior to the manual examination of the genotype information, the internal size standard had to be checked to make sure that all the peaks were detected. The results were checked for undefined and incorrect peaks made by the genotyping software. In addition to the edited alleles, stutter peaks were removed from the final data output. These can be defined as PCR amplification products which are one repeat unit shorter or longer than the main allele and is caused by strand slippage. Stutter peaks of tetranucleotide repeat loci are often recognizable for their intensity is generally below 10% of the main band (Walsh 1996). However, with the degradation of ancient DNA, complicated situations can occur where it is not clearly interpretable if a low peaks represents an actual allele or stutter peak. Strict guidelines for data interpretation were determined to minimalize individual bias issues. Therefore, a peak detection threshold was established. Alleles which could possibly be attributed to stutter or technological artifacts were considered as 'uncertain alleles'.

Observation of additional peaks

In evaluating the data, it must be determined whether the source of the DNA in the archaeological sample is from one or more individuals. The occurrence of additional peaks in the DNA profile can be an indication for contamination. However, additional peaks can also be the result of technological artifacts or have a biological cause.

2.6.6 Consensus Profiles

In order to exclude possible contamination and limit the possibility of including false alleles in profiles, especially when testing low amounts of DNA, multiple PCR amplifications are performed and a consensus profile was developed. The consensus profiles were constructed for each of the replicate samples from the ESI and two ESX series of reactions. An allele had to be seen at least once in the two separate STR profiles of one particular sample to be confirmed, and therefore included as a reliable allele in the consensus profile. Alleles which were interpreted as uncertain alleles, because they could possibly be attributed to stutter or technological artifacts, were excluded from the consensus profiles.

2.7 Reconstructing family relations

2.7.1 Statistical calculation of the match significance

Following the confirmation of the autosomal and Y-chromosomal STR profiles of each individual from the 'family grave' and matrix A, the profiles have to be compared to each other to reconstruct possible family relations. Statistical methods have to be applied to provide more insight into the relevance of the resemblance for a possible biological relationship. The relevance of the resemblance between two or more individuals is based on the mutation rate of the genetic marker loci and the allele frequencies within a specific population.

The mutation rate of the different STR markers is a factor which influences the relevance of a match. The mutation of a specific genetic marker locus between two generations will result in a false exclusion, due to the occurrence of differences between parent and offspring. Nevertheless, a higher mutation rate also implies more variability within the locus. A higher degree of polymorphism means a higher level of discrimination between individuals, which is useful for kinship analysis (Kayser and Sajantila 2001).

When two individuals share alleles which are common within the population, the possibility that the samples match by chance increases. Therefore, the allele frequency determines the rarity of a DNA profile. Two different databases which include allele frequencies were used for the statistical analysis with autosomal STR data: a database with allele frequencies from present-day people in the Netherlands (Westen *et al.* 2014) and an allele frequency database constructed from all typed alleles of the archaeological samples from Oldenzaal. It is unlikely that both databases represent the exact allele frequencies at any point in time the cemetery was in use. The degree to which the individuals, where archaeological samples were collected from, represent the population of Oldenzaal and the surrounding area is unclear.

It is also unclear to what extent the variability of the modern database has changed as a result of mutations and the migration of people. In addition, the modern database provides a representative overview of the allele frequencies from the entire contemporary Dutch population instead of only a relatively small population such as Oldenzaal. This might also influence the allele frequency, as well as the variation in sample size used to construct the databases. However, these two databases are the only available datasets useful for this analysis. The use of these two databases might also provide interesting information regarding the variation that occurs between the results of both databases, such as a change in the genetic

composition of a population over time. This change can be the result of migrations or mutations.

2.7.2 Relpair v2.0.1

For the statistical analysis in this research, the Relpair v2.0.1 software was used to calculate the likelihoods of family relationships based on the obtained autosomal STR profiles. This analysis has only been performed with the alleles from the consensus profiles. Incomplete STR profiles, due to degradation of the ancient DNA, will result in less powerful profile matching. Therefore, the minimum number of genotyped markers was set to eight. As a result of this threshold, individuals V3038 (6 markers) and V2904 (7 markers) from matrix A were not incorporated in the statistical analysis.

The Relpair v2.0.1 software tests for eight different relationship types: unrelated (UN), monozygotic twins (MZ), full siblings (FS), half siblings (HS), parent-offspring (PO) and second-degree relatives such as grandparent-grandchild (GG), avuncular (AV) and first cousins (CO) (Epstein *et al.* 2000). The only family relation that can be reconstructed with certainty is the parent-offspring relationship. Because offspring will inherit half of their parent's DNA, it is statistically possible that two children inherit the exact opposite half of the genetic information from their parents and as a result do not share any alleles. Therefore, the statistical calculations regarding the relationship other than parent-offspring between two individuals yield no reliable results. However, on average the DNA of biologically related individuals will match more compared to the DNA of unrelated people. For this research it was decided to incorporate the other scenarios as well. The statistical analysis consists of a calculation of the probability and likelihood of the different relationship types between all possible pairs from and between the 'family grave' and matrix A. The software requires three input files: a control file, locus file and pedigree file.

Control file

The software requires an input of the assumed per-allele genotyping error rate of the obtained data. Since ancient DNA is often highly degraded, alleles can be missing, resulting in originally heterozygote markers appearing as homozygote markers (Caragine *et al.* 2009). This can influence the calculated likelihood of relationship between two individuals as the software cannot make distinctions between an individual with a homozygous marker and an individual with a homozygous marker which possibly misses an allele. Therefore, the

incorporation of a one, two and five percent genotyping-error rate was performed for this statistical analysis.

Besides the error rate the software requires additional parameters for the control file. The critical value is a parameter that determines the minimum level of the likelihood ratio in order to be reported in the output. This likelihood ratio depicts the number of times it will be more likely that matching DNA profiles are the result of a biological relationship than by chance. The likelihood ratio will depend on the allele frequencies in the different databases. For this statistic analysis it was decided to use a critical value of 1000 following the standards used by the FLDO. However, there are no strict forensic guidelines for this value. The minimum number of shared genotyped markers between two individuals, and the allele frequencies are also incorporated into the control file and was set at six and eight for varying parameters.

Locus file

The locus file contains information regarding marker names and positions on the autosomal chromosomes, as well as allele names and frequencies. The used markers influence the calculation based on the frequency in which they occur in a given population. The position of the markers on the chromosomes can have an impact on the likelihood because of the linkage disequilibrium phenomenon. This means that markers which are located close to each other are more likely to be inherited together (Jobling *et al.* 2013, 140). Therefore, most markers used in this research were located on different chromosomes.

Pedigree file

The pedigree file lists gender, possible parent and monozygotic twin status and genotypes. However, because there is no a priori information available concerning possible parents and the status of twinship this is left blank. Relpair v2.0.1 shows the likelihood ratio of the inferred relationship over the assumed relationship. For this research the assumed relationship is always considered unrelated.

2.7.3 Familias v3.0

Because the statistical analysis performed by the Relpair v2.0.1 software did not take the mutation rates into account, the Familias v3.0 software was used for an additional calculation of the probabilities and likelihoods of possible family relationships. The software requires the following input files: the General DNA data file with the allele systems, the Persons file with gender and age, Known Relations with the already known relations, Case Related DNA Data

file with the genotypes of the individuals for all available alleles, the Pedigrees file and finally the DNA measurements and mutation parameters. The Persons and Known Relations files were not possible to enter because there was no data available concerning the age, gender and already known family relations for all the individuals. For this statistic analysis it was decided to lower the critical value to 500 in order to test for additional relationships and compare the results with the Relpair data.

2.7.4 Harris matrix analysis

The individuals from the 'family grave' were interpreted as having possible family relations because they were closely positioned to each other in a single burial context. Once kinship relationships in matrix A were obtained from the statistical analysis, possible spatial associations within the cemetery of these biologically related individuals can also be examined in order to provide an additional indication for a kinship relation between two individuals. The Harris matrix from matrix A was used for this purpose.

3 Results

3.1 PCR Results

3.1.1 Results of the Quantification test

The Applied Biosystems 7500 Real Time PCR System with the Quantifiler® Duo kit yielded no matches for the 'family grave' samples V1215, V1237 and V1282. The male specific sex-determining region SRY yielded no matches for all the 'family grave' samples. Because all the quantification results of the 'family grave' samples yielded a DuoHuman concentration lower than 0.02 ng/µl, it was decided that all the PCRs were performed with an input of 5 µl DNA extract for every sample from the 'family grave'.

Both detectors of the Quantifiler® Duo kit yielded no results for 17 samples from matrix A. Twenty-nine samples show a concentration for the DuoMale sex-determining region SRY. The quantification results of the samples from matrix A yielded a DuoHuman concentration lower than 0.02 ng/µl for 54 samples. It was decided that the PCRs for these low concentration samples were performed with an input of 5 µl DNA extract. For the remaining 27 samples with a concentration higher than 0.02 ng/µl it was decided that the PCRs for these samples were performed with an input of 2 µl DNA extract. The negative controls of the extractions from the samples of the 'family grave' and matrix A showed no DNA concentration for both detectors of the Quantifiler® Duo kit.

3.1.2 The Powerplex ESX 16 and ESI 16 results

The positive controls gave the expected results. The negative controls of the PCRs as well as the negative controls of the extractions showed no results. This provides an indication for the absence of contamination during the lab procedures. However, this cannot rule out the possibility that contamination is present in one of the samples. Extreme differences in allele peak heights within one sample and the occurrence of additional alleles can also give indications for possible contamination. Extreme allele peak height variation within the DNA profiles of each sample did not occur. However, sample V1100 from matrix A has an additional allele at marker D18S51 but this can probably be attributed to allelic drop-in caused by a technological related artifact or biological cause. However, over the entire dataset from Oldenzaal no STR profiles had multiple markers with more than two alleles. All the STR profiles showed a decrease of the amplification product with the increase of the

fragment length. This is a clear indication for degraded DNA and contributes to the authenticity of the results (figure 7).

In addition, no clear resemblances were demonstrated with the DNA from the laboratory staff. Based on these results, all the DNA profiles could be considered authentic and reliable and therefore were defined as from a single contributor.

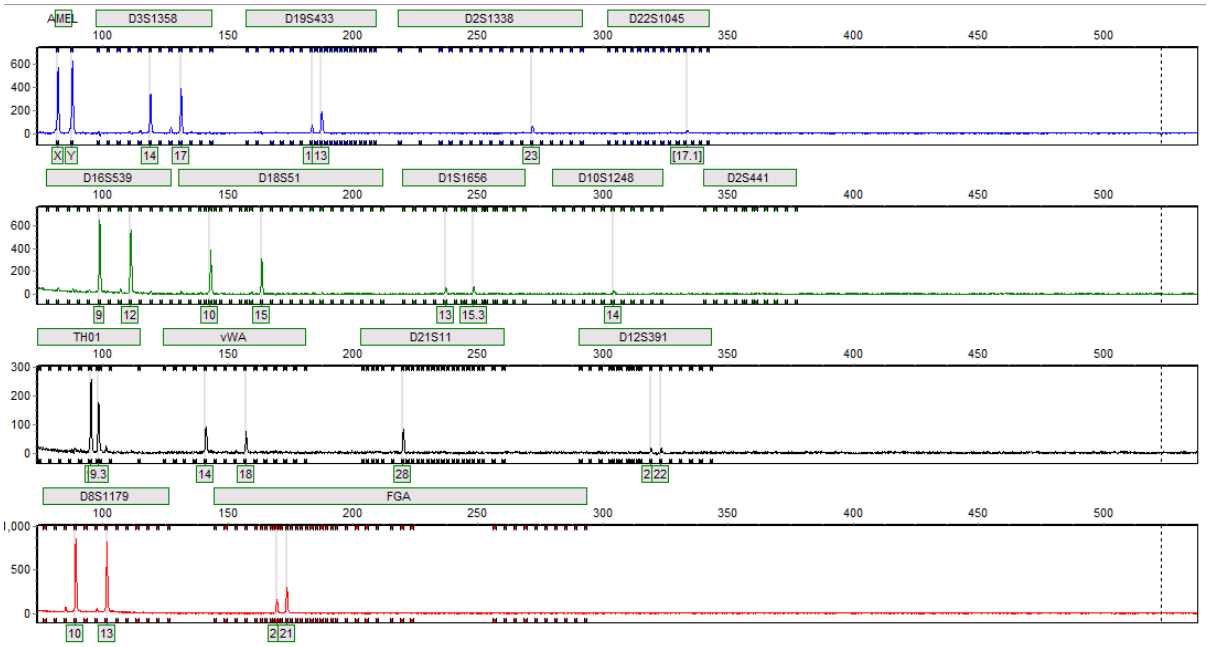

Figure 7. ESI analysis with the autosomal STR profile from sample V0870, viewed using GeneMarker 2.4.0. Each peak represents the amount of amplification product for a specific allele, with an increase in fragment length from left to right.

For the ‘family grave’, the Powerplex ESX and ESI results yielded the full set of 16 auSTR markers for the samples V1286, V1282 and V1307. For the samples V1215 and V1214 a total of 15 auSTR markers could be typed and for sample V1237 only 13 auSTRs. The amelogenine results of the Powerplex ESX and ESI demonstrated that samples V1215 and V1237 were from male individuals. The auSTR consensus profiles of the ‘family grave’ samples can be found in table 1.

For matrix A, the Powerplex ESX and ESI results yielded the full set of 16 auSTR markers for 68 samples; 84% of the total analyzed samples from matrix A. For five samples a total of 15 auSTR markers could be typed and eight samples yielded 14 or less typed auSTRs. The amelogenine results of the Powerplex ESX and ESI demonstrated that 35 samples within

Table 1. The consensus profiles of the individuals the 'family grave'

Samplenr.	Amelogenine	D1S1656	D2S1338	D2S441	D3S1358	D8S1179	D10S1248	D12S391	D16S539	D18S51	D19S433	D21S11	D22S1045	FGA	TH01	vWA
V1214	X	12-16	{18}	13-14	16	12-14	14	18-18.3	10-{13}	{12}-13		30	16	21-{25}	9.3	17-19
V1215	X-Y	12		11-14	14-15	11-13	15-16	21-22	9-13	13	{13}-{14.2}	28-31	11-16	20-{21}	6-8	16
V1237	X-Y	{12}		{12}-{14}	9.3	12-14	12		{11}-{12}	{12}	{13}	31			9.3	
V1282	X	11-12	{17}-24	10-14	14-16	13	14-15	18.3-19	{11}-{12}	{13}	{13}-{14}	31.2	15-16	{21}	7-9	14-17
V1286	X	11-18.3	23-{24}	10-13	15-17	8-13	13	15-19	9-12	{12}-{13}	{15}	31.2	12-16	{21}-{22}	{9}-9.3	14-16
V1307	X	12-17.3	{24}-{25}	11	16-18	12-14	15	17.3-18	13	15	{15}	30	11-15	24-{25}	6-9	16-17

An overview of the autosomal STR Markers from the 'family grave' individuals. The alleles enclosed by {} denote the alleles which were picked up in a single ESI or ESX.

matrix A were from male individuals. The auSTR profiles of the individuals V1166, V2381, V2904 and V3038 contained less than five typed markers in every single ESX or ESI analysis and were therefore considered indeterminate. The 42 auSTR profiles which did not yield an Y allele for the amelogenine marker were considered to be from female individuals. The auSTR consensus profiles of the 81 samples from matrix A can be found in table 2.

All the samples from the 'family grave' and matrix A revealed a decline in amplified STRs with an increase in fragment length, which corresponds with the degradation of the DNA into smaller fragments (Handt *et al.* 1994, 527).

3.1.3 The Powerplex Y23 results

The two male individuals within the 'family grave' yielded for the total 22 ySTR markers only nine typed ySTR markers for sample V1215 and two for sample V1237. Besides the low number of typed ySTR markers, only the ySTR marker DYS635 contained results for both samples. The two samples share the same allele at this marker. However, the possibility to compare only one marker between these two male individuals will provide no reliable indications for a possible patrilineal relationship. The ySTR consensus profiles of these two 'family grave' samples can be found in Appendix I.

For eight of the 35 male individuals from matrix A all 22 markers were typed. The samples V1233, V1350, V1376 and V1420 contained less than six typed markers and were therefore not incorporated in the comparison analysis between the various ySTR profiles. The comparison of the ySTR profiles yielded two possible patrilineal relationships: V0218-V0922 with nine shared alleles and V1443-V1509 with 12 shared alleles.

Table 2. The consensus profiles of the individuals from matrix A

Samplenr.	Amelogenine	D1S1656	D2S1338	D2S441	D3S1358	D8S1179	D10S1248	D12S391	D16S539	D18S51	D19S433	D21S11	D22S1045	FGA	TH01	vWA
V0218	X-Y	14-16.3	{17}-19	10	16-18	12-13	15	17.3-21	{13}-14	12-15	14-16.2	30-31.2	15	23-27	9	17
V0256	X-Y	16	20-24	10-12	15	10-13	13-14	19-20	11	12	13-16	31-32.2	11-16	20-22	6-9.3	15-17
V0449	X	15-16	17-19	10-12	14-16	13-14	15	18-20	11-12	12-14	13-15	29-30	15	23-25	8-9.3	17-19
V0700	X-Y	16-18	18-{26}	11	16	13	14-16	17-21	{12}-{13}	{16}-21	13	29-30	11-14	19-21	9.3	14-15
V0781	X-Y	11-15	20-26	11	15	11-13	13-14	17-22	11	13-15	14	31	11-14	19-26	8-9.3	17
V0863	X	15-15.3	{23}	11-14	15-16	12	13-14	17.3-22	{11}-{13}	{12}-14	13-14	28-30	15-16	20-23	6-9.3	14-16
V0865	X-Y	12-13	20-25	11	18	13-14	14-15	18-21	9-11	14-19	15-16.2	28-29	11-16	22	6-7	14-17
V0870	X-Y	13-15.3	23-25	11	14-17	10-13	13-14	21-22	9-12	10-{15}	12-13	28	15-17	20-21	9-9.3	14-18
V0899	X-Y	11-17.3	17-25	11-11.3	16-17	10-13	13-14	17.3-22	10-11	11-17	13-16	29-31.2	16	23-24	7-9.3	16
V0922	X-Y	{15}-15.3		10	15-16	12-13	13-15	16-21	11-13	12-15	13-16.2	{30}	15-16	23-27	9	17-19
V1100*	X	11-16.3	{20}-21	14	14-15	13-14	14-15	24-25	9-11	{14}-17-19	15	29-30	11-15	19-20	7-9.3	18
V1101	X	12-18.3	{17}-18	14	14	11-13	13-15	18-19	11-12	16-17	14	29-30	11-15	20-23	6-9	17-19
V1104	X-Y	14-18.3	17-20	11	15-18	12-14	13-15	18-22	11-12	14-18	13-14	30	11-15	21-27	7-9.3	18
V1105	X-Y	16-17.3	20-23	10	15-18	9-15	14-16	17-19.3	9-11	12-14	13-14	30	{15}-16	21-22	8-9.3	14-15
V1123	X	15.3-18.3	19-20	11.3-15	15-16	9-12	13-16	18-23	11-13	12-13	13	27-30	14-16	22-24	9-9.3	16-18
V1147	X	11-17	17-24	10-11	18-20	10-13	14	17.3-20	11-12	13-19	14-15	32.2	11	23-24	6-9.3	16-20
V1166	X			10	15	12-{14}	15		13			{30}			{9}	{17}
V1201	X-Y	15-18.3	20-23	13-14	16-17	13-14	13-15	20-21	9-12	{12}-15	13-14	30-31	15-16	20-24	6-8	16-18
V1229	X	11-18.3	17	11-12	16	15-16	13-14	18-24	13	12-14	12-15	29-32.2	15	21-23	6-8	18-19
V1233	X-{Y}	{18.3}		11.3-14	14-16	{11}-{12}	13		{12}	{13}	{14}	{30}-{31}	{15}-{17}		{9}-9.3	
V1274	X	{16}		11-14	17-{18}	13-{14}	15	{18}-{21}	11-12	{14}-{17}	{14.2}	{32.2}	15-{16}		7-9	{15}-18
V1278	X-Y	11-16.3	{19}	12-14	17	10-12	13	15-20	{10}-11	14-16	13-15.2		15-16	20-25	7-9	16
V1293	X-Y	16	{18}-25	10-11	15-17	10-11	13-14	17.3-18	10-12	13-19	14	31-31.2	16-17	24	7	15-17
V1297	X	16-18	16-18	11-14	14-17	13	13	18-22	11-12	17-{21}	15-16	30	15-16	23-24	9-9.3	18-19
V1320	X-Y	12-16	{17}-{19}	11-14	18	10-14	15-17	16-21	{10}-11	17	14-15	29-30	16	22	7-9	15-19
V1350	Y	17.3		11-14	{15}-{18}	{15}	{15}	{21}-23	12-{13}	14	{15}	{29}-30	{15}	{25}	7-9.3	14-17
V1376	X-{Y}	{18.3}	17	11-15	{16}-17	13		{17.3}-22	{12}-13	{14}			11-{16}	{22}	{8}-{9}	14-{19}
V1388	X-Y	12-18.3	18-23	10-14	14-18	13	15-16	17.3-19	11-12	16-17	14	29-30	11-17	24-26	6-9.3	17-18
V1400	X	15.3-17.3	20-23	11-14	15-16	13	14-15	20.3-24	12	14-19	14-15	28-32.2	16	21-24	6-9.3	14-18
V1408	X	12	21-24	11-12	14-18	8-14	13	19-19.3	9-12	16-18	14-16	28-32.2	11-16	22	8-9.3	14-16
V1416	X	14-15	21-{25}	10-11	15	12-{13}	14-16	22-24	12-13	13-15	13	28-32.2	11-16	{24}-25	7-8	15-16
V1420	X-Y	12-{17.3}		{11}-12	16	11-12	13-14	17-18	11-13	12-16	12	{30}	11-16	20	6-9.3	15-18
V1435	X	12-18.3	18	14	14-16	13	15-16	18-19	9-12	16-17	14-16	27-28	15-17	24-26	6-9	17-19
V1436	X-Y	16.3-18.3	19-25	13-14	16-17	13-16	13-14	20	12-13	13-19	13	27-28	15	20-22.2	9	17-19
V1443	X-Y	12-15	20-{25}	11	14-17	10-13	14-16	16-22	11-12	16-{18}	13-{14}	28-31	11-16	20-{27}	6-7	17-18
V1454	X	17.3-18	19-23	14	17	10-12	16	20	12	16-17	14-15.2	28-30	11-16	21	6-8	15-16
V1476	X	12-15	19-25	11-14	14-16	12	14-16	16-22	12-13	15-16	14-15	27-31.2	11-15	22-27	6-9.3	17
V1484	X	17-17.3	17-20	11	16	12-13	15-16	21-22	9-12	14-15	13-13.2	31-31.2	16	21	9-9.3	17
V1488	X	12-17.3		11-11.3	16-18	12-13	14-18	18	11-12	{14}-16	15.2-16	30-31	11-17	22-23	8-9	15-19

V1509	X-Y	14-16.3	16-21	10-11	15-16	12-15	13-15	16-22	11	12-14	13-16.2	29-30	15-16	20-25	8-9	15-16
V1513	X	13-17.3	20-21	10-14	17-18	10-14	14-16	19	11-12	14-16	13-14	27-30	16-18	{23}-25	9.3	14-18
V1517	X	11-15	{20}	11.3-14	17-20	10-11	14-15	20	9-{10}	{13}-{16}	14-15	{31}	14-15	21	8	19
V1521	X-Y	11-17.3	20	11-14	15-19	13-16	13	19-21	11-13	16-18	13-14	29-30.2	16	20-22	9.3	17
V1550	X	12-17	17-26	10-13	16	11-13	13-16	17-18	11-12	14-15	13-15	29-30	15-18	19-22	8-9.3	14-16
V1584	X	16-17.3	17	10-14	17-18	13-14	11-16	18-22	11	14-16	13-14.2	28-32.2	16	21-22	9.3	15-16
V1585	X	15-17.3	{17}-20	10-14	14	12	14-15	19.3-23	11	12-20	14-{15}	28-29	15-16	22-23	9-9.3	17-18
V1673	X	15-17.3	17	10-12	15-16	10-14	13-15	22	10-12	10-14	14-14.2	31-31.2	16-17	21-{24}	7-9.3	15-18
V1682	X-Y	15.3	17-20	11	15-16	13-15	11-14	17-19	12-14	13-16	14-16.2	31-33.2	15	17-22	8-9.3	15-17
V1812	X-Y	15.3-18.3	16-17	11-15	15-17	13	14-15	15-18	10-13	13-15	12-14	28-29	11-16	19-23	6-9	17-18
V1863	X	12-17.3	17-24	11	16	8-12	15	17-18	13	14-15	11-14	28-30	15-16	20-24	7	17-19
V1901	X-Y	12-13	24	10-14	18	11-15	14	17-21	12-13	14-17	14	30	11-16	21-24	9.3	16-17
V1990	X	12-14	17-19	10-13	15-16	13	14	18-23	11-12	13-16	14-15	28	15-16	19-21	8-9.3	14-19
V2003	X-Y	12-18.3	20-21	11	16-18	12-15	13-15	21-23	10-12	12-15	15-16	31.2	16	21	7-9.3	17
V2017	X	12-16	{19}-{25}	11	14-15	14	13-16	{18}-20	10-11	12-19	{14}-15	30	15	{24}	8-9.3	16-17
V2061	X-Y	11-14	20-24	11-15	14-17	10-14	13	17-17.3	{11}	{14}-{21}	14	28-39	14-15	{21}-{23}	8-9	15-16
V2064	X	11-17.3	18-23	10-11	15-17	10-12	14	15-22	10-12	13	14-15	31.2-32.2	15-18	23-26	9	15-16
V2076	X	15-16	23-25	11-14	14-15	13-14	13-14	18	12	12-13	13-{15.2}	27-29	14-16	23	8	18-19
V2119	X	12-16.3	17-20	11-14	15-17	13-15	13-15	15-19	10-11	13-17	13	27-28	11-16	22-24	7	16-19
V2127	X-Y	17.3-18.3	{19}-23	11-13	16-17	14-15	13-14	23-27	12-13	14-18	13-14	30-31.2	11-16	20-21	6-9.3	17-19
V2196	X	15.3-17	18-24	10-11	16-18	11-13	13-16	21-23	9-12	12-19	14.2-15	28-32.2	15-16	20-22	9.3	14-17
V2205	X	12-17	17-20	11-14	16	12-16	13	21-22	9-14	12	14-15	28-{30}	15-16	20-21	9.3	19
V2268	X-Y	12-14	17-21	11-15	16-18	12-{14}	13-17	18	11-12	{16}-{17}	14-16	30-30.2	11-18	{22}-{23}	6	17-19
V2293	X-Y	12-15	19-{24}	11-14	15-18	13-14	13-14	20-21	11-12	13-16	14-15	26-31	14-17	18-23	8-9.3	16-17
V2318	X			{14}	{14}-{15}	{14}		{17}	{12}			{31}			{9}	
V2362	X	11-17.3	17-21	14	18	15	13	17.3-18	11-13	14-19	14-16	28	11-15	19-24	6-9	14-16
V2367	X	13-16.3	24	11-15	14-18	12-16	12-13	17-23	11-{12}	14-17	13-14	30-32.2	15-16	22-24	8-9.3	18-19
V2399	X	14-17	20-22	11.3	17	13	14-15	18	11-13	19	14	29-31.2	15-16	19-22	8-9.3	15-18
V2470	X	11-16	17-20	10-14	14-17	13	14-17	18-20	11	14-19	15	30.2	11	22-25	9.3	17
V2503	X		17	{11}-14	16-17	14-15	{13}-{16}	16-22	11		{14}-{15}		{11}-{17}	20	9.3	{17}
V2554	X	11-14	19-21	11-14	14	8-13	15-16	15-17	12-13	12	13-15	28-30	15-17	20-21	7-9.3	18-19
V2558	X-Y	17-18.3	17	11-11.3	15-16	13-15	13-14	22-23	9-11	13-16	13.2-16.2	28-32	14-17	24	6-9.3	17-18
V2643	X	11-17.3	19-24	11-14	17	13-15	13-16	23-25	10-11	15-17	14-15	27-30	15-16	20-26	6-9.3	15-17
V2686	X-Y	11	23	10	15-16	12-15	13-16	21-{22}	9-10	15-{19}	12-14	29	11-15	20-23	8-9	15-19
V2729	X	16.3-19.3	{17}-{24}	10-11	16-17	15	13-16	16-22	{9}-11	14-17	{13}	28-31	11-17	{20}-{25}	6-8	17
V2737	X	15-17.3	20-23	11-15	15-17	11-13	13-16	18.3-22	11-12	13-17	13-14.2	28	11-16	22-23	8-9	17
V2748	X	15-16	17-20	14	17-18	9-13	14-16	16-19	11-12	13-15	12-14	30-31	15-16	20-21	5-9	14-19
V2761	X-Y	13-14	17-18	11-14	11-17	13-15	13-16	17-18	12	16-17	14-15	26-28	15	20-24	9.3	15-16
V2873	X-Y	12-19.3	17-{21}	1011.3	14-18	14-15	13-16	23	11-12	14-23	14-16	30	15-16	20-{27}	8-9.3	16-17
V2893	X	11-15	{21}-{23}	14	15-17	12	13-15	17-18	11	12	13-17	28-31.2	11-17	22-23	9-9.3	17
V2904	X								{13}	{12}	{15}	{30}		24		
V3038	X			{11}			16			17		{32}				

An overview of the autosomal STR markers from the individuals within matrix A. The alleles enclosed by {} denote the alleles which were picked up in a single ESI or ESX. (* an extra allele in profile)

Because the pairs contained incomplete profiles, the amount of shared alleles is equal to the amount of typed markers of the most incomplete profile. Nevertheless, two individuals that have a similar ySTR haplotype are not necessarily related to each other. The ySTR consensus profiles of the samples from matrix A can be found in Appendix I.

3.2 Potential Kinship Relations

3.2.1 Relpair v2.0.1 and Familias v3.0 statistical results

Relpair data

Six possible full sibling relationships and one parent-offspring relationship have a likelihood ratio above 1000 for all the various parameters with the allele frequencies from Oldenzaal. An additional full sibling relationship was added to the results when the allele frequencies of the modern day people from the Netherlands were used. The likelihood ratio results are depicted in table 3 for the Oldenzaal allele frequencies and table 4 for the modern day allele frequencies from the Netherlands. The results yielded no differences between a minimum number of six and eight shared genotyped markers between all possible relationships at varying assumed genotyping error rates of one, two and five percent. Marker D18S51, which has an additional allele in the auSTR profile of sample V1100, was left blank in the Relpair statistical analysis to avoid the incorporation of a false allele.

Table 3. The statistical results from Relpair v2.0.1 based on the Oldenzaal allele frequencies

relationship pair	possible relationship	RUN 4		RUN 7		RUN 1		RUN 9		RUN 5		RUN 11	
		6*	1%**	8	1%	6	2%	8	2%	6	5%	8	5%
V0922 -V0218	FS	475859		475859		446001		446001		358939		358939	
V1388-V1435	FS	153424		153424		145452		145452		121088		121088	
V1435-V1101	FS	59958		59958		60035		60035		57630		57630	
V2503-V2729	FS	12833		12833		12941		12941		12714		12714	
V1388-V1101	FS	4278		4278		4102.4		4102.4		3540.8		3540.8	
V1166-V0922	PO	3197.3		3197.3		2903		2903		2169.1		2169.1	
V1166-V0218	FS	1438.6		1438.6		1404.9		1404.9		1280.8		1280.8	

An overview of how many times more likely one possible relationship is over the assumed unrelated scenario (FS = Full siblings and PO = Parent-offspring).
*The minimum number of shared genotyped markers between the pairs.
**The assumed genotyping error rate.

Table 4. **The statistical results from Relpair v2.0.1 based on the modern day allele frequencies from the Netherlands.**

relationship pair	possible relationship	RUN 3		RUN 8		RUN 2		RUN 10		RUN 6		RUN 12	
		6*	1%**	8	1%	6	2%	8	2%	6	5%	8	5%
V0922 -V0218	FS	> 10^6		> 10^6		> 10^6		> 10^6		> 10^6		> 10^6	
V1388-V1435	FS	316459		316459		298915		298915		246025		246025	
V1435-V1101	FS	153521		153521		153038		153038		144922		144922	
V2503-V2729	FS	37302		37302		37353		37353		35935		35935	
V1388-V1101	FS	10296		10296		9807.7		9807.7		8299		8299	
V1166-V0922	PO	5639		5639		5101.2		5101.2		3834.7		3834.7	
V1166-V0218	FS	4409.8		4409.8		4281.5		4281.5		3769.1		3769.1	
V1476 -V1443	FS	1627		1627		1728.4		1728.4		1979.7		1979.7	

An overview of how many times more likely one possible relationship is over the assumed unrelated scenario (FS = Full siblings and PO = Parent-offspring).
*The minimum number of shared genotyped markers between the pairs.
**The assumed genotyping error rate.

The results yielded two scenarios in which three individuals show relationships with each other. Individuals V1101, V1388 and V1435 are all related to each other as full siblings. Individuals V0218, V0922 and V1166 show an unlikely scenario in which individuals V0922 and V1166 have an parent-offspring relationship and the rest of the pairwise comparisons yield full sibling relationships.

Familias data

As with the statistical results from Relpair, the results from Familias yielded only two different scenarios: full-siblings and parent-offspring. Compared to the Relpair results, one additional pair was added when the allele frequencies from Oldenzaal were used. This pair contained the individuals V1476 and V1443 and has a likelihood ratio under 1000. This explains why it did not showed up in the Relpair results (table 5). Relationship V1388-1435 has a likelihood ratio above 500 for both the full-sibling and parent-offspring relationships. The Relpair data determined

Table 5. **The statistical results from Familias v3.0.based on the Oldenzaal allele frequencies.**

Person 1	Person 2	Relationship	LR
V0922	V0218	Siblings	402041
V1388	V1435	Siblings	134873
V1435	V1101	Siblings	31986.7
V2503	V2729	Siblings	6889.91
V1388	V1101	Siblings	3718.69
V0922	V1166	Siblings*	1736.42
V1166	V0218	Siblings	1470.05
V1476	V1443	Siblings	622.366
V1388	V1435	Parent-Child	537.473

An overview of the possible relationships with the number of times of which one possible relationship is more likely over the assumed unrelated scenario (LR =Likelihood Ratio and * = the parent-offspring relation in Relpair)

this pair only as full-siblings.

For the allele frequencies of the modern day people from the Netherlands, the Familias results yielded V1166-V1307 as an additional pair compared to the results of Relpair, of which V1307 is an individual within the 'family grave'. Similar to the results with the Oldenzaal allele frequencies, pair V1388-1435 has a likelihood ratio above 500 for both the full-sibling and parent-offspring relationships. Considering the different critical values for the likelihood ratio between the statistical analysis of Relpair and Familias, the results show the same relationships pairs.

Table 6. The statistical results from Familias v3.0.based on the modern day allele frequencies from the Netherlands.

Person 1	Person 2	Relationship	LR
V0922	V0218	Siblings	3.8383^6
V1388	V1435	Siblings	278273
V1435	V1101	Siblings	81147.8
V2503	V2729	Siblings	19563.9
V1388	V1101	Siblings	8985.45
V1166	V0218	Siblings	4534.44
V0922	V1166	Siblings*	3940.6
V1476	V1443	Siblings	3276.02
V1388	V1435	Parent-Child	517.893
V1166	V1307	Siblings	516.29

An overview of the possible relationships with the number of times of which one possible relationship is more likely over the assumed unrelated scenario (LR =Likelihood Ratio and * = the parent-offspring relation in Relpair)

3.2.2 Comparing the ySTR and auSTR results

The two possible patrilineal relations within matrix A, provided by the ySTR comparisons, can be compared to the possible family relationships from the statistical analysis with Relpair. Pair V0218-V0922 appears in the results of the statistical analysis as a possible full sibling relationship. The statistical analysis gave no likelihood ratio above 1000 for one of the eight relationship types regarding pair V1443-V1509. In summary, out of the 87 of analyzed individuals eight appear to be biological kin: seven full-siblings and one parent-offspring.

3.2.3 Spatial connections within matrix A

The individuals which are likely to have kinship relations, based on the Relpair and Familias calculations, were checked in the Harris matrix from matrix A for possible spatial connections. The burials of pairs V0922-V1166 and V1388-V1435 were directly laying on top of each other. Individuals V1443 and V1476 were also spatially closely related to each other with only one burial between them, unfortunately no DNA samples were taken from this burial (V1365). The Harris matrix from matrix A can be found in figure 8.

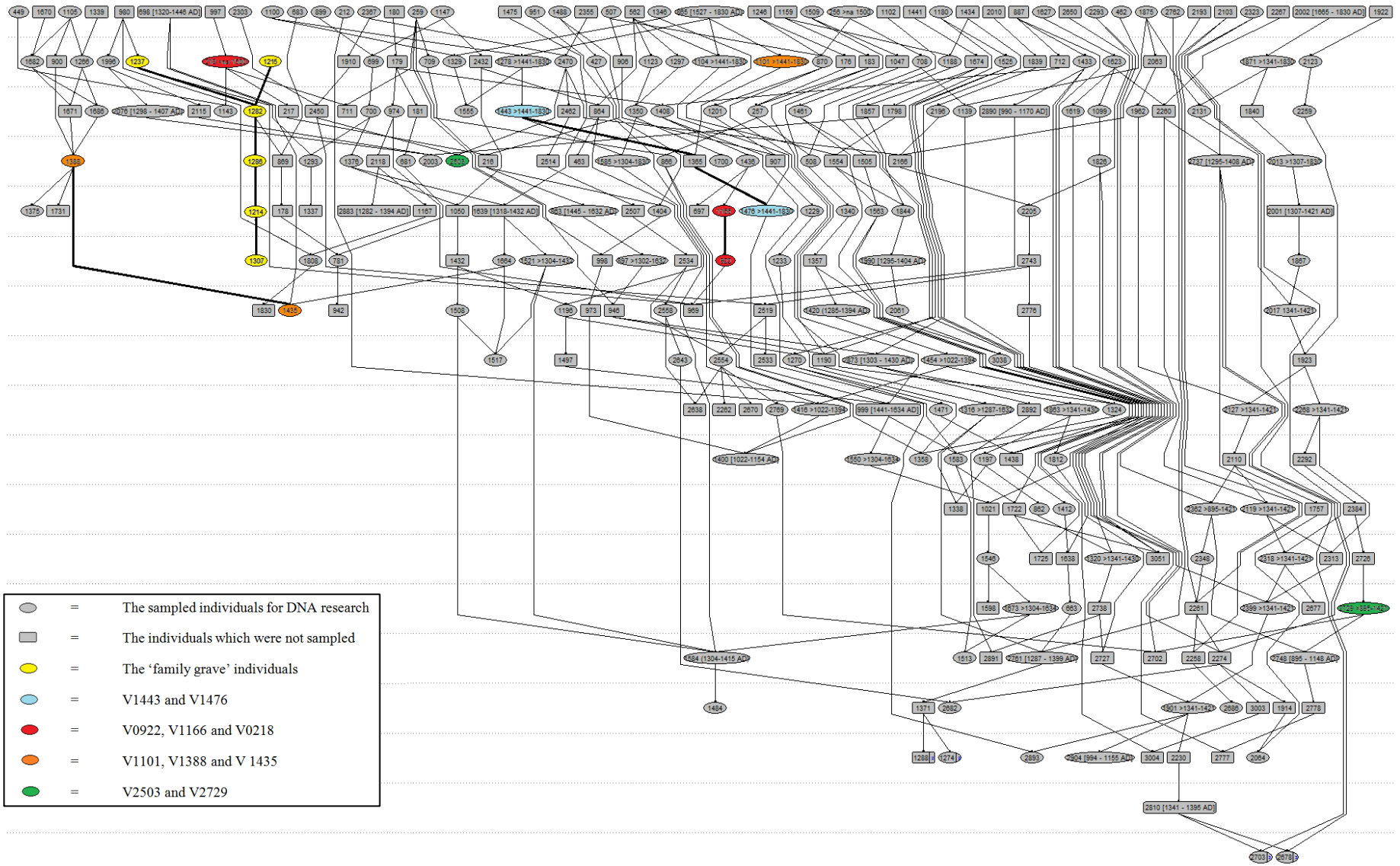

Figure 8. The Harris matrix with the relative positions of all the individuals from Matrix A; including the 'family grave' individuals.

4. Conclusion

4.1 Conclusion

Due to the absence of gravemarkers and written grave registers with names of the buried individuals at the Oldenzaal cemetery, DNA analysis was performed in order to determine whether or not the individuals found in the interpreted ‘family grave’ are related to each other. The sampled individuals from matrix A were also incorporated for the determination of possible additional kinship relationships. Because samples taken at archaeological excavations most often yield degraded DNA, precautions were taken to avoid contamination during the sampling at the excavation and extraction in the DNA laboratory. The large amount of typed auSTR and ySTR markers for the individuals from the ‘family grave’ and matrix A, revealed the relatively good quality and quantity of the ancient DNA. A third allele was picked up in one of the STR profiles, which can give an indication for possible contamination. However, considering the fact that this appeared at only one specific marker in one auSTR profile it can be assumed that this was probably the result of allelic drop-in caused by a technological or biological related artifact. Therefore, the results are considered to be authentic.

The results of the statistical analysis on the pairwise comparisons of the auSTR profiles gave no genetic indications for kinship relations between the six individuals of the purported ‘family grave’. However, the ySTR profiles of two male individuals from the ‘family grave’ did not provide enough typed markers to reconstruct a possible patrilineal relationship. Based on these results it cannot be excluded that these two individuals were biologically related to each other. Relationships between people which are not biologically, such as husband-wife couples cannot be detected with DNA analysis. However, this burial context contained six individuals which cannot all be ascribed to this relationship.

In contrast to the ‘family grave’, the results of the statistical analysis on the auSTR profiles from matrix A did produce possible kinship scenarios. At various parameters, six full sibling relations and one parent-offspring relation were reconstructed with the use of the Oldenzaal allele frequencies in Relpair. The results yielded an additional full sibling relation when the modern allele frequencies from the Netherlands were applied. This means that the two databases with allele frequencies contain sufficient amount of differences to provide different numbers of possible kinship relations.

The scenario provided by Relpair in which individuals V0922 and V1166 have a parent-offspring relationship, and the pairs V0218-V1166 and V0218-V0922 yielding full sibling relationships, is probably due to the incomplete auSTR profile of individual V1166, where seven markers did not yield any typed alleles. In Familias these three pairs are all described as full-sibling.

The Relpair results showed a full-sibling relationship for pair V1388-V1435, where Familias showed a parent-offspring relationship. However, when comparing the two consensus profiles, both individuals share no alleles for marker D21S11. Because both individuals are heterozygous for this marker, a parent-offspring relationship is unlikely.

Although the 'family grave' did not yield any genetic indications for possible kinship relationships genetic indications for kinship relations within matrix A did emerge from this research. In addition, the Harris matrix analysis revealed that three pairs of possibly biologically related individuals were spatially linked to each other; an aspect upon which the interpretation of the 'family grave' was based. Thus, in some instances at Oldenzaal family grave areas do exist, but we must be cautious to not assume all communal grave areas are composed of family members, as often they are not, not only at Oldenzaal, but at other Dutch archaeological sites as well.

4.2 Future Research

DNA research can give answers to a broad range of cultural questions behind the determination of biological similarities. Physical anthropology research is important for culturally related questions because it can provide information on sex, age-at-death, stature, ethnicity, health and disease, and possibly even the cause of death of an individual. This also enables the comparison of sex determination based on morphological features in the skeleton, with the sex determination based on DNA data. However, the physical anthropological information is still lacking at this phase of the Oldenzaal research project.

To certify the authenticity of the results, it would be necessary to reproduce the analyses with the remaining extract. MtDNA analysis will also be performed at a later stage of the Oldenzaal research project in order to determine possible matrilineal relationships.

Despite the large amount of complete STR profiles, the presence of unconfirmed alleles can influence the reliability of the results. The statistical analysis could not compare all the markers between the individuals. Therefore, additional STR analysis can be performed on the

samples which lack typed alleles at certain markers, in an attempt to confirm more alleles. This might clarify the parent-offspring/full sibling scenario of the individuals V0218, V0922 and V1166.

A different approach is the use of other commercial STR kits in order to analyze complementary markers. An extraction could also be performed on the second tooth. However, it might be more valuable when other techniques can be applied on the biological material in order to compare different techniques.

4.3 Research contribution

This research contributed to our understanding of the reconstruction of family relationships within a cemetery, based on ancient DNA obtained from skeletons. Ancient DNA analysis is a relatively new research approach within the field of archaeology and can provide insight into the social structures in past populations, migration patterns and medical-genetic conditions within a population. Currently, there is no published work regarding the demonstration of kinship relations from a burial contexts within the Netherlands based on ancient DNA analysis. This research shows the potential of DNA research, which can provide information other archaeological research techniques are not capable of.

Abstract

During the excavation at the St. Plechelmus church in Oldenzaal between 2011 and 2013, six skeletons were found in a single burial context and were interpreted to be family members buried in a 'family grave'. Ancient DNA recovered from these individuals yielded information that is used to determine if there are genetic indications which can prove or disprove this interpretation. Furthermore, 81 surrounding skeletons were incorporated in this research for the determination of possible additional family relationships from a larger area of the cemetery. Short tandem repeat amplifications for 16 autosomal markers and 22 y-chromosomal markers provided reliable polymerase chain reaction products with no contamination. With allele frequency data from present-day Dutch people, and allele frequencies from all the Oldenzaal samples, statistical analyses were performed with the programs Relpair and Familias to reconstruct kinship relations. The results found no genetic indications for possible kinship relations between the six individuals from the 'family grave'. However, this cannot exclude that these individuals were biologically related to each other. For the surrounding 81 skeletons, the results indicate eight possible kinship relations. A Harris matrix showing all the excavated skeletons revealed that three out of the eight possible kinship relation pairs were spatially linked to each other; an aspect upon which the interpretation of the 'family grave' was based in the first place. This thesis demonstrates the successful use of aDNA from the Oldenzaal cemetery to reconstruct family relationships.

Appendix I

Table 7. Overview of the sampled individuals

Burial context	Individual	Sampled tooth	Teethpowder (g)	Sex*	Age**
'Family grave'	1214	2.6	1.88	Female	1339-1397 AD
	1215	6.4	0.63	Male	
	1237	1.7	1.55	Male	- 1500 AD
	1282	5.5	1.09	Female	
	1286	5.5?	0.82	Female	- 1500 AD
	1307	1.6	1.67	Female	-1387 AD
Matrix A	0218	1.7	1.61	Male	1500 AD -
	0256	3.4	0.81	Male	1500 AD -
	0449	1.3	0.99	Female	
	0700	4.3	1.34	Male	1441 AD -
	0781	4.7	1.91	Male	
	0863	1.8	1.14	Female	1446-1632 AD
	0865	3.3	0.84	Male	1527-1830 AD
	0870	4.7	1.94	Male	
	0899	4.5	1.16	Male	1441 AD -
	0922	1.4	1.26	Male	
	1100	4.3	0.91	Female	1441 AD -
	1101	4.8	1.78	Female	1441 AD -
	1104	3.5	1.38	Male	1441 AD -
	1105	4.7	0.94	Male	
	1123	2.6	2.25	Female	1304 AD -
	1147	4.4	0.71	Female	1441 AD -
	1166	3.5	1.89	Indeterminate	
	1201	3.4	0.69	Male	
	1229	4.8	1.35	Female	
	1233	1.8	1.25	Male	
	1274	5.5	1.17	Female	- 1399 AD
	1278	1.8	2.22	Male	1441 AD -
	1293	3.7	1.56	Male	- 1446 AD
	1297	3.7	1.12	Female	1304 AD -
	1320	4.7	1.75	Male	(1341-1430 AD)
	1350	2.7	1.93	Male	1303 AD -
	1376	3.7	1.69	Male	1441 AD -
	1388	3.5	0.89	Male	
	1400	3.7	1.47	Female	1022-1154 AD
	1408	2.8	1.03	Female	
	1416	3.7	1.65	Female	(1022-1394 AD)
	1420	1.7	1.49	Male	1285-1394 AD
	1435	1.7	2.45	Female	- 1432 AD
	1436	1.7	1.34	Male	
	1443	2.8	1.61	Male	1441 AD -
	1454	2.5	1.65	Female	(1022-1394 AD)
	1476	4.7	2.09	Female	1441 AD-

1484	2.6	1.47	Female	- 1415 AD
1488	1.7	1.78	Female	1445 AD -
1509	8.5	0.86	Male	1441 AD -
1513	3.7	1.60	Female	- 1634 AD
1517	3.7	1.76	Female	- 1432 AD
1521	3.7	2.04	Male	(1304-1432 AD)
1550	2.8	0.98	Female	(1304-1634 AD)
1584	2.6	1.70	Female	1304-1415 AD
1585	2.2	0.46	Female	1304 AD -
1673	3.7	1.75	Female	(1304-1634 AD)
1682	3.5	0.54	Male	
1812	3.7	1.88	Male	(1341-1430 AD)
1863	4.7	1.95	Female	(1341-1430 AD)
1901	3.8	1.69	Male	(1341-1421 AD)
1990	4.5	0.82	Female	1295-1404 AD
2003	3.7	1.37	Male	- 1407 AD
2017	4.7	1.37	Female	(1341-1421 AD)
2061	4.8	1.85	Male	- 1404 AD
2064	4.8	1.45	Female	- 1408 AD
2076	1.7?	1.87	Female	1299-1408 AD
2119	4.7	1.98	Female	(1341-1421 AD)
2127	3.3	1.00	Male	(1341-1421 AD)
2196	3.6	2.05	Female	
2205	1.3	1.06	Female	
2268	3.7	1.83	Male	(895-1421 AD)
2293	4.4	1.43	Male	895 AD -
2318	4.7	1.73	Indeterminate	(1341-1421 AD)
2362	3.7	1.69	Female	(895-1421 AD)
2367	3.3	0.86	Female	
2399	3.7	1.28	Female	(1341-1421 AD)
2470	4.6	1.42	Female	
2503	4.1	0.53	Female	
2554	3.7	2.03	Female	
2558	1.4	0.49	Male	
2643	4.7	1.30	Female	
2686	6.4	1.02	Male	- 1408 AD
2729	4.3	0.65	Female	(895-1421) AD
2737	3.7	1.59	Female	1295-1408 AD
2748	4.7	1.33	Female	895-1148 AD
2761	4.7	1.64	Male	1286-1401 AD
2873	2.6	2.60	Male	1303-1430 AD
2893	3.7	1.90	Female	- 1421 AD
2904	3.8	0.77	Indeterminate	994-1155 AD
3038	1.3	0.79	indeterminate	- 1404 AD

*Sex determination is based only on DNA analysis.

**Most of the individuals were dated indirectly; based on the relative position towards dated skeletons.

Appendix II

	Samplenr.	DYS391	DYS389 I	DYS439	DYS389 II	DYS438	DYS437	DYS19	DYS392	DYS393	DYS390	DYS385	DYS576	DYS448	DYS481	DYS549	DYS533	DYS570	DYS635	DYS643	DYS458	DYS456	GATA_H4
'Family'	V1215	11	{13}							{13}			{20}	{19}	{22}			18	{23}		{17}		
	V1237															{13}			{23}				
Matrix A	V0218	11	13			{13.1}*	{15}			13	24	12-14	18	19	22	13	{12}	17	23		17		
	V0256	11	14	12	30	12	15	14	13	13	23	11-14	17	19	22	14	12	17	23	10	16	17	11
	V0700	10	11							13	{24}	11-13	17	19	22	13		18	24		17	{15}	
	V0781	10	13	12	29	12	15	14	13	13	24	11-14	18	19	23	13	11	17	24	11	18	16	12
	V0865	11	13	12	29	12	{15}	14	13	13	23	11-14	17	19	22	13	12	17	24	10	17	{17}	11
	V0870	9	14	10	30	10		{13}	{11}	13	24	13-14	18	20	25	12	11	22	21		18	15	12
	V0899	11	13	11	28	12	15	{14}	{13}	13	24	11-14	17	19	20	13	12	17	23	{10}	16	15	12
	V0922	11	{13}							13		12-14	18		{22}		{12}	17			{17}		
	V1104	10	13	12		12	{14}			13	24	11-14	19	18	22	13	12	18	23		17		{11}
	V1105	11	13	13	29	12	{15}	14	13	13	24	11-14	17	19	24	12	11	17	24	{10}	17	16	{13}
	V1201	11	13	11	{29}	12		{14}		13	24	11-14	18	19	22	13	13	18	23		15	15	
	V1233	{10}											{18}										
	V1278	10	13							14	{25}	10-14	18	20	23	{12}		19	23		{16}		
	V1293	{11}	{13}	{11}						13	{24}	{11}-{14}	17	19	22	{13}		{18}	{23}		{18}		
	V1320	10	13		{29}		15			13	{24}	{11}	17	{19}	22	13	{11}	16	24		{18}		
	V1350	{10}													{23}			{17}			{17}		
	V1376	{11}								{13}			{15}		{23}								
	V1388	11	12							13	{22}	13-15	17	20	24	12	11	19	21		15		
	V1420	10											19					18			16		
	V1436	10	14	11	31	9	15	14	11	12	23	13-16	18	20	22	12	11	15	21	10	18	16	11
	V1443	10	14							15	{23}	{14}-{15}	16	20	26		12	20	21		{16}		
	V1509	10	14	11	32	10	14		12	15	23	14-15	16	20	26	12	12	20	21	{13}	16	15	11
	V1521	11	13	12	29		15	{14}	{13}	12	24	12-15	18	19	23	12	12	17	23		17	15	
	V1682	11	13	11					{13}	13		11-14	17	19	22	13	12	17	23		16		
	V1812	11	12	{11}	{28}		15	{16}	{11}	13	25	13	15	21	23	13	11	20	22		17		
	V1901	11	14	12	30	12	15	14	13	13	23	11-14	19	19	22	12	12	17	23	10	17	17	12
	V2003	10	11		{27}	10	{16}		{11}	13	22	{14}-15	15	20	25	12	11	21	23		16	{15}	{11}
	V2061	10	12							13			16		24			21	{22}		{16}		
	V2127	10	13			{10}				13	24	16-17	17	20	22	13	{12}	22	22		15		{12}
	V2268	10	12	11						13	22		15	{20}	23	12	{10}	20	23		{15}		
	V2293	12	13	12	{29}	12			13	13	23	11-14	18	19	22	14	12	16	23		17	{17}	{11}
	V2558	11	13	12	29	12	15	14	13	13	24	11-14	17	19	22	12	12	17	23	10	16	15	12
	V2686	10	13		{29}					13			17	19	22	12		16	{23}				
	V2761	11	12	14	28					13	23	12-13	19	19	23	14	12	16	23		17	{16}	{12}
	V2873	11	13	{14}	29	{12}	15	{14}		13	23	{11}-{14}	17	19	22	12	{12}	17	23		17		

Table 8. An overview of the ySTR markers from the individuals within matrix A and the 'family grave'. The alleles enclosed by curly brackets denote the alleles which were picked up in a single powerplex Y23.

Appendix III

The ABI 3100 Genetic Analyzer

In order to accurately genotype STR markers using multicolor fluorescence detection, a separation and detection technique must be performed. The ABI 3100 Genetic Analyzer from Applied Biosystems is a Capillary Electrophoresis instrument which is used to distinguish the different DNA molecules present within the PCR product. During the analysis a high positive voltage is applied over 16 capillaries, which results in migrating negatively charged DNA fragments through these capillaries (figure 9). The capillaries are filled with POP-4 polymers which provide a sieving matrix for the separation of single-stranded DNA. Smaller fragments will migrate faster through the polymer-filled capillaries than larger fragments. PCR products are separated by size and fluorescent dye labeled primers using electrophoresis, followed by laser-induced fluorescence with multi-wavelength detection. The quantity of injected DNA depends on the electric field, the injection time, the concentration and ionic strength of the sample, the area of the capillary opening and the buffer (Butler *et al.* 2004).

The internal standards are added to each sample for calibration purposes; sizing the DNA fragment peaks. The ILS 500 and ILS 600 internal standards contain DNA fragments of known size and are labeled with a different dye color so that it can be spectrally distinguished from the STR alleles which are labeled with other colors (Butler 2005, 37

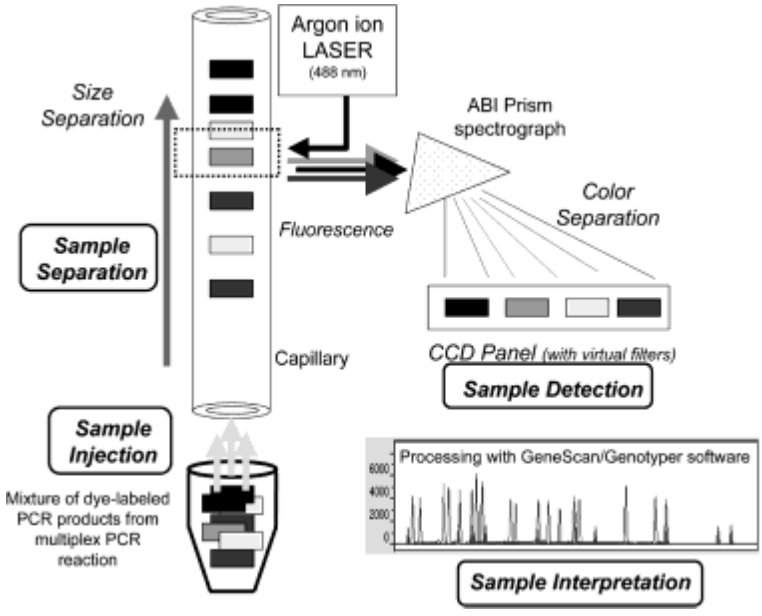

Figure 9. Schematic illustration of the separation and detection of STR alleles with an ABI 3100 Genetic Analyzer (Butler et al. 2004).

The collected data in the form of multicolored electropherograms can be analyzed by software that automatically determines STR allele sizes based on a standard curve provided by the internal size standard.

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