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碩士論文

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College of Medicine

National Taiwan University

Master Thesis

牙齒 DNA 之人別鑑定

Individual Identification from Dental DNA

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致謝

在法醫所 6 年不為學位，不為工作，只為興趣。很多人問我這樣做值得嗎？這很難說，如果把錢和時間算進去，實在不划算，但是這段人生旅程所收穫的東西是無法用錢去衡量的，只能說我很高興有做這件事，如果沒做的話，我會一直想。

能讀法醫所本身就是一件不可思議的事，更何況要完成論文寫作。入學時已近 50 歲，體力與記憶力已大不如前。還好所上李俊億老師、華筱玲老師、翁德怡老師、孫家棟老師、吳木榮老師、陳冠元老師、張晉誠老師及客座老師們認真教學傾囊相授，使我獲益匪淺，大開眼界，聞所未聞，看所未看，真是令人嘆為觀止。法醫領域浩瀚如海。

在思考論文題目時，想跟自己原本專業結合，牙齒+DNA 就誕生了。謝謝所上淑芬助教的幫忙、實驗室玉貞助教的鼓勵、法務部法醫所林俊彥組長實驗上的協助以及李俊億老師不厭其煩的指導 還有那些捐贈牙齒的無名英雄，沒有他們，這篇論文完成不了。同時也謝謝一起上課、實習的同學易霖、文杰、子誠等等，往事歷歷在目，真是回味無盡。

最後謝謝我的父母、岳父母、老婆和小孩。他們默默的支持與體諒讓我堅持下去完成自己的夢想，謝謝你們。

魏義峰 謹致

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中文摘要

牙齒可以作為人類遺骸基因組 DNA 的可靠來源。使用牙齒組織作為個體識別的 DNA 來源來自於其抵抗環境攻擊的特徵，例如焚燒、浸泡、創傷和肢解。從牙齒中提取 DNA 的傳統方法需要對牙齒進行完全機械破壞。雖然這方法簡單可靠，但對於希望進一步證據目的或人類學研究來說並不理想。牙齒細胞豐富的區域是牙髓 (pulp)和牙骨質(cementum)。 本研究分別採用牙髓和牙骨質樣本兩種對牙齒破壞最小的方法提取 DNA 進行個體鑑定。牙髓取樣是用牙科手機在牙齒的咬合面鑽孔，然後用牙銼取出牙髓和成牙本質細胞，然後用複合樹脂將孔回填。牙骨質取樣是用刀片刮牙根外表面的牙骨質。牙骨質的損失非常小，幾乎無法發現 本研究測試了 58 個埋藏和未埋藏的脫落牙齒樣本，以及 4 個法醫牙齒樣本。 將 DNA 產量和 STR 成功率與這兩種採樣方法進行了比較。 結果表明，牙骨質取樣在 DNA 質量和效率方面優於牙髓取樣。 這說明微生物在降解核 DNA 和降低 DNA 產量方面有至關重要的影響。門齒、小臼齒和大臼齒之間的 STR 基因分型成功率表明多牙根比單牙根提供更好的 DNA 數量和質量。在牙髓採集的樣本中，有蛀牙的牙齒的 DNA 產量非常少，而在牙骨質採集的樣本中則不然。如果牙齒型態保存是重中之重，那牙骨質取樣是一種理想的 DNA 提取方法。如果對病原菌 DNA 感興趣的話，從咬合方向鑽孔取牙髓可能是一種很好的替代方法，這兩種方法都可以在不破壞牙齒結構的情況下製備 DNA。

關鍵詞 : 人別鑑定、牙髓(pulp)、牙骨質(cementum)、DNA、STR、牙齒型態。

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ABSTRACT

The teeth could serve as a reliable source of genomic DNA of human remains. Using dental tissues as a DNA source of individual identification is due to its particular character of resistance to environmental assaults such as incineration, immersion, trauma, mutilation and decomposition. The traditional method to extract DNA from teeth requires mechanical crush of the entire tooth. Although this is easy and reliable, it is not ideal for further evidence purpose or anthropological study. Cell-abundant areas of the tooth are pulp and cementum. Two methods of minimum destruction on tooth for pulp and cementum samplings respectively were used in this study to extract DNA for individual identification. About the pulp sampling, a dental handpiece was used to drill a hole on occlusal surface of the tooth, and then dental files were used to retrieve pulp and odontoblasts, after that the hole was filled back with composite resin. As for the cementum sampling, blades were used to scrape cementum which is on the outer surface of the root . The loss of cementum was so small that could hardly be found. Fifty-eight fallen tooth samples were buried and unburied, and 4 forensic tooth samples were tested in this study. DNA yields and STR success rates were compared with these two sampling methods. It showed that cementum sampling outperformed pulp sampling in DNA quality and efficiency. It indicated that microbes played an imperative role in degrading

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the nuclear material and reduced DNA yields. The success rates of STR genotyping among incisors, premolars, and molars showed multirooted teeth provided better DNA quantity and quality than single-root teeth. The DNA quantity of teeth with cavities were seriously affected in pulp samples, while the cementum was not observed in this matter. Cementum sampling is an ideal method for DNA extraction if tooth morphological preservation is the highest priority. If pathogen DNA is of interest, occlusal approach to retrieve pulp may serve as a good alternative to prepare DNA without destruction of the tooth structure.

Keyword: individual identification, pulp, cementum, DNA, STR, tooth morphology.

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Chapter 1 Introduction

There are many sources for DNA extraction in forensic science, such as blood, muscle, semen, hair, bones, saliva, etc. [1]. However, after a long post-mortem interval, for example, 3 months or longer, it may only leave bones and teeth available for analysis. Usually such remains were compared with previous dental records or bone X-ray. If pre-death records were missing, positive identification often has been impossible [2]. During the past few years, DNA analysis methods have been applied to forensic cases. Compared with bones, teeth are a better source of genomic DNA due to their special composition and location in the jawbone which can provide additional protection to DNA. Using dental tissues as a DNA source of individual identification is due to its particular character of resistance towards physical or chemical aggression. Teeth are the hardest tissue in the human body [3] and are resistant to adverse conditions such as wetness, high temperature, UV light and microbial invasion, therefore a very good source for DNA collection. DNA extracted from teeth is often less prone to contamination and higher quality than DNA extracted from bones [4]. Several studies show the reliability of teeth as a source of genomic DNA in human identification [5]. For example, Milos et al. reported 82.7% success of 6963 teeth pulverized in obtaining nuclear DNA profiles from victims of armed conflicts that occurred between 1992 and

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1999 [6]. With the assistance of polymerase chain reaction or PCR, forensic laboratories may make using traces of DNA possible. Understanding microanatomy of the tooth is very helpful for better gaining DNA from dental tissues. Pulp, dentine powder, cement powder can be used as samples for collection. The dentine-cement powder proved to be the best sample for isolation of either genomic or mitochondrial DNA (mtDNA), because the DNA in it remains well preserved and there is rarely contamination [2].

Human teeth can be divided into two parts: the crown and the roots. The crown contains enamel, dentin and pulp and is showed up in the mouth, while the roots are composed of cementum, dentine and pulp and positioned in the sockets of jawbone (Fig.1). Studies show tooth roots are able to yield more DNA than tooth crowns [7].

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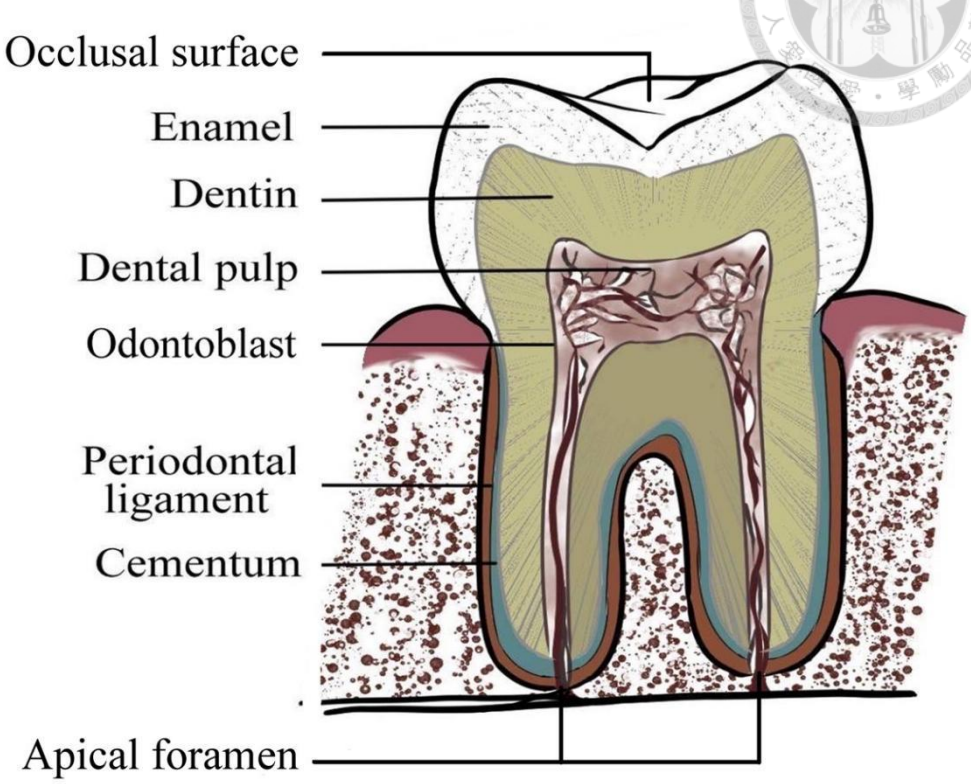

Fig. 1 Tooth structure. The illustrated image shows a sectional view of a human mandibular molar.

Enamel on the outer surface of the crown is composed of 96% (by weight) mineral and is the hardest tissue in the human body. Enamel is acellular and contains no DNA. It provides an excellent physical barrier protection from environmental assault such as heat, UV light, wetness and microbes [8]. Although enamel has limited permeability in vivo [10], the size of the pores is extremely small that molecules larger than water being

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prevented coming through. This is important in preventing the access of microbes and exogenous contaminants from coming inside the tooth.

In the pulp chamber, there are several cell types and microvessels. For example, odontoblasts (cells that form dentine), fibroblasts, nerve cells, plasma cells, macrophages and undifferentiated mesenchymal cells etc. [10]. Microvessels include capillaries, arterioles and venules. The odontoblasts which beneath the dentin circumscribe the outer part of the pulp and their cytoplasmic processes extend through the dentine matrix [11-12]. In the pulp odontoblasts are the most numerous cells [13]. They have been estimated at 11,000 per mm^2 [12] and fibroblasts, which have been estimated at 1000 per mm^2 [12]. Since about 80 diploid cells can yield the minimum DNA quantity required for STR typing, pulp obviously is a good source of DNA [14]. Pulp interconnects microvessels via the root apex to the surrounding alveolar bone [15]. The high cellularity of pulp provides valuable source of DNA in teeth. However, pulp may be absent or in little quantity in aged and/or diseased teeth [16]. Dental pulp has been an important source of information about ancient diseases because pathogens are prevalent in the blood stream and can be detected in dental pulp vessels [17].

Dentine, which is between pulp and enamel in the crown and pulp and cementum in the root is composed of 65-70 % mineral in the form of hydroxyapatite, collagen, and

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water. Usually, dentine is devoid of nucleus, however, odontoblasts can sometimes be entrapped in the dentine during the formation of tertiary dentine (a response to dental caries). Dentine is a unique tissue, densely arranged by parallel tubules, running through the dentine to the pulp chamber [18]. These tubules contain odontoblastic cell processes and nerve fibers. Odontoblastic processes have mitochondria along their length, making the tubules rich in mtDNA [15]. However, dentine is not a good source of nuclear DNA.

Cementum is on the outer surface of the roots and is an avascular mineralized tissue with a laminated structure. It is composed of 45-50% (by weight) mineral content(hydroxyapatite). There are two types of cementum according to the presence or absence of cementocytes. One is cellular cementum which is on the lower half of the root, and another is acellular cementum which is around cervical third of the root. Cementocytes embedded in the extracellular matrix are a source of nuclear DNA, as osteocytes in osteolytic lacunae [19]. Cellular cementum is similar to bone in physical and chemical content but is different in structures and functions [20]. They are avascular, have no innervation and contain fewer inorganic salts [21]. Unlike bone, cementum does not undergo remodeling but does increase in thickness throughout life [22]. Cellular cementum is mainly seen on the apical third of the roots and in the furcation area of posterior teeth [23].

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In summary, pulp and cementum are obviously the best sources of nuclear DNA in the tooth and both these tissues and dentine are good sources of mtDNA [24]. Apical area within 2mm of the root has the richest cellularity which contains pulp, odontoblast and cementocyts. Enamel is important in the protection of dentine and pulp but is free of DNA. If enamel is sampled together with the other tooth tissue, it will have a dilution effect and the minerals, including calcium, necessitating purifying steps and complicating the extraction process and inhibiting PCR amplification.

The DNA content of teeth varies between individuals and varies between different teeth in the same person. The factors that influence DNA content include tooth types, age of the individual, and health status of the tooth. Each factor will have an impact on the DNA quantity in the crown and root, and in the pulp, dentine and cementum [24].

There are three types of human teeth, they are molars, premolars, and incisors (including canines) representing three roots, two roots and one root respectively. They differ in form and size but have a similar histological structure [24]. Studies of DNA content among different tooth types showed the larger pulp chamber providing more pulp cells and more DNA yield [25]. It also indicated that multi-rooted teeth had more chances to extract DNA than single-rooted teeth, probably because multi-rooted teeth had larger pulp volume and the increased root surface area could provide more

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cementum [26]. Teeth retained in the sockets are better protected by the alveolar bone [27] and have less chance to be contaminated.

With the increasing age there are several changes. These changes will affect DNA content. The most obvious negative change is the decrease on pulp volume due to the normal continuous deposition of dentine. Pulp not only decreases in volume over time but also decreases in cellularity and becomes more fibrous [28]. A positive change is the amount of cellular cementum increases with age [17]. Pulp volume decreases through natural attrition, and dentine decreases in porosity because of occlusion of the tubules [25]. Overall, increasing age leads to a decrease in DNA content of the tooth.

Dental diseases have an adverse effect on the DNA content of teeth [26]. For example, dental caries, a microbial disease origin, results in localized dissolution and destruction of the morphology of the teeth [24]. If bacteria enter to the pulp, it will result in cell death. In response to caries, the pulp retracts, and tertiary dentine is deposited [26]. This tertiary dentine exhibits a less organized structure than primary and secondary dentine. Ultimately caries can lead to a complete dissolution of pulp cells. Cellular cementum can only be affected by caries if root caries happens. [24]. As cementocytes are embedded in the cementum and protected by the matrix, cementum becomes an important tissue for recovery of DNA from pulpless teeth [24].

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Periodontal disease is a plaque-induced inflammatory disease affecting the supporting tissues of the tooth [26]. As the disease progresses the alveolar bone is destroyed resulting in a reduction of the height of the gingiva around the tooth and exposure of cementum to the oral cavity [26]. Cementum affected by periodontal disease becomes coated with plaque and calculus [26]. Advanced periodontal disease may cause pulp necrosis via microbial invasion through apical foramen and make the teeth fall off the sockets completely.

DNA degradation of teeth has been shown to be time dependent and is greatly affected by environmental factors. A dry environment will promote desiccation of pulp, protecting the DNA from hydrolytic dissolution, while a wet environment will allow putrefaction and complete destruction of pulp cells [23]. Alvarez Garcia et al. examined nuclear DNA yields from teeth separated from the jaws that were subjected to varying environments [27]. This study examined some teeth crushed whole and some teeth used pulp tissue by conventional endodontic access or cut by a transversal section. It reported that fallen teeth kept on the surface yielded more DNA than those buried under the ground and those teeth submerged in water gave the poorest results [27]. Because environmental conditions such as temperature, humidity, pH and UV exposure do not appear in isolation, it is difficult to separate their individual effects, but it is important to

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note that different rates of success are expected from different environment conditions and the length of exposure [24].

An important consideration when dealing with human DNA from skeletal remains is the potential for contamination [24]. Contamination with exogenous DNA has been reported in forensic DNA analysis and is a well-known problem in ancient DNA research [28]. The most frequently reported decontamination technique for teeth is the use of bleach [29]. It is generally considered that bleach destroys exogenous DNA while leaving endogenous DNA unaffected. A study, however, suggests that bleach treatment appears to create a depurination-associated fragmentation similarly to previously described endogenous sequences [30]. No studies have demonstrated the depth of penetration of bleach through the tooth [29]. Cementum on the surface of the root may be affected by bleach and reduce the DNA yield [24]. Before the true influence of decontamination methods on the DNA content being established, it is better to avoid the use of bleach or other chemicals that may damage DNA [24]. Simple cleaning method is all likely that is required before DNA sampling. Simple cleaning technique involves brushing or light scraping to remove debris and /or wiping with DNA free water [24].

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Chapter 2 Literature Review

Methods currently used for obtaining DNA from dental tissues are: (1) pulverizing the entire tooth [31], (2) horizontal sectioning through the cementoenamel junction [32], (3) vertical split of the tooth [33], (4) retrograde access to pulp chamber [34], (5) orthograde or conventional occlusal access to pulp chamber, using dental files to retrieve pulp and odontoblasts [35], (6) scraping cementum by blades [29].

2.1 Pulverizing the entire tooth

This “classical” method of DNA extraction includes steps of freezing, crushing and grinding the entire tooth and subsequent decalcification. PCR inhibition can be caused by tooth components itself, such as calcium and collagen, and/or by some products used in the extraction process, such as ethylenediaminetetraacetic acid (EDTA), phenol, chloroform [31]. These pre-extraction steps are time-consuming and labor-intensive. More importantly, the tooth no longer existed. Tilotta et al. compared the nuclear DNA yield from teeth pulverized whole to those only pulp retrieved endodontic occlusal access (via a hole drilled in the crown) and showed that a greatly higher DNA yield came from the more conservative sampling method. The non-destructive method would allow

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further anthropological study and for return to the families, which is especially important in cases where limited remains are recovered [24].

2.2 Horizontal sectioning through the cementoenamel junction

Another method that can avoid whole tooth destruction was introduced by Shiroma [32]. This technique cut through cementoenamel junction horizontally (Fig.2) and collected pulp and dentine via drilling from the inside of the tooth, and after that the two halves of tooth were glued back together giving the appearance of an intact tooth.

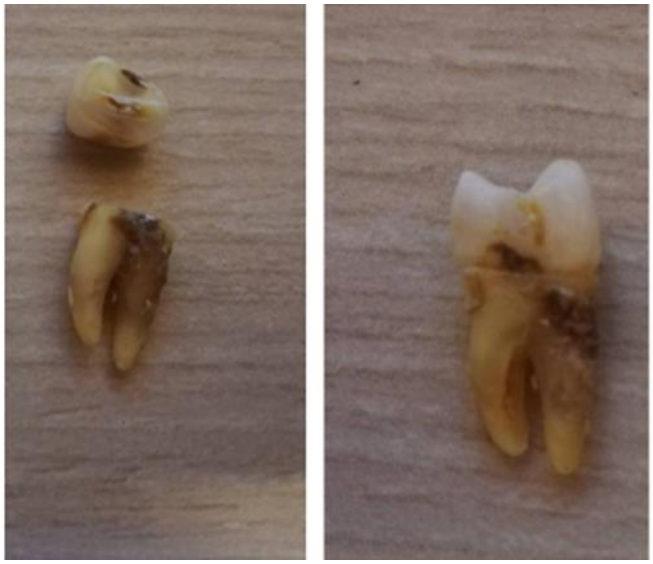

Fig. 2 left: horizontal section of the tooth; right: restore the tooth morphology with glue

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2.3 Vertical split of the tooth

Another method is vertical split of the tooth (Fig.3). A 1mm deep and 1mm wide furrow was cut in the coronal apical axis with a carborundum disk. Afterwards, the tooth was split by bi-bevel chisel, the pulp cavity was exposed along with part of the root canal [33]. After sampling the two halves of tooth were glued back to its original appearance.

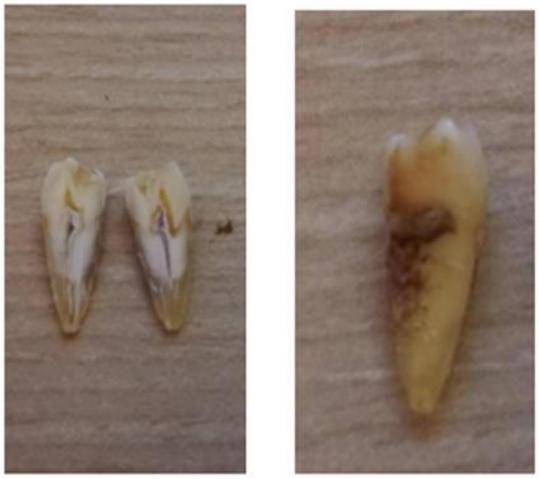

Fig. 3 left: vertical split of the tooth; right: restore the tooth morphology with glue

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2.4 Retrograde access to pulp chamber

Another grinding-free method to extract DNA from teeth is retrograde approach (Fig.4) to the pulp. This method uses endodontic dental files to access the root canals and pulp cavity via the apical end of the roots to avoid mechanical damage of the crown morphology. Although this method spares transection, splitting or destruction of the occlusal surface to gain access to the DNA, it was more laborious (need 2-3 h) [34]. The quality and quantity of DNA by this method outperformed the grinding of the entire tooth method, but it was not practical in routine forensic cases.

Fig. 4 retrograde access to pulp chamber

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2.5 Orthograde or conventional occlusal access

Another grinding-free method is “orthograde entrance technique” (Fig.5) or “conventional endodontic occlusal access” does not need much time to retrieve pulp and odontoblasts. The entire sampling process takes about 30-40 min. The collected powders do not need to be purified and can be processed with commercial kit. Thus, this “orthograde entrance technique” shows extremely advantageous in terms of cost, time, quality, and quantity of DNA [35].

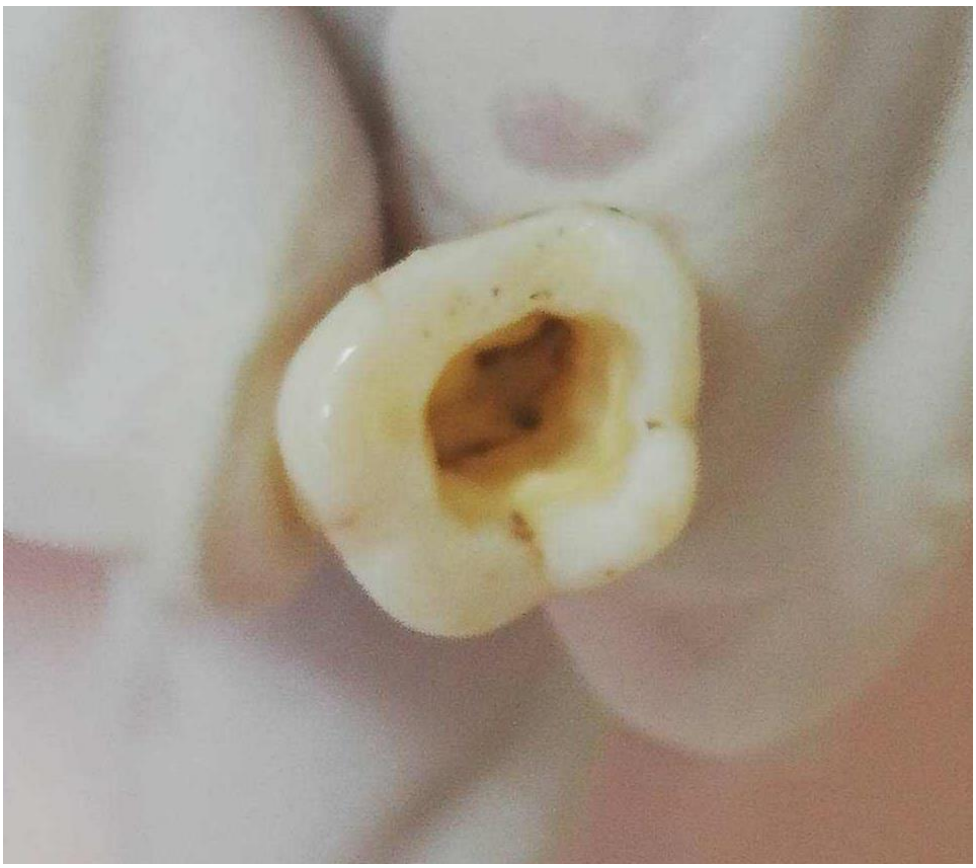

Fig. 5 Orthograde or conventional endodontic access to pulp chamber

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2.6 Scraping cementum by blades

Another minimally destructive ideal protocol for DNA extraction from ancient teeth was proposed by Harney et al. This method targeted the cementum of tooth roots (Fig.6) [29]. The cementum is 20-50 um thick at the cementoenamel junction and 150-200 um thick at the apex of the root. Cementum resembles the petrous bone that are thought to preserve high level of DNA [28]. Petrous bone and cementum are thought to serve as repositories of ancient DNA [28]. Cementum does not undergo remodeling, but it continues to accumulate throughout life [36]. In a direct comparison between cementum and petrous bone samples, Hansen et al. [37] find that cementum and petrous bone yield a comparable amount of endogenous DNA in well-preserved samples, but in poorly preserved individuals, the petrous bone yields a higher proportion of endogenous DNA.

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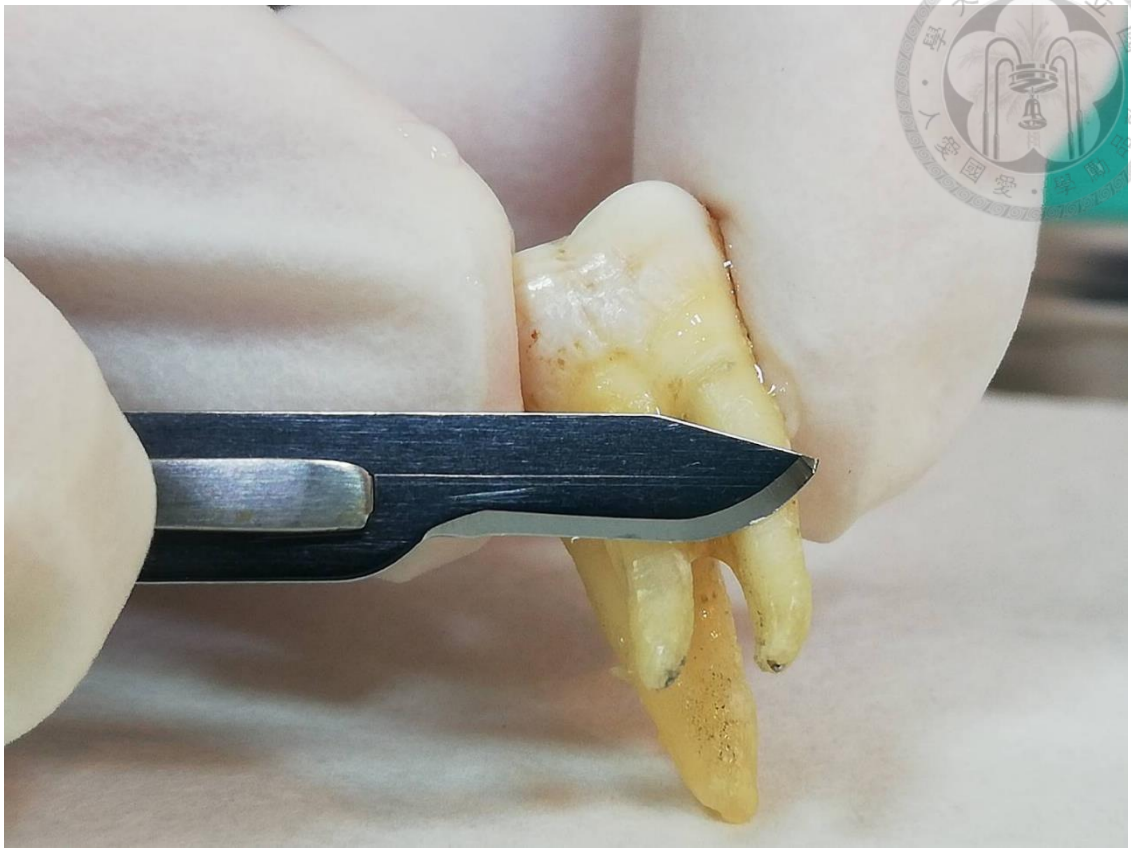

Fig. 6　Surgical blade 15# used to scrape cementum on Charta paper.

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Chapter 3 Materials and method

Since preserving tooth structures is our major concern, we chose to target pulp and cementum. For sampling pulp, we used direct approach opening by drilling a hole on occlusal surface of the tooth and retrieving pulp and odontoblasts with dental files. For sampling cementum , we used disposable blades to scrape cementum .We compared the quantity and quality of DNA of the same tooth with these two methods without use of bleach, because using sodium hypochlorite (bleach) to decontaminate exogenous DNAs would damage the nuclear material and reduce DNA yields from cementum by an order of magnitude even with bleach solution as diluted as 2.5% for as little as 1min [29].

3.1 Sample preparation

The total tooth number was 58. They were diagnosed either with advanced periodontitis or large decay which meant hopeless to be preserved and extracted by the local dentist. These teeth were donated to the Department and Graduate Institute of Forensic Medicine, College of Medicine, National Taiwan University. Informed consent was obtained as per the National Taiwan University Hospital Ethics Committee approval (202006155RINB). We separated these teeth into five groups (Table1) : group A, 9 teeth, intact, kept in the air ; group B ,10 teeth (incisor), intact, buried in the soil ; group C, 8

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teeth (premolar), intact, buried in the soil ; group D ,13 teeth (molar) intact, buried in the soil ; group E ,18 teeth with obviously large cavity, buried in the soil. Teeth were buried in the soil for 92 days, they were kept in the garden outdoors (Fig 7), during which 10 days were rainy (Table 2). We wanted to know the influences between tooth types, tooth condition, and the preserved ways.

Table 1 Teeth classified into five groups. Tooth type "mixed" means it includes incisor, premolar and molar. Preserved condition "soil "means it was buried in the soil for 3 months while "air" means it was kept dried in the air.

	Tooth type	Tooth number	Tooth condition	Preserved condition
Group A	Mixed	9	Intact	Air
Group B	Incisor	10	Intact	Soil
Group C	Premolar	8	Intact	Soil
Group D	Molar	13	Intact	Soil
Group E	Mixed	18	Caries	Soil

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Table 2　Precipitation was shown from Oct1 to Dec31 2020 .Average temperature of Oct, Nov and Dec of 2020 is 25.5°C, 22.6°C, 18.7°C respectively.

date	Precipitation (mm)	date	Precipitation (mm)	date	Precipitation (mm)
2020/10/1	×	2020/11/1	×	2020/12/1	×
2020/10/2	×	2020/11/2	×	2020/12/2	×
2020/10/3	×	2020/11/3	×	2020/12/3	×
2020/10/4	×	2020/11/4	×	2020/12/4	×
2020/10/5	×	2020/11/5	×	2020/12/5	×
2020/10/6	×	2020/11/6	×	2020/12/6	×
2020/10/7	×	2020/11/7	1.0	2020/12/7	×
2020/10/8	×	2020/11/8	×	2020/12/8	×
2020/10/9	×	2020/11/9	×	2020/12/9	11.5
2020/10/10	×	2020/11/10	1.0	2020/12/10	0.5
2020/10/11	×	2020/11/11	×	2020/12/11	12.0
2020/10/12	×	2020/11/12	×	2020/12/12	×
2020/10/13	×	2020/11/13	1.0	2020/12/13	×
2020/10/14	×	2020/11/14	×	2020/12/14	×
2020/10/15	×	2020/11/15	×	2020/12/15	×
2020/10/16	×	2020/11/16	×	2020/12/16	×
2020/10/17	×	2020/11/17	×	2020/12/17	×
2020/10/18	×	2020/11/18	×	2020/12/18	×
2020/10/19	×	2020/11/19	×	2020/12/19	×
2020/10/20	×	2020/11/20	×	2020/12/20	×
2020/10/21	×	2020/11/21	×	2020/12/21	×
2020/10/22	×	2020/11/22	×	2020/12/22	×
2020/10/23	0.5	2020/11/23	×	2020/12/23	5.0
2020/10/24	×	2020/11/24	×	2020/12/24	0.5
2020/10/25	×	2020/11/25	×	2020/12/25	×
2020/10/26	×	2020/11/26	×	2020/12/26	×
2020/10/27	×	2020/11/27	×	2020/12/27	1.0
2020/10/28	×	2020/11/28	×	2020/12/28	×
2020/10/29	×	2020/11/29	×	2020/12/29	×
2020/10/30	×	2020/11/30	×	2020/12/30	×
2020/10/31	×			2020/12/31	×

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Fig. 7 These teeth were buried in the soil for 3 months outdoors.

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After digging out, all teeth were brushed to remove debris and wiped cleanly with running water and then the calculus on the root surface were removed by dental ultrasonic device (Fig.8-10). We did not use bleach or UV-light to decontaminate exogenous DNA.

Fig. 8 The teeth were dug out.

Fig. 9 The teeth were washed with running water.

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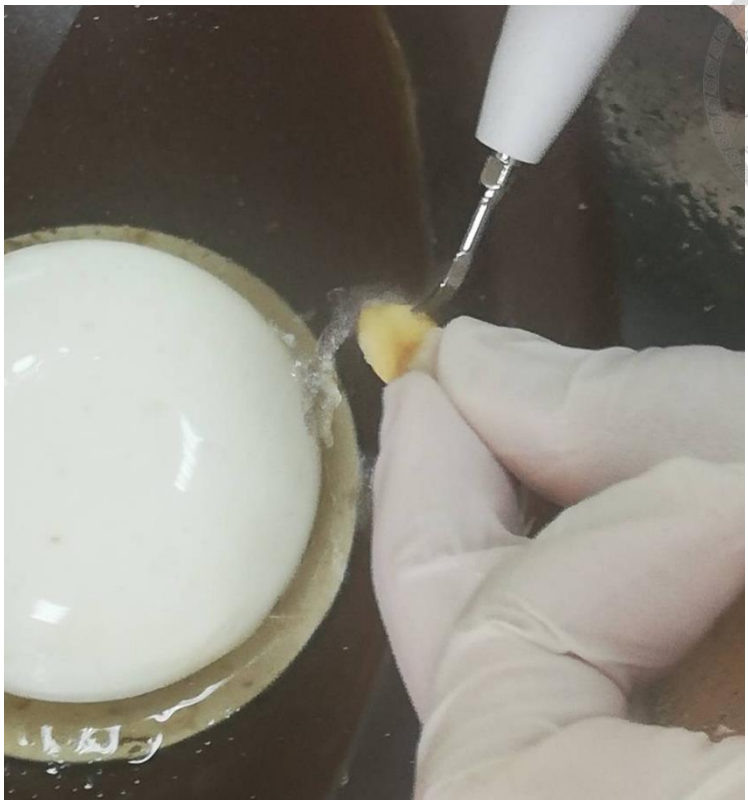

Fig. 10 The calculus of the root surface was removed with dental ultrasonic device.

Several precautions were taken to prevent contamination in this study. The teeth samples were brushed to remove debris with running water after being dug out. The whole surface of the teeth must be cleaned, and without any residual tissue or calculus left by visual inspection. In every step, disposable gloves and masks were used. Non-disposable dental instruments used to retrieve pulp were sterilized in sterilizator (STURDY® Industrial CO., LTD, SA-252F, ISO13485 certified) set at 121 ℃ for 30 min. DNA extraction, quantitation, amplification and data analysis were performed according to the laboratory protocols.

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3.2 Pulp (including odontoblastic powder) sampling

On the crown surface of the tooth, a hole was dug and entrance access was prepared by using two round diamond burs, 0.5 and 1.0 mm diameter respectively. Burs were connected to the dental hi-speed handpiece (TTbio® evo500WT, Taiwan) at 250,000—300,000 rpm with cooling system (Fig11). It is necessary to use water cooling because the heat generated by the hi-speed handpiece would damage the DNA. Using long fissure 1.5mm diameter diamond bur connected to the dental handpiece continues opening chamber to gain direct line access to root apex that afterward the dental files can passively reach the apical foramen. This is important because it can avoid dental files breaking and make pulp and odontoblastic powder easily collected. Using the barbed broaches (MANI® BARBED BROACHES) to retrieve pulp tissue (Fig.12-14), the barbed broaches with pulp tissue was transferred to the sterile DNA collecting tubes .

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Fig. 11　Dental hi-speed with water spray connected to 0.5mm diameter of bur to open chamber on the occlusal surface of the tooth.

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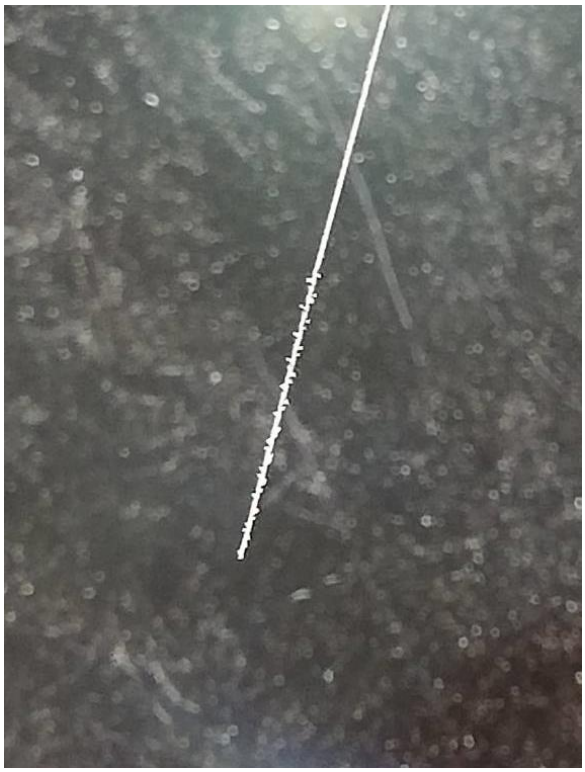

Fig. 12 barbed broaches.

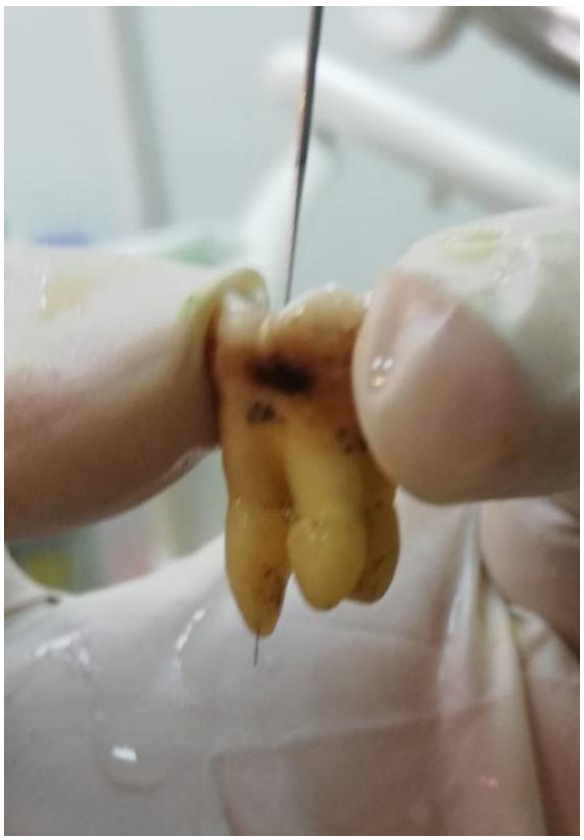

Fig. 13 Barbed broaches through the opening to retrieve pulp tissue.

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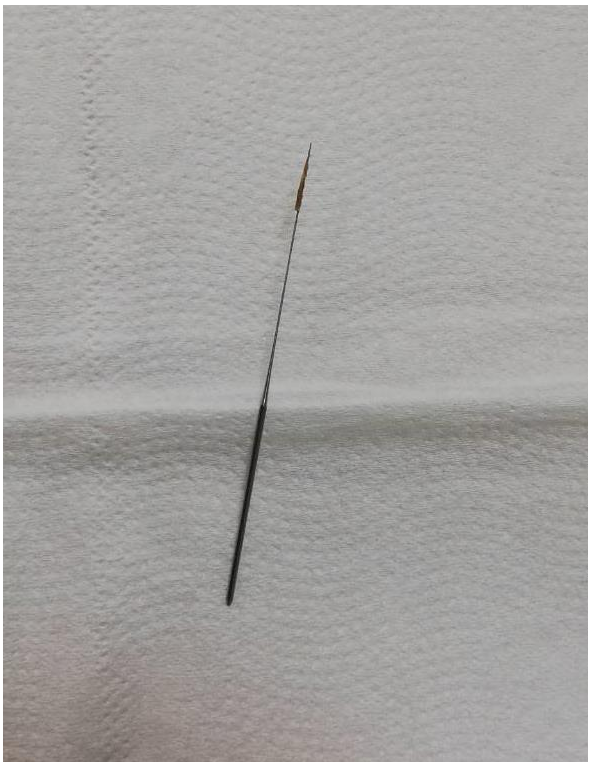

Fig. 14 Pulp tissue on the barbed broaches

Pulp and odontoblastic powder were continually collected by using series of dental endodontic K-files (sizes 10-80) (Fig.15). If the whole process was done manually, it would take about 60min. If with the help of rotary machine (Analytic Quantec® ETM, Taiwan, low-speed handpiece 18:1 at 340 rpm, no need water cooling), it could only take 30 min to finish the sampling (Fig.16). We collected pulp and odontoblastic powder to maximize the DNA quantity. The endodontic K-files were used to 80# that meant apical foramen was enlarged to 0.8mm which might cause some curved root tips to break. The weight of the collected powder varied from 5mg to 10mg. Armamentarium for retrieving

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pulp and odontoblastic powder was shown in Fig.17. They were all packed and sterilized in the autoclave.

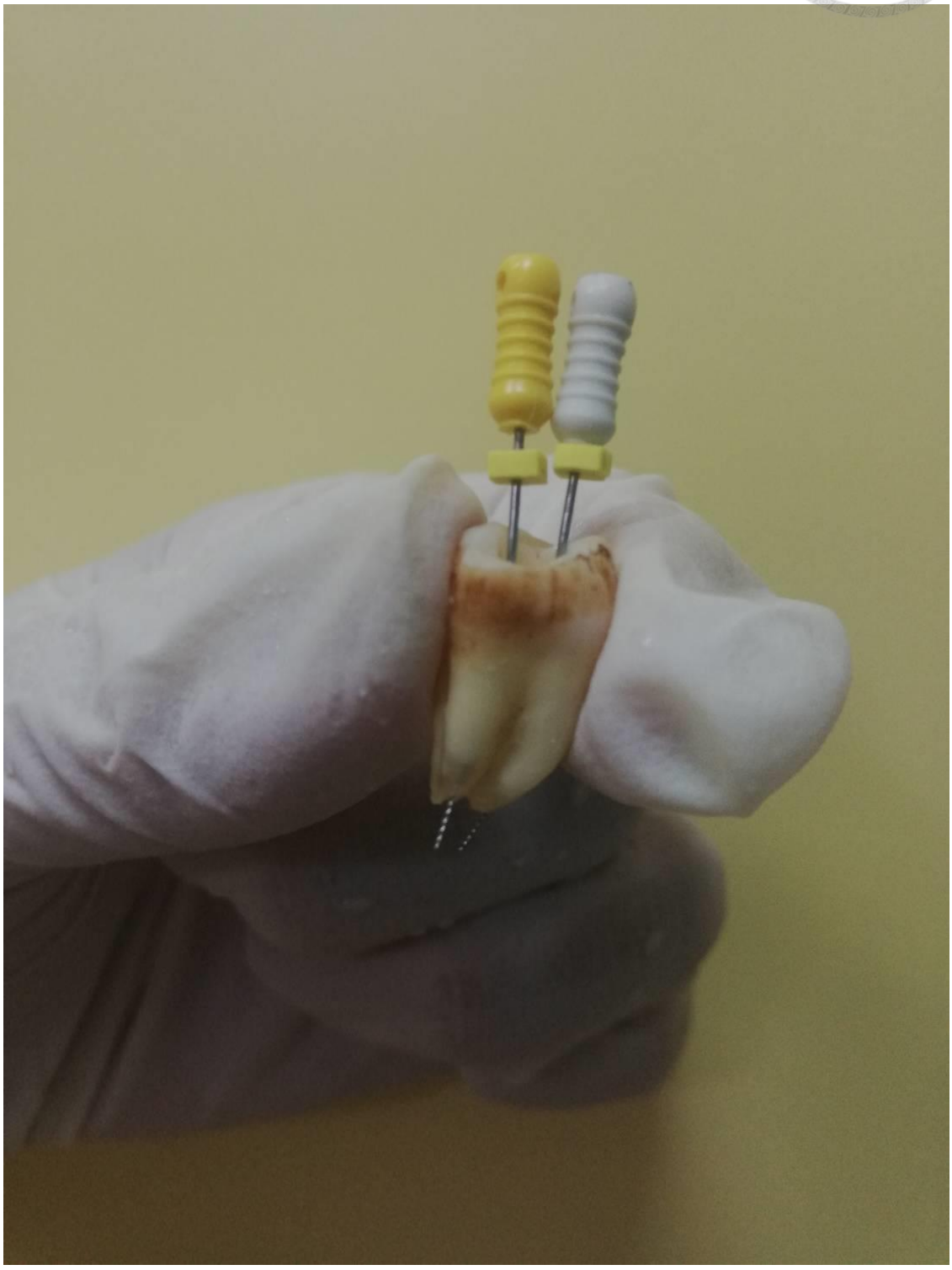

Fig. 15 The k-files used to collect pulp and odontoblastic powder.

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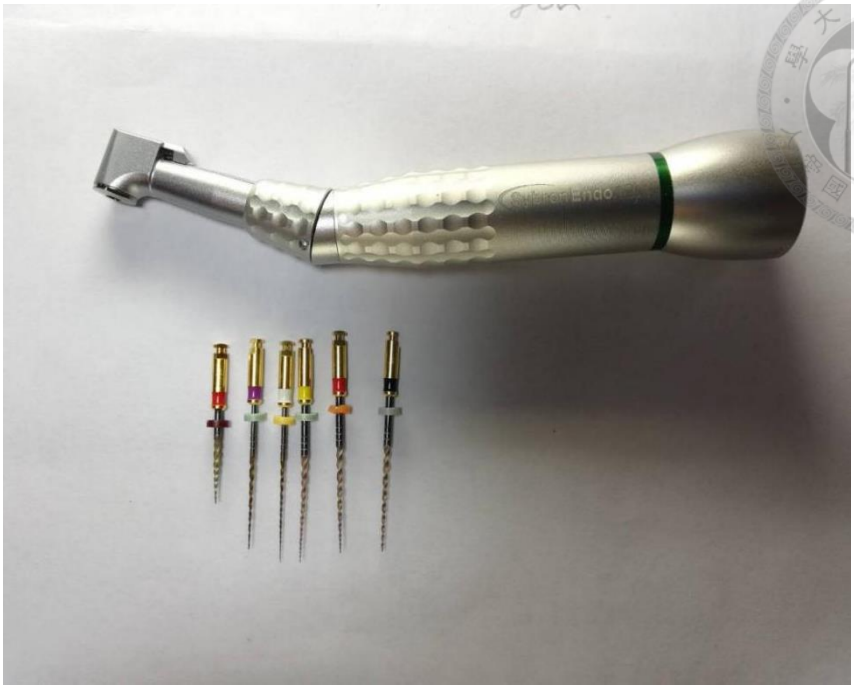

Fig. 16 The rotary machine (Analytic Quantec® ETM, Taiwan, low-speed handpiece 18:1 at 340 rpm) used to collect odontoblastic powder.

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Fig. 17 Armamentarium in retrieving pulp tissue and odontoblastic powder include round bur, fissure bur, hi-speed and low-speed handpiece, barbed broaches, dental files and Charta paper. All were packed and sterilized in autoclave.

3.3 Cementum sampling

On the same tooth we used blades (#15) to scrape cementum on Charta paper. The cementum powder was transferred to Eppendorf for extraction process. The weight varied from 5 mg to 10mg. This method of cementum sampling took about only 5 to 10 min. The loss of cementum was so small that could hardly be found.

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3.4 Restore tooth morphology

Composite resin can be filled back in the cavity after pulp sampling in order to restore its original morphology. A sterilized piece of cotton was put in the floor of the pulp chamber before being filled. This makes reopen chambers easily if needed in the future. 37% Phosphoric Acid Gel (DenFil® Etchant-37) was applied in the cavity to form a micro-etched surface. On the etched surface bonding agent (G-Premio BOND®, GC, Japan) was applied and cured with halogen light (Ultra Lite 1000E®, S/N A041166). Finally, the cavity was filled with a composite resin material (BEAUTIFIL®, SHOFU INC, Japan) maintaining the original state of the teeth. The restored teeth could hardly be found differences unless careful inspectors (Fig.18).

Fig. 18 left: occlusal opening on the tooth surface. right: filling back with resin.

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3.5 DNA extraction, amplification and STR genotyping

DNA was extracted using the QIAamp DNA Investigator Kit (Qiagen®, Maryland, USA). There were pulp tissue and odontoblastic powder (5 -10 mg) in one Eppendorf and cementum powder in another. They were processed in the same way as follows. Adding 360 uL Buffer ATL and 20 uL Proteinase K (20mg/mL) in the Eppendorf, be sure the samples were totally soaked in the buffer. Put the Eppendorf in a shaking incubator at 56°C overnight, then 300uL Buffer AL and 1uL Carrier RNA were added and returned to the shaking incubator at 70°C for 10 min. Samples were centrifuged at 20,000 g for 1min and the supernatant was transferred to new Eppendorf and mixed with 150 uL 99% Ethanol and added to spin columns. The columns were centrifuged at 6,000 g for 1min. The filter was washed with 500 uL AW1 buffer and centrifuged again. The filter was washed with 700 uL AW2 buffer and centrifuged again at 6,000 g for 1min. Adding 700 uL 99% Ethanol to the filter and centrifuged at 6,000g for 1min, and the filter was centrifuged at 20,000 g for another 1 min. The columns were transferred to the new Eppendorf, keep caps open to let residual Ethanol evaporate thoroughly at 56°C for 3min. Adding 50 uL pure water in the Eppendorf at room temperature for 1min and centrifuging at 20,000 g for 1min, the final elute DNA was 50uL and was put in the 4°C refrigerator for quantification and STR-typing .

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The quantification was performed using 7500 Real-Time PCR System (Thermo Fisher Scientific®, Maryland, USA) according to Applied Biosystem Quantifier® Human DNA Quantification Kit recommended protocol. PCR Amplification was performed using the Applied Biosystems 9700 thermal cycler (Thermo Fisher Scientific) using the GlobalFiler® PCR Amplification Kit (Thermo Fisher Scientific, Maryland, USA). PCR reaction preparation was as follows: GlobalFiler® Master Mix reaction volume 3.0 uL, GlobalFiler® Primer Set reaction volume 1.0 uL, double distilled H_2O (dd H_2O) reaction volume 2.0 uL, DNA sample input volume 4.0 uL (0.1 ng/ul), made a total volume of 10.0 uL. Replication condition: 95°C incubation 1min, in 29 cycles, 94°C denature 10 s, 59°C anneal/extend 1.5 min, 60°C final extension 60 min, forever 15°C. PCR products were electrophoresed using the Applied Biosystems 3500XL Genetic Analyzer (Thermo Fisher Scientific) , sized using the GeneScan® (Thermo Fisher Scientific) software and genotyped using the GeneMapper® ID-X Software (Thermo Fisher Scientific) , with a threshold of 500 relative fluorescence units (rfu) for heteozygous and 1500 rfu for homozygous. Data below this threshold were qualitatively noted, but not included in the data as observed/detected alleles. The number of alleles were calculated by manual inspection. Standards were as follows: green qualified, orange and red checked manually. The GlobalFiler® PCR Amplification Kit is a single

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multiplex assay that amplifies a set of 24 markers, with an amplification time of approximately 45-80 min [34]. The full complement of loci in the GlobalFiler® Kit are: D3S1358, vWA, D16S539, CSF1PO, TPOX, Yindel, Amelogenin, D8S1179, D21S11, D18S51, DYS391, D2S441, D19S433, TH01, FGA, D22S1045, D5S818, D13S317, D7S820, SE33, D10S1248, D1S1656, D125S391, D2S1338. In rare cases, the absence of the Amlogenin-Y allele from an unknown sample could lead to gender misidentification. The absence of the Y allele from a known male sample could be due to either a primer binding site variant or deletions of the allele on the Y-chromosome [36]. The GlobalFiler® Kit alleviates this risk by including two additional male identification markers, a Yindel and the Y-STR, DYS391, both located on the q arm of the Y-chromosome, while the Amelogenin gene in on the p arm of the Y-chromosome [38].

3.6 Case study

Four teeth of two forensic cases with seriously decomposed bodies of drowning were tested with the developed methods in this study. Samples were labeled as cases TA and TB.

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Chapter 4 Results

The DNA quantities of group A of the 9 teeth samples were between 0.5 ng of minimum and 8690 ng of maximum for pulp sampling, and 12 ng of minimum and 2035 ng of maximum for cementum sampling. These teeth were intact, no cavities and kept dried indoors. The DNA quantity and quality of group A were good in both samplings. The success rate of positive amplification of this group is 100% (24/24). The results show no DNA degradation via microbial invasion through root apical foramens. The variation of the DNA contents is between individuals and between teeth health conditions (Table 3). Pulp sampling and cementum sampling of genotypes of A4 tooth were shown on Fig.19 and Fig.20 respectively.

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Table 3 The quantity of DNA and success rates of STR typing of group A in pulp and cementum sampling. N=9, mixed teeth, air-dried, 3 months. * means root tip fracture

sample	FDI tooth position notation	DNA quantity(ng) pulp	STR-typing (GlobalFiler® kit) pulp	DNA quantity(ng) cementum	STR-typing (GlobalFiler® kit) cementum
A1*	#16	43.5	24/24	2035	24/24
A2	#45	890	24/24	15	24/24
A3*	#17	8690	24/24	720	24/24
A4	#17	8.35	24/24	169.5	24/24
A5	#21	0.5	24/24	556.5	24/24
A6	#37	448	24/24	445	24/24
A7	#47	1.95	24/24	140	24/24
A8	#16	929.5	24/24	12	24/24
A9	#26	3300	24/24	1740	24/24
mean		1590	24/24	648	24/24

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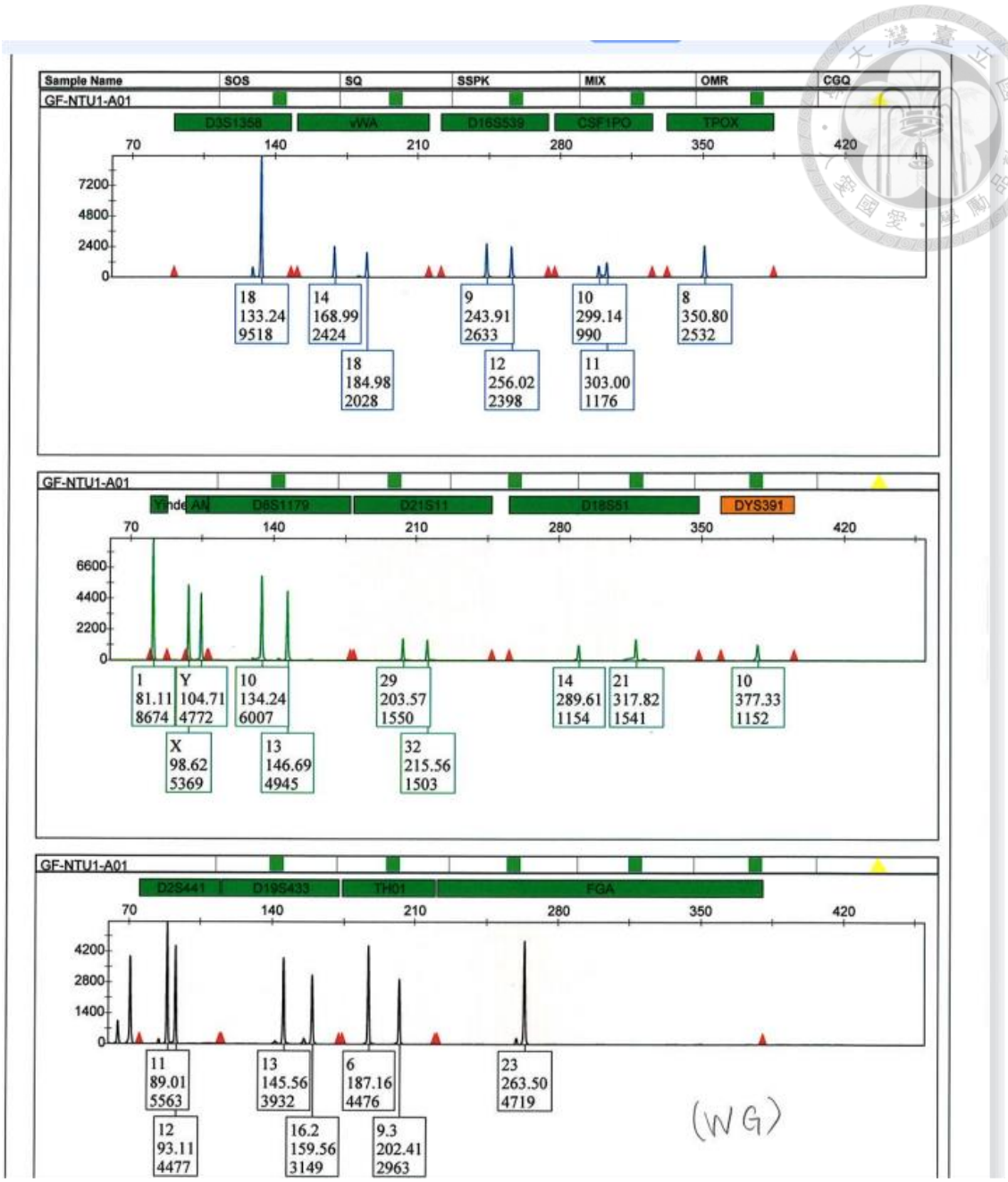

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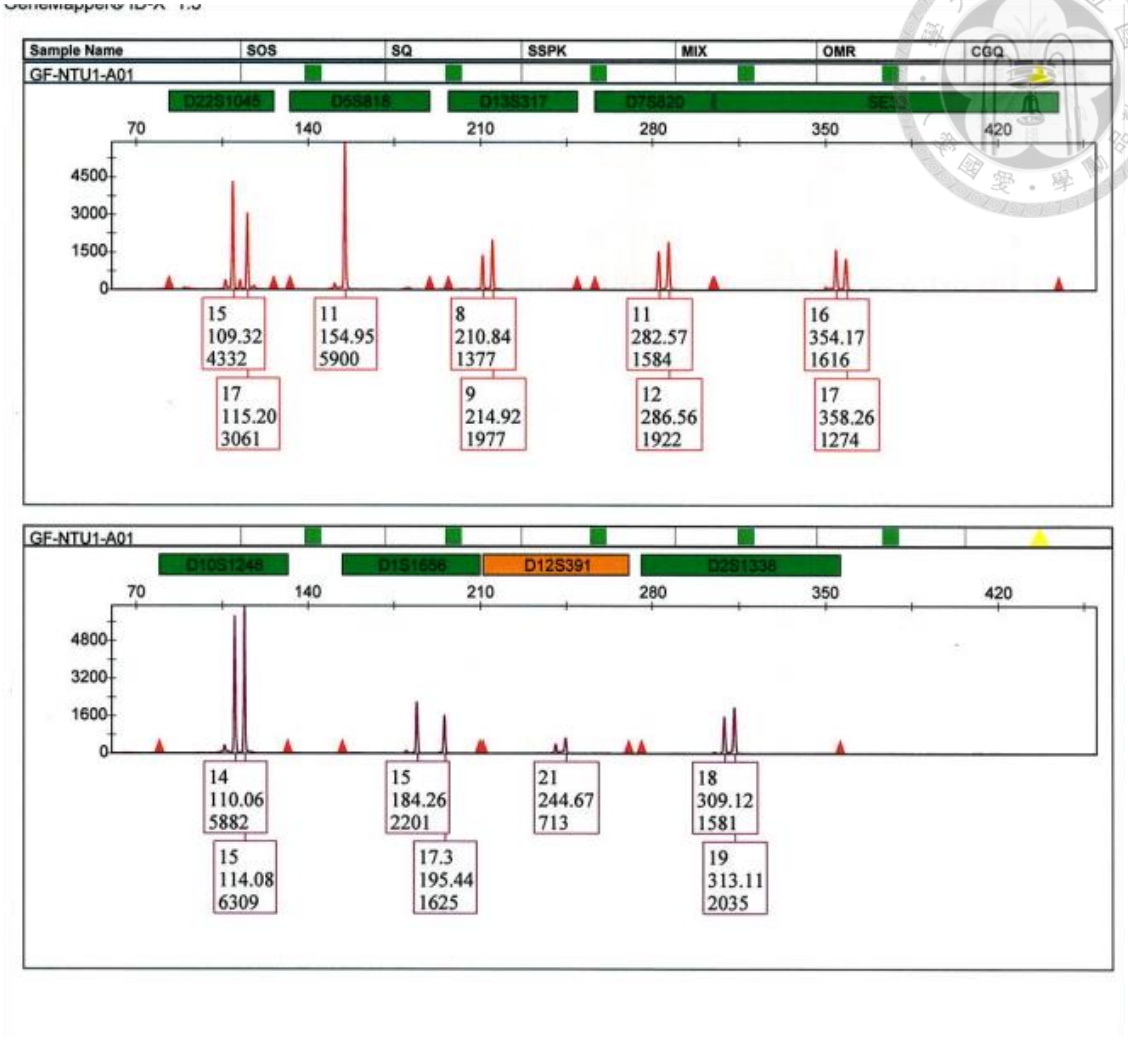

Fig. 19 GlobalFiler® electropherogram of STR genotyping from pulp sampling of sample A4 (NTU-1).

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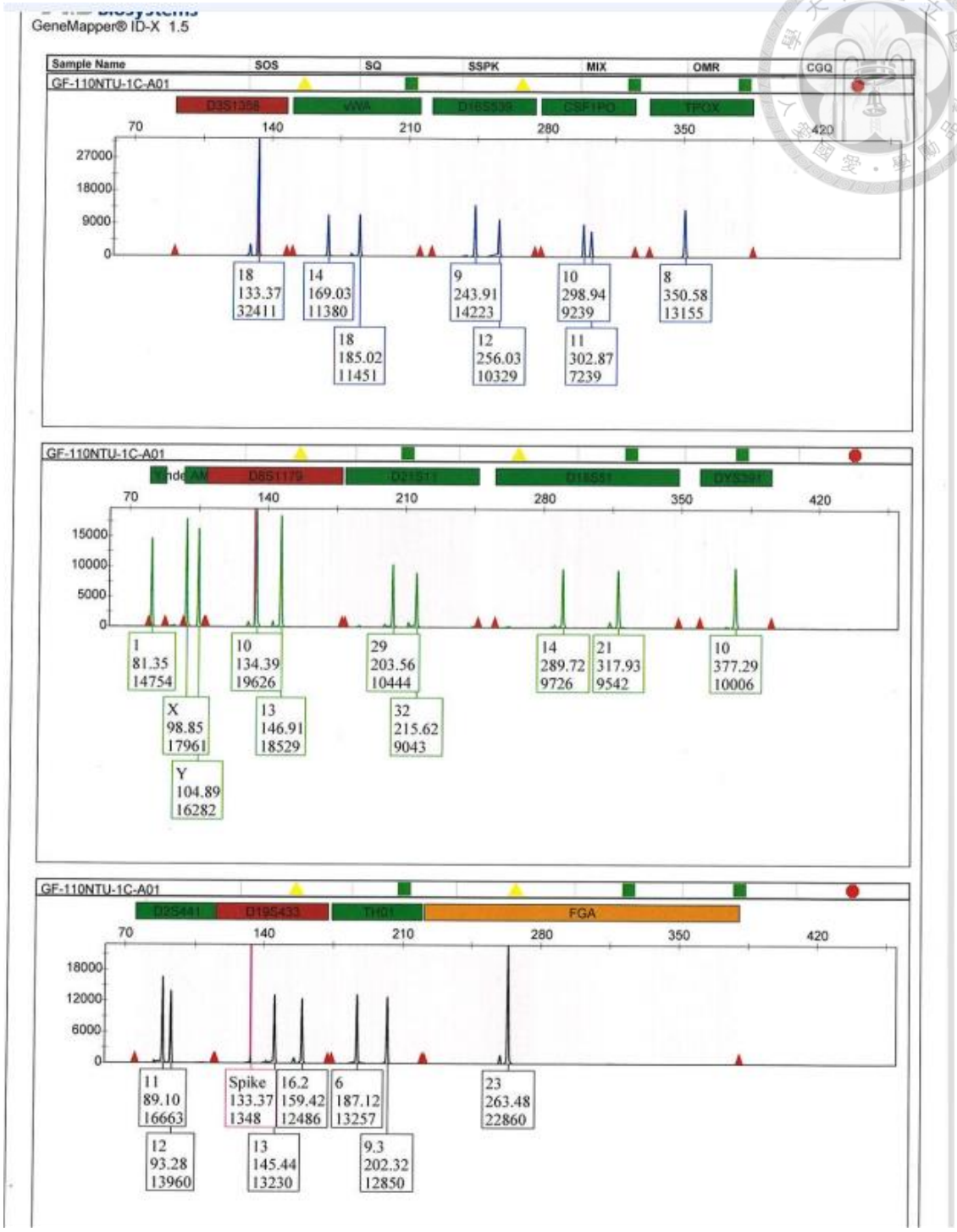

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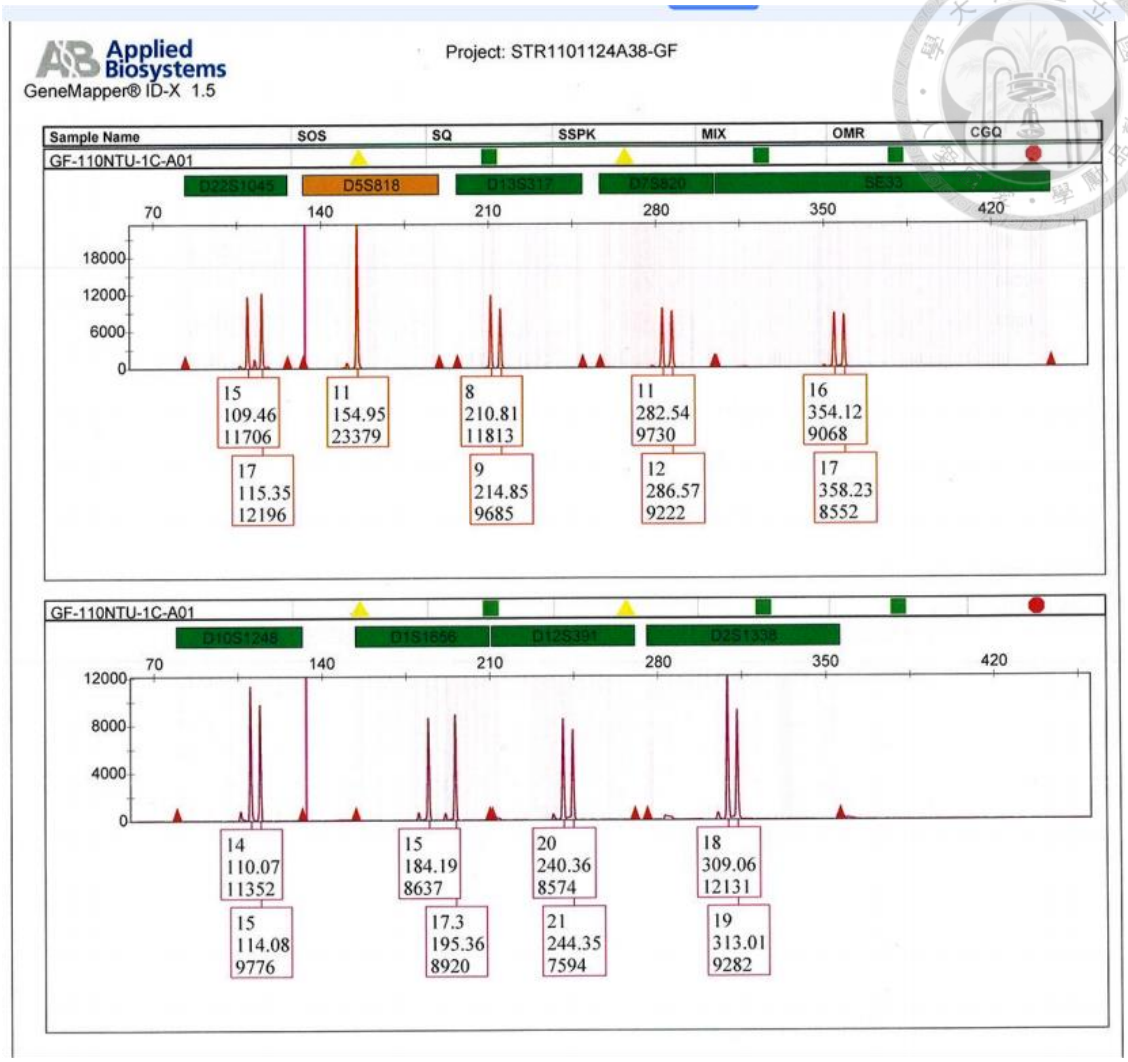

Fig. 20 GlobalFiler® electropherogram of STR genotyping from cementum sampling of sample A4 (NTU-1).

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The DNA quantities of group B of the 10 samples changed between undetected (UD) and 6.0 ng of maximum for pulp sampling and 0.05 ng of minimum and 0.5 ng of maximum for cementum sampling. Of them B2 and B4 the tooth canals were obstructed that meant pulp and dentin powder collection could not be done. This group of teeth were incisors (including canine) and were buried in the soil for 3 months. These teeth were intact, no cavities were notified. The mean success rate of positive amplification of this group is 68% (16.2/24) for pulp sampling and 100% (24/24) for cementum sampling (Table 4). Pulp sampling and cementum sampling of genotypes of B10 tooth were shown on Fig.21 and Fig.22 respectively.

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Table 4 The quantity of DNA and success rates of STR typing of group B in pulp and cementum sampling. N=10. * means root tip fracture (2-3 mm). UD: undetected.This group B is incisors.

sample	FDI tooth position notation	DNA quantity(ng) pulp	STR-typing (GlobalFiler®kit) pulp	DNA quantity(ng) cementum	STR-typing (GlobalFiler® kit) cementum
B1*	#13	1.5	24/24	0.4	24/24
B2	#32	UD	0/24	0.5	24/24
B3*	#11	1.0	18/24	0.5	24/24
B4	#21	UD	0/24	0.15	24/24
B5*	#23	1.5	24/24	0.05	24/24
B6	#42	3.5	24/24	0.05	24/24
B7*	#13	1.0	24/24	0.1	24/24
B8*	#11	3.75	24/24	0.1	24/24
B9	#11	UD	0/24	0.05	24/24
B10	#11	6.0	24/24	0.2	24/24
mean		1.83	16.2/24	0.22	24/24

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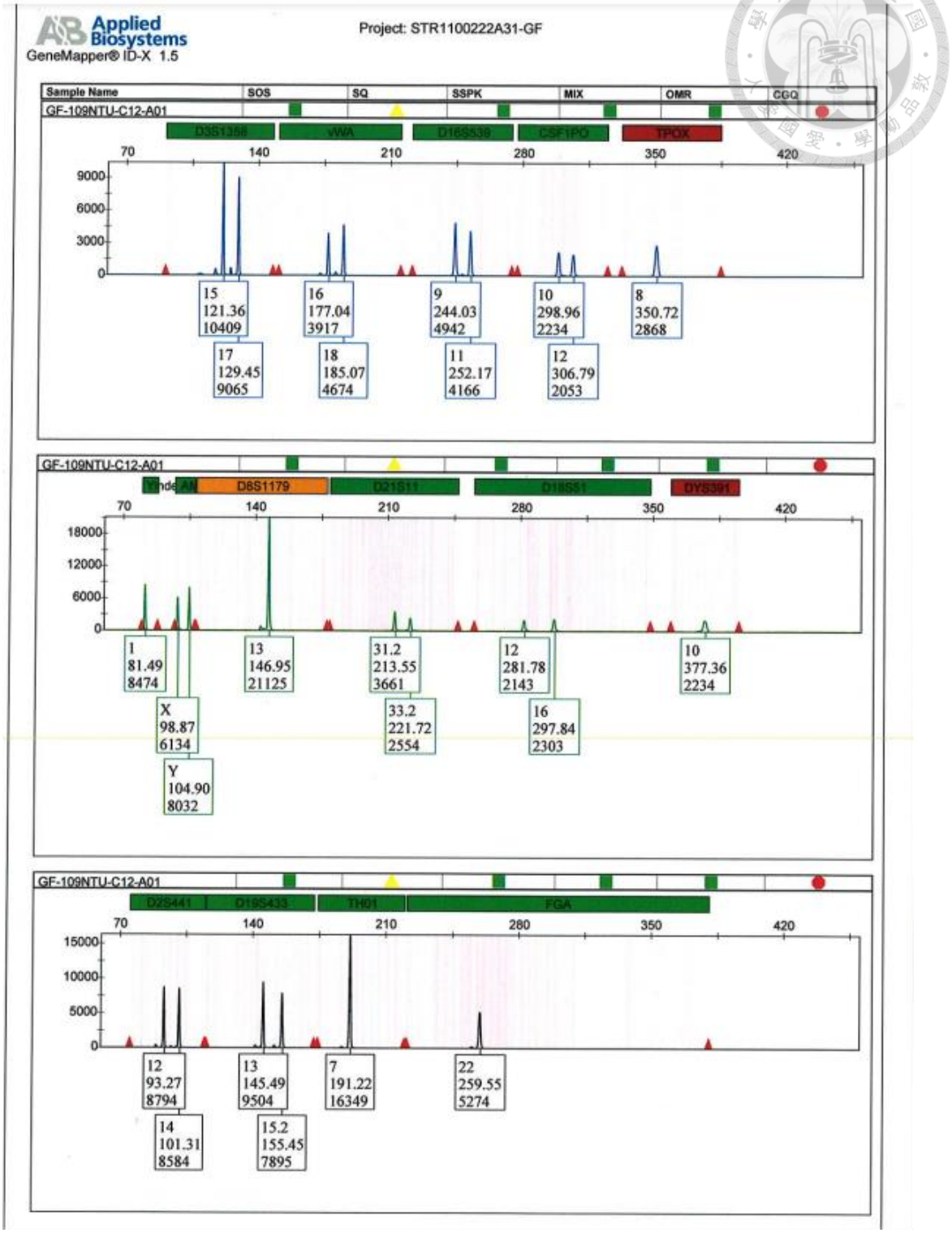

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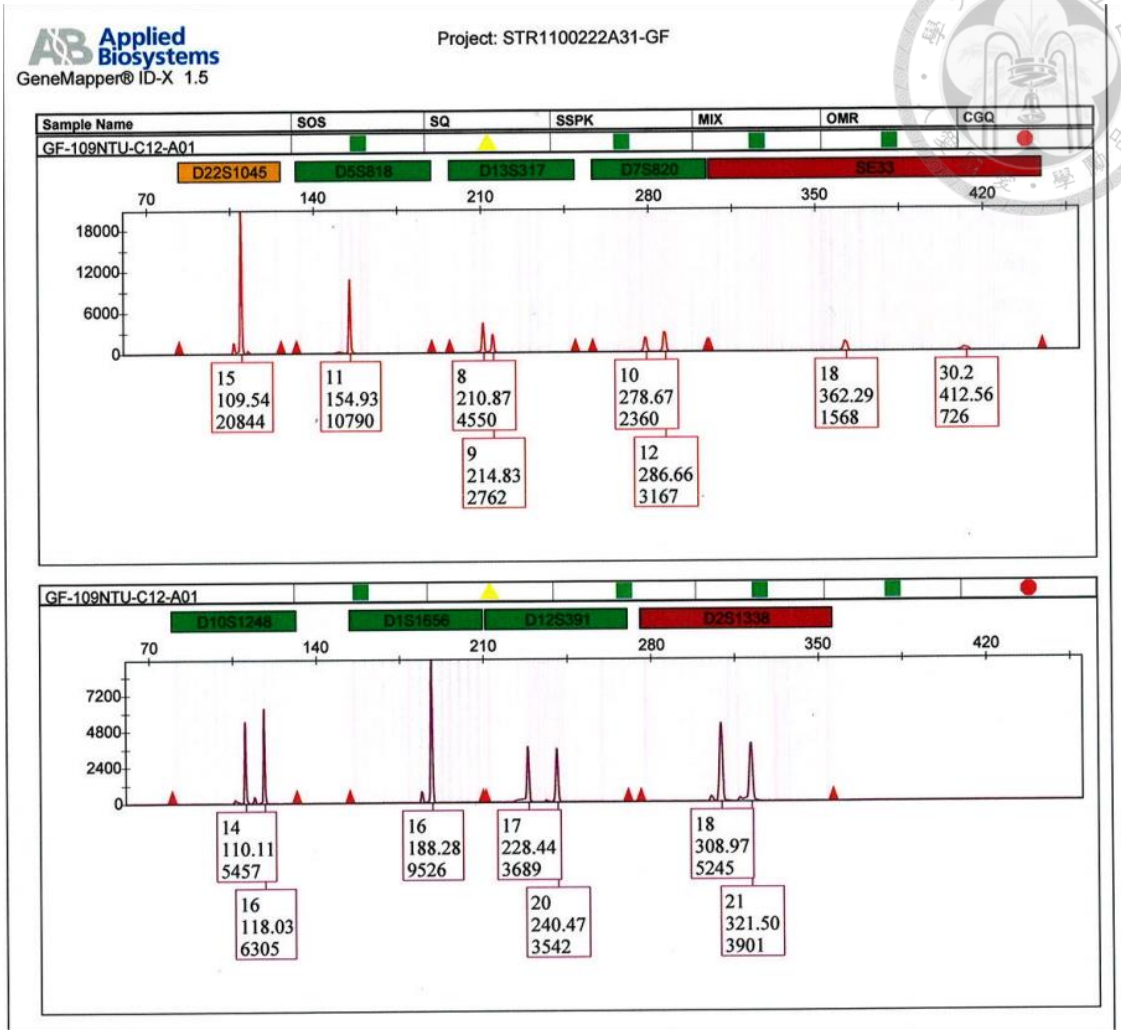

Fig. 21 GlobalFiler® electropherogram of STR genotyping from pulp sampling of sample B10 (NTU-C12)

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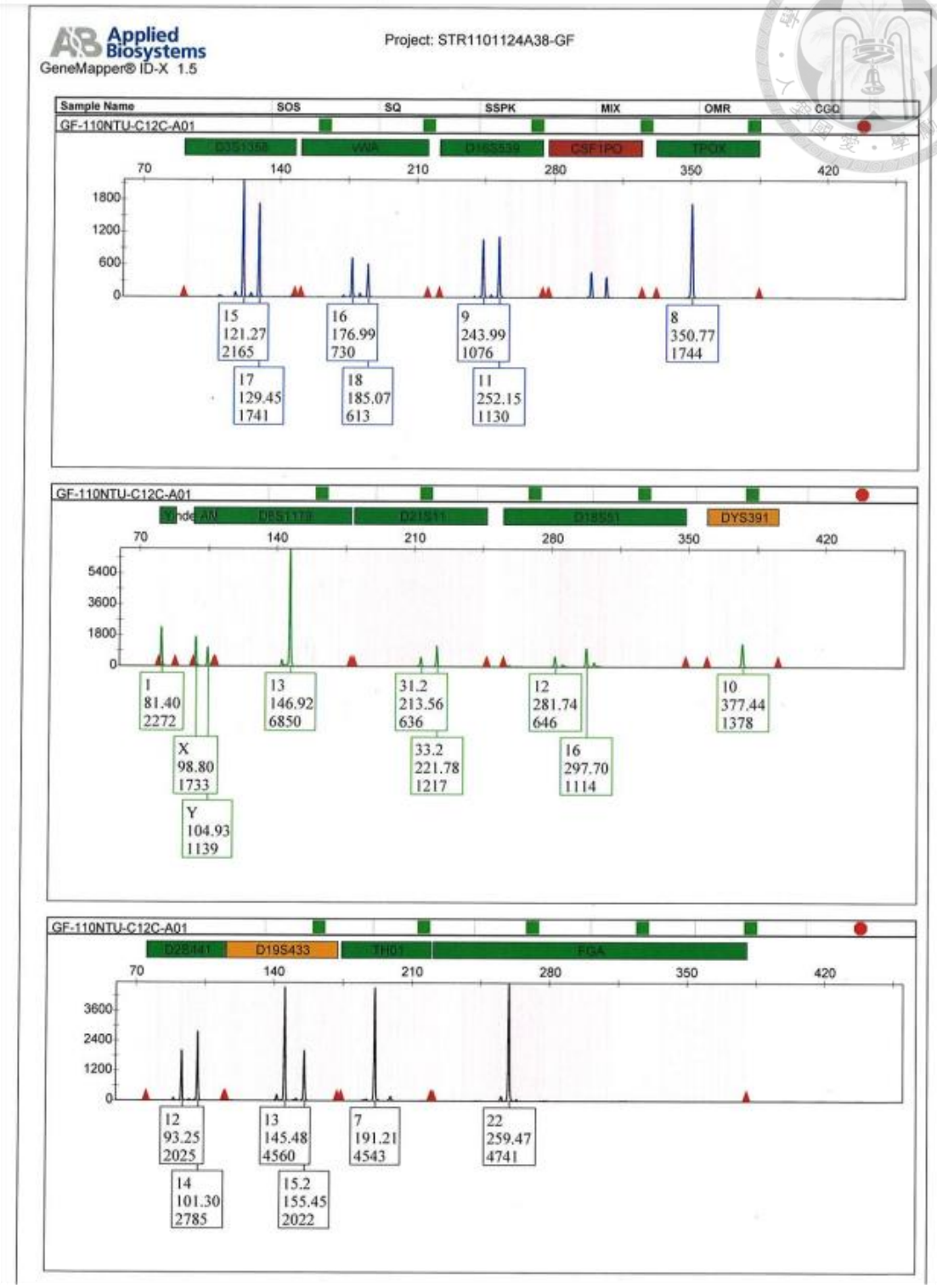

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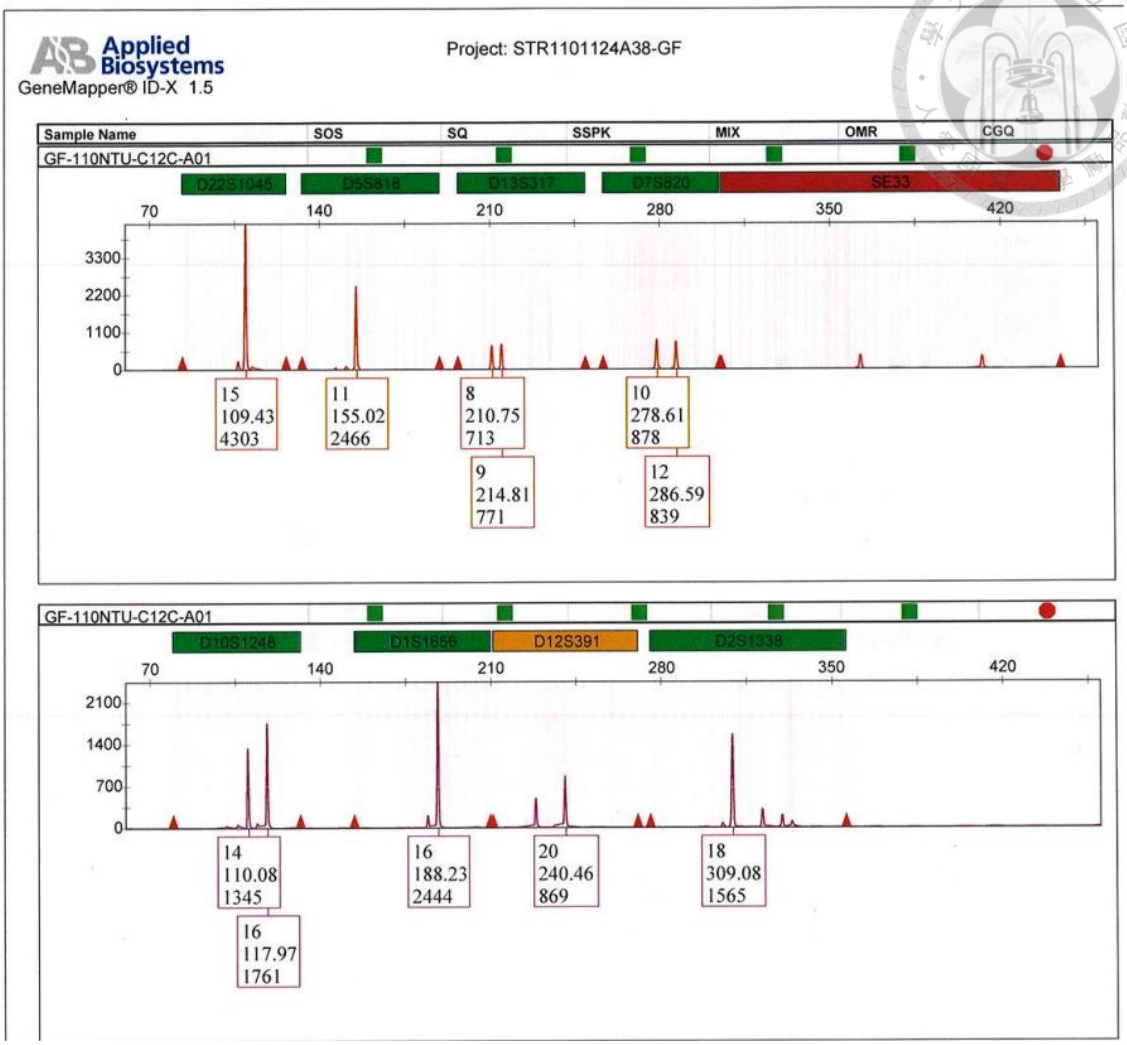

Fig. 22 GlobalFiler® electropherogram of STR genotyping from cementum sampling of sample B10 (NTU-C12)

doi:10.6342/NTU202301086

The quantities of DNA in group C varied between UD and 70 ng of maximum in pulp sampling, and UD of minimum and 1.3 ng of maximum in cementum. This group of teeth were premolars and buried in the soil for 3 months. They were intact, no cavities were detected. The success rate of positive amplification of this group in pulp sampling is 77% (18.5/24) and 100% (24/24) in cementum sampling (Table 5). Pulp sampling and cementum sampling of genotypes of C8 tooth were shown on Fig.23 and Fig.24 respectively.

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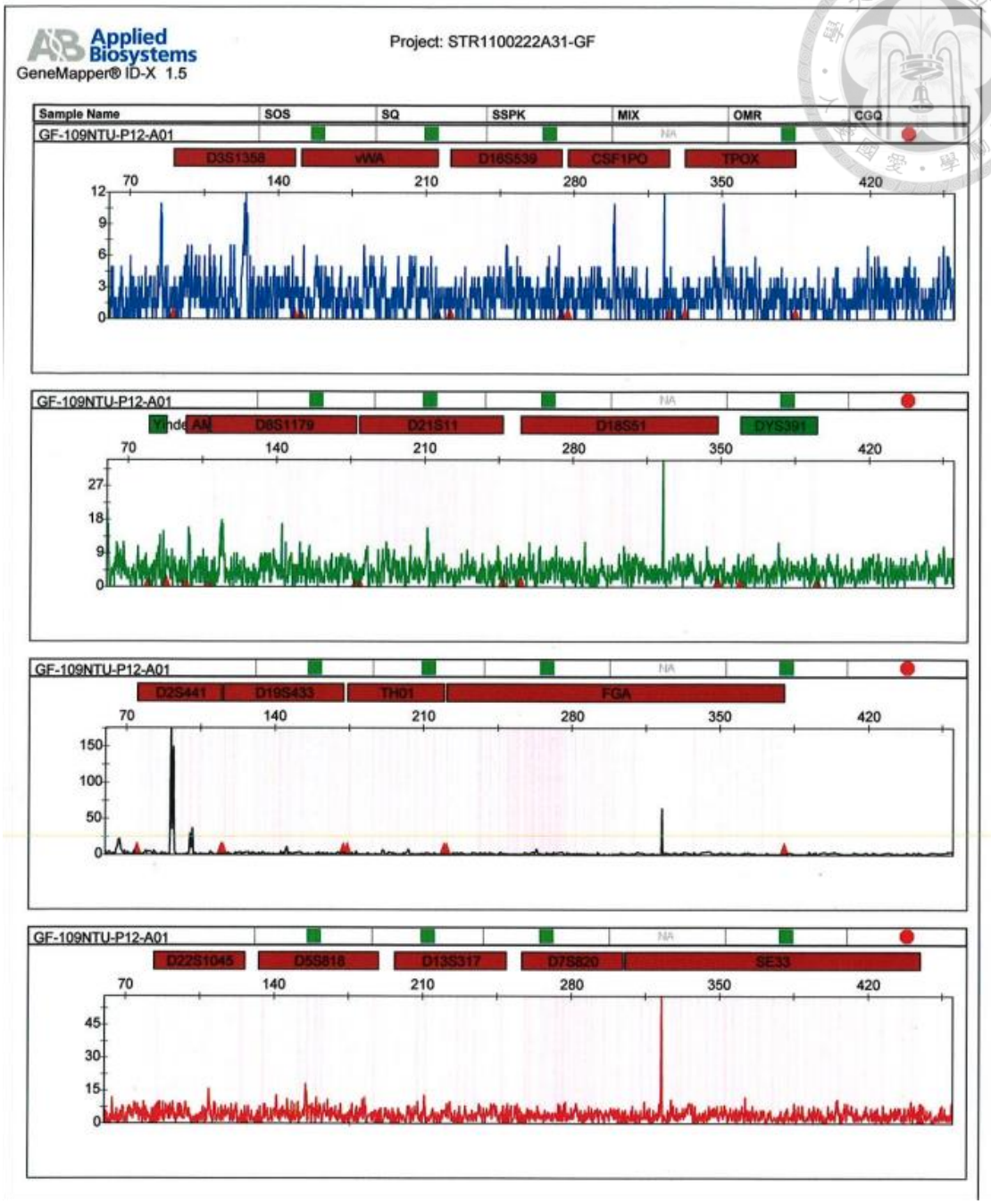
Table 5 The quantity of DNA and success rates of STR typing of group C in pulp and cementum sampling. This group C is premolars, buried in the soil for 3 months, N=8 , * means root tip fracture. UD: undetected.

sample	FDI tooth position notation	DNA quantity(ng) pulp	STR-typing (GlobalFiler® kit) pulp	DNA quantity(ng) cementum	STR-typing (GlobalFiler® kit) cementum
C1*	#45	13	24/24	0.4	24/24
C2	#44	1.5	24/24	0.9	24/24
C3*	#45	1.5	24/24	UD	24/24
C4*	#14	UD	4/24	0.05	24/24
C5	#45	23	24/24	0.35	24/24
C6	#15	60	24/24	1.3	24/24
C7	#14	70	24/24	0.3	24/24
C8	#14	0.5	0/24	0.45	24/24
mean		21.4	18.5/24	0.47	24/24

doi:10.6342/NTU202301086



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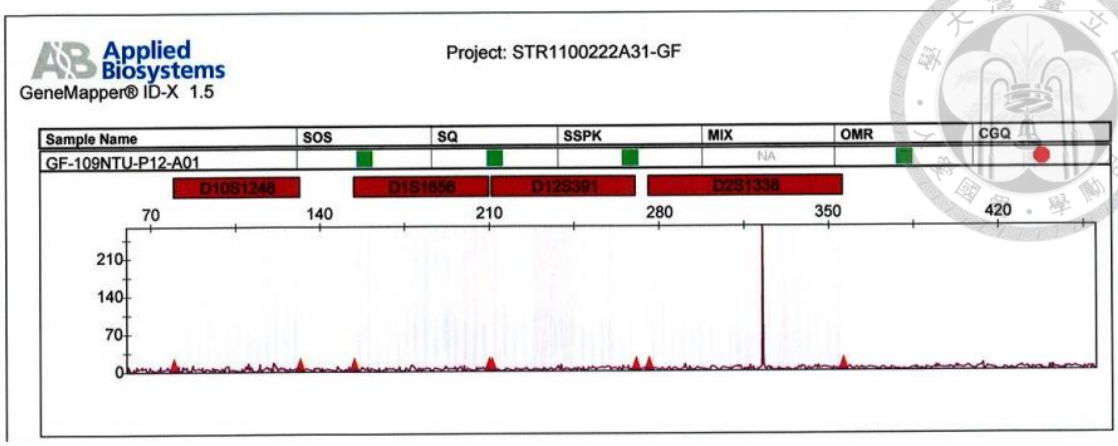

Fig. 23 GlobalFiler® electropherogram of STR genotyping from pulp sampling of sample C8 (NTU-P12)

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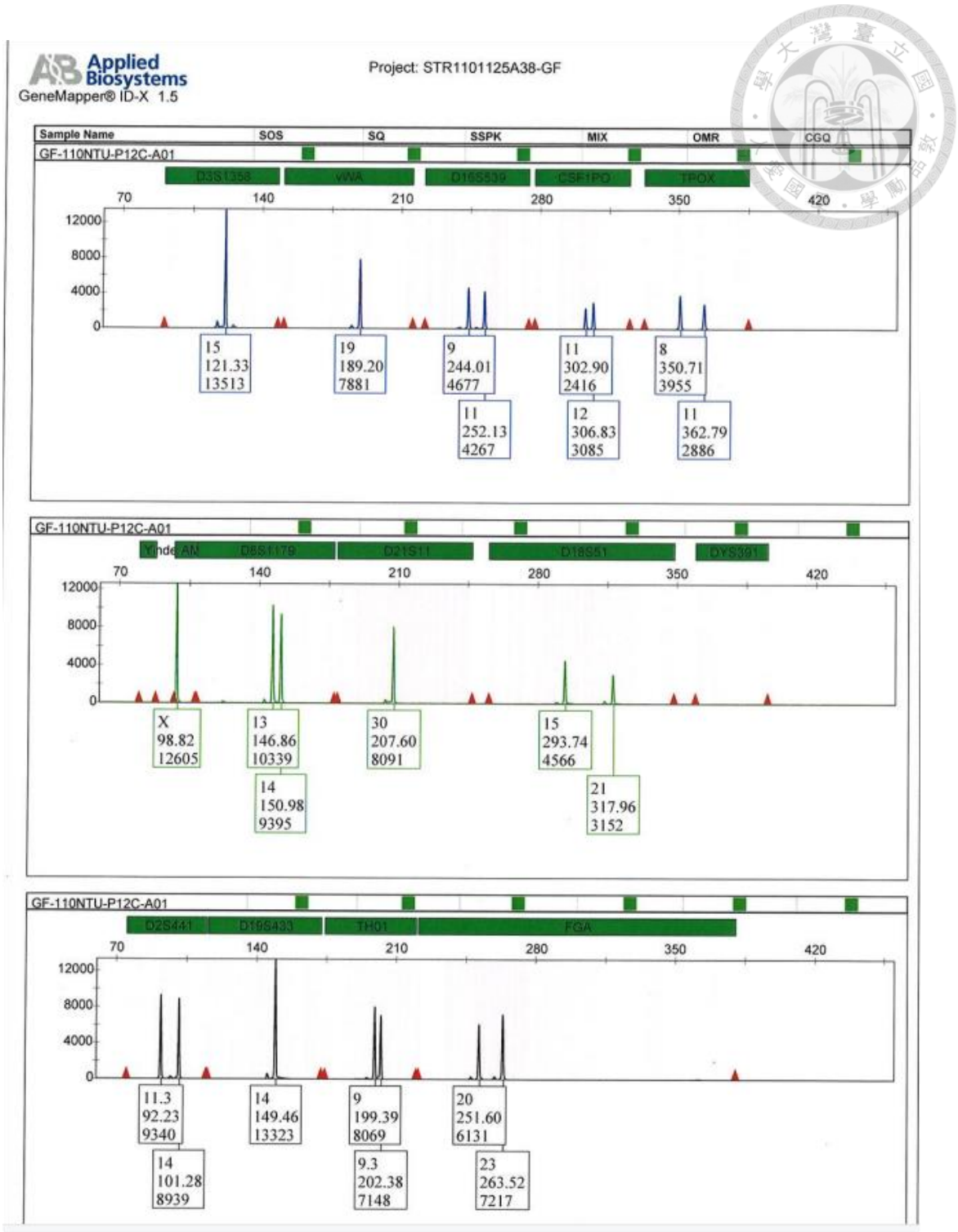

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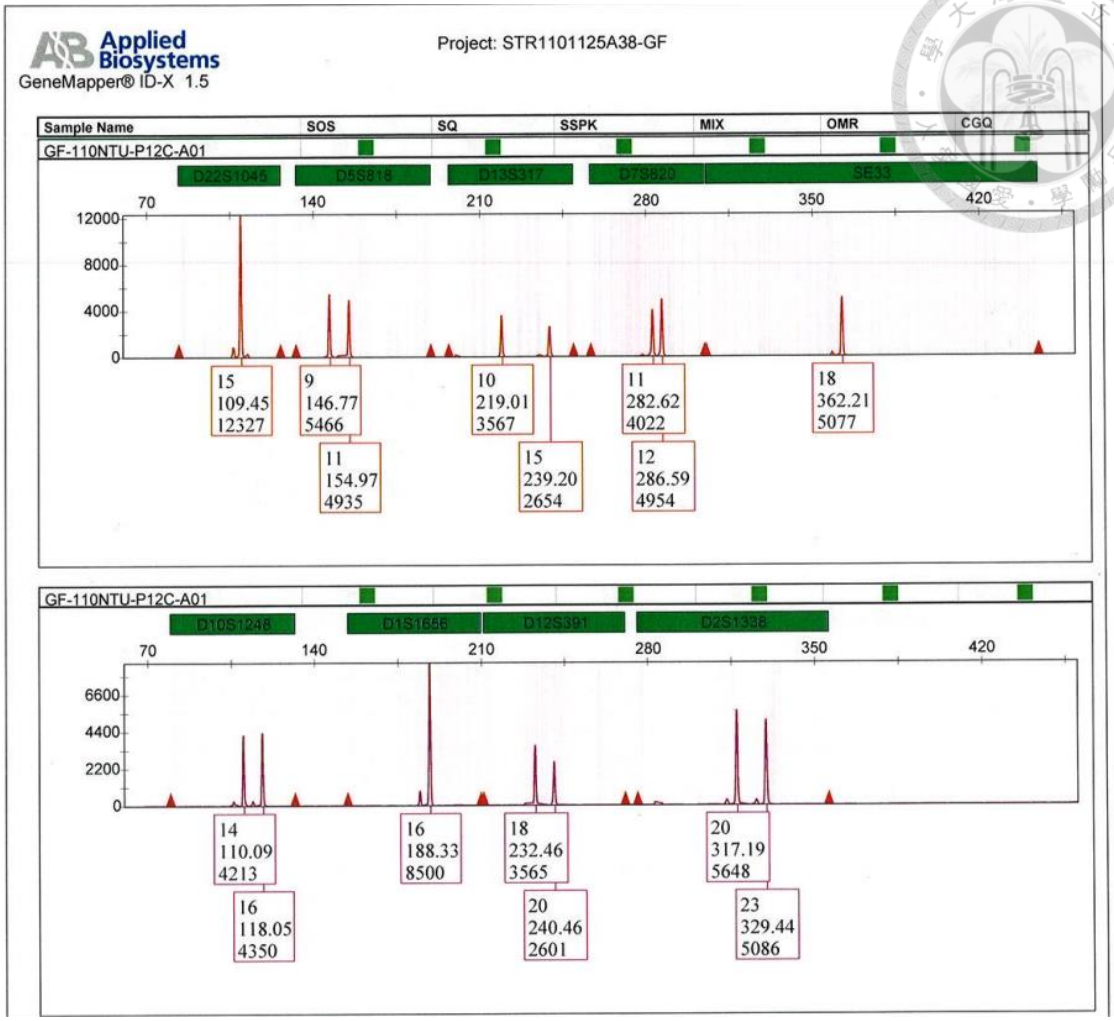

Fig. 24 GlobalFiler® electropherogram of STR genotyping from cementum sampling of sample C8 (NTU-P12)

doi:10.6342/NTU202301086

The DNA quantities of group D of the 13 samples (Table 6) changed between UD and 92.5 ng of maximum in pulp sampling, and UD of minimum and 5.1 ng of maximum in cementum sampling. This group of teeth were molars and buried in the soil for 3 months. They were intact, no cavities were noted. The success rate of positive amplification of this group is 87% (20.9/24) in pulp sampling, and 98% (23.6/24) in cementum sampling. Pulp sampling and cementum sampling of genotypes of D10 tooth were shown on Fig.25 and Fig.26 respectively.

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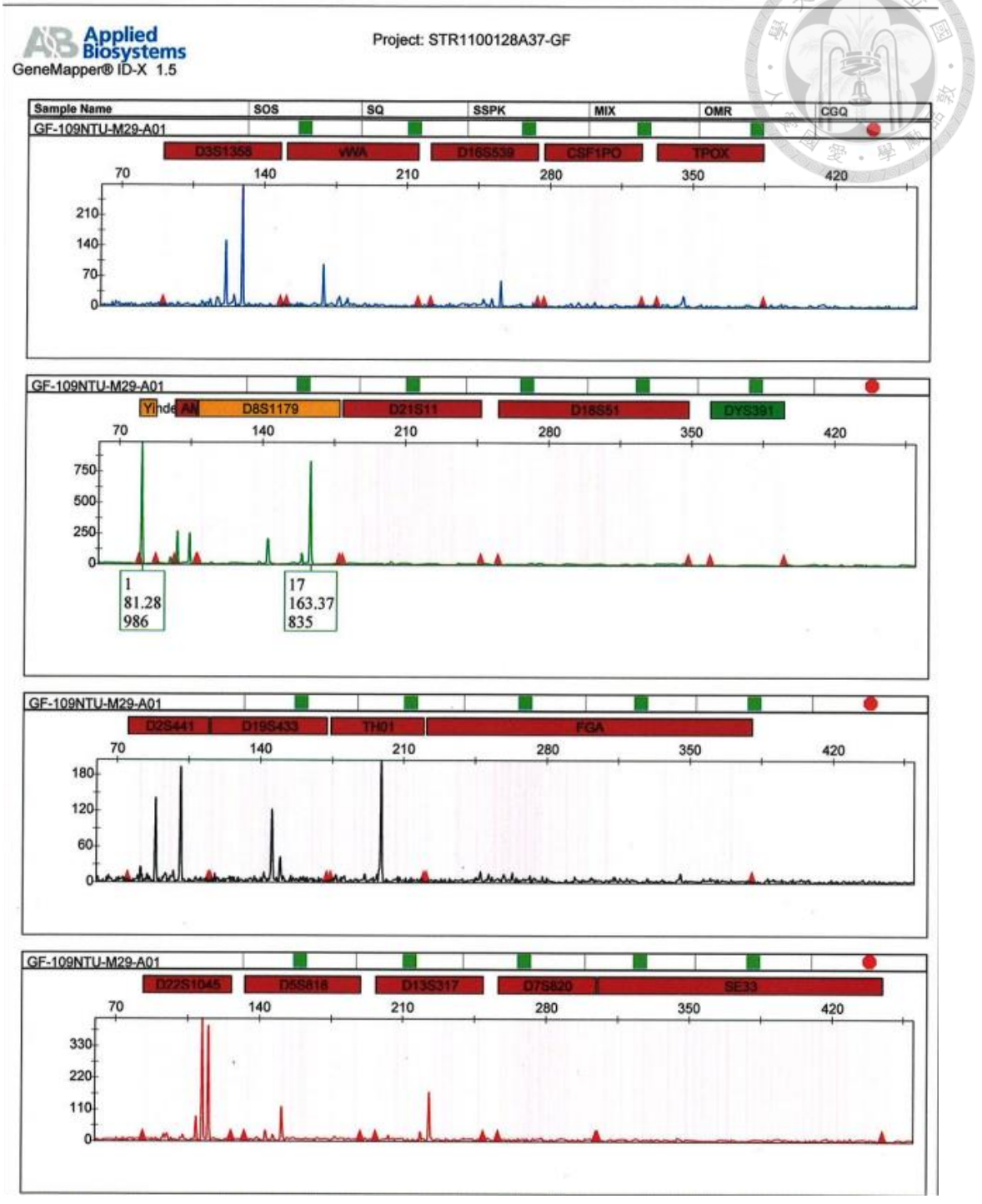
Table 6 The quantity of DNA and success rates of STR typing of group D in pulp and cementum sampling. This group D is molars , N=13. * means root tip fracture . UD: undetected.

sample	FDI tooth position notation	DNA quantity(ng) pulp	STR-typing (GlobalFiler® kit) pulp	DNA quantity(ng) cementum	STR-typing (GlobalFiler®kit), cementum
D1*	#18	7.5	24/24	4.5	24/24
D2*	#47	13	24/24	1.4	24/24
D3	#16	32	24/24	1.8	24/24
D4	#18	92.5	24/24	0.6	24/24
D5*	#18	1.5	2424	0.45	24/24
D6*	#48	4.5	24/24	5.1	24/24
D7	#18	1.0	24/24	0.3	24/24
D8*	#48	0.5	10/24	1.6	24/24
D9	#36	UD	18/24	UD	19/24
D10	#46	UD	14/24	0.5	24/24
D11*	#47	UD	13/24	3.95	24/24
D12*	#36	1.5	21/24	0.1	24/24
D13	#26	0.75	24/24	0.5	24/24
mean		11.9	20.9/24	1.6	23.6/24

doi:10.6342/NTU202301086



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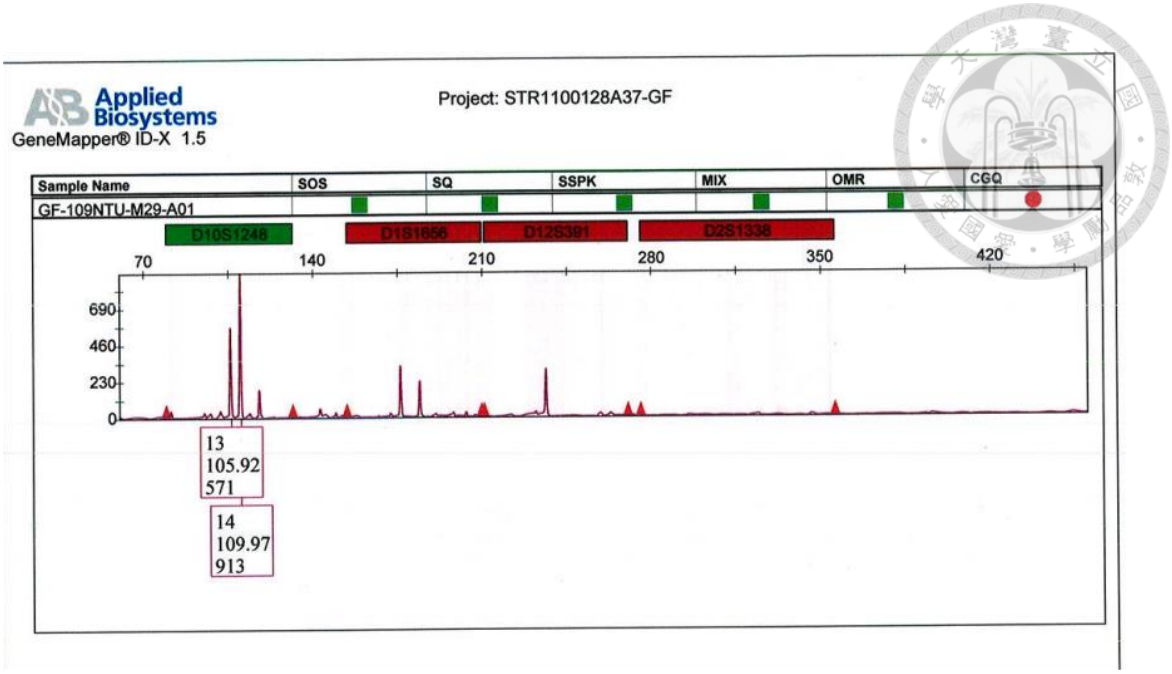

Fig. 25 GlobalFiler® electropherogram of STR genotyping from pulp sampling of sample D10 (NTU-M29)

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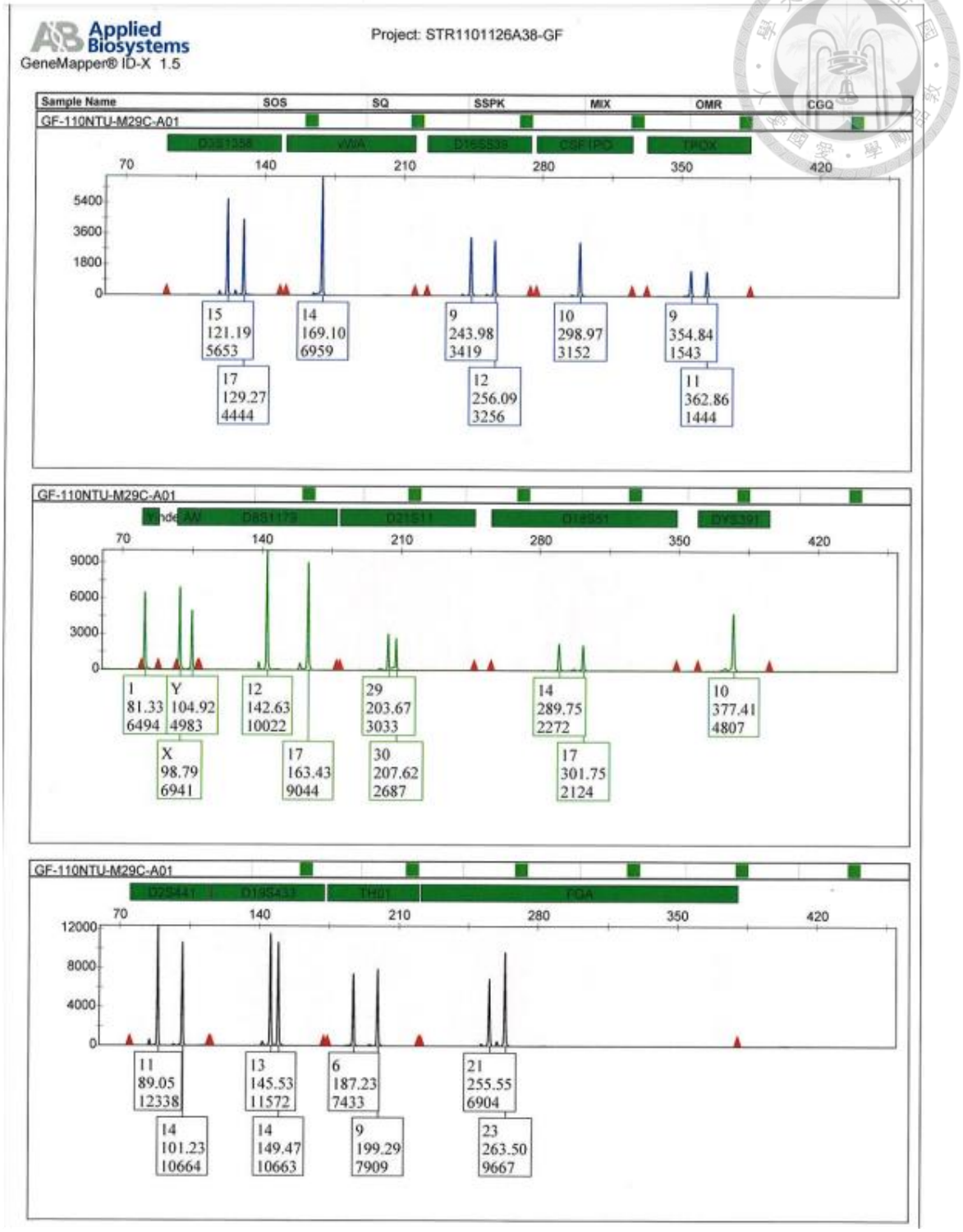

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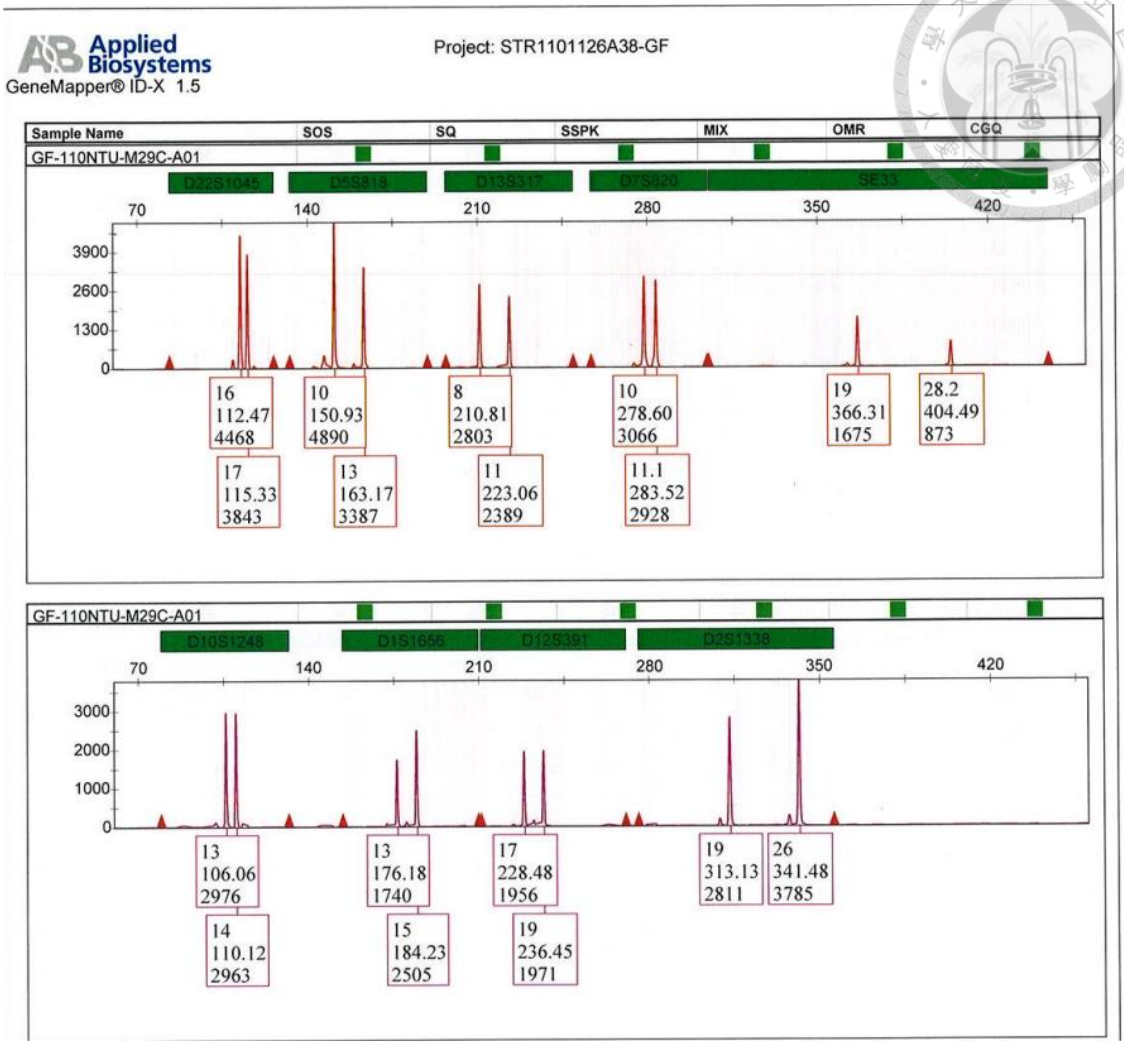

Fig. 26 GlobalFiler® electropherogram of STR genotyping from cementum sampling of sample D10 (NTU-M29)

doi:10.6342/NTU202301086

The DNA quantities of the group E of 18 tooth samples (Table 7) were from UD of minimum to 1.7 ng of maximum in pulp sampling and from UD of minimum to 15.6 ng of maximum in cementum sampling. These teeth included all tooth types with obvious caries involved pulp, were buried in the soil for 3 months. The success rate of positive amplification of this group in pulp sampling was 62% (14. 9/24), while 100% (24/24) in cementum sampling.Pulp sampling and cementum sampling of genotypes of E9 tooth were shown on Fig.27 and Fig.28 respectively.

doi:10.6342/NTU202301086

Table 7 The quantity of DNA and success rates of STR typing of group E in pulp and cementum sampling. In this group E, there are 18 teeth including incisor, premolar, and molar. They have cavities, buried in the soil for 3 months. * means root tip fracture. UD: undetected.

sample	FDI tooth position notation	DNA quantity(ng) pulp	STR-typing (GlobalFiler® kit) pulp	DNA quantity(ng) cementum	STR-typing (GlobalFiler®kit) cementum
E1*	#11	1.0	24/24	0.7	24/24
E2	#35	0.5	24/24	0.1	24/24
E3	#16	0.5	24/24	2.65	24/24
E4*	#38	UD	20/24	1.25	24/24
E5	#46	UD	0/24	1.4	24/24
E6*	#16	UD	11/24	0.15	24/24
E7	#46	UD	9/24	0.8	24/24
E8	#28	UD	15/24	14.35	24/24
E9*	#36	UD	18/24	1.0	24/24
E10	#26	0.5	13/24	1.4	24/24
E11*	#46	UD	4/24	UD	24/24
E12	#16	1.7	24/24	5.8	24/24
E13	#46	1.2	11/24	0.35	24/24
E14	#16	0.75	16/24	0.1	22/24
E15*	#48	0.75	7/24	15.6	24/24
E16	#28	0.25	9/24	2.55	24/24
E17*	#48	0.2	16/24	0.45	24/24
E18	#35	0.3	24/24	6.25	24/24
mean		0.42	14.9/24	3.0	24/24

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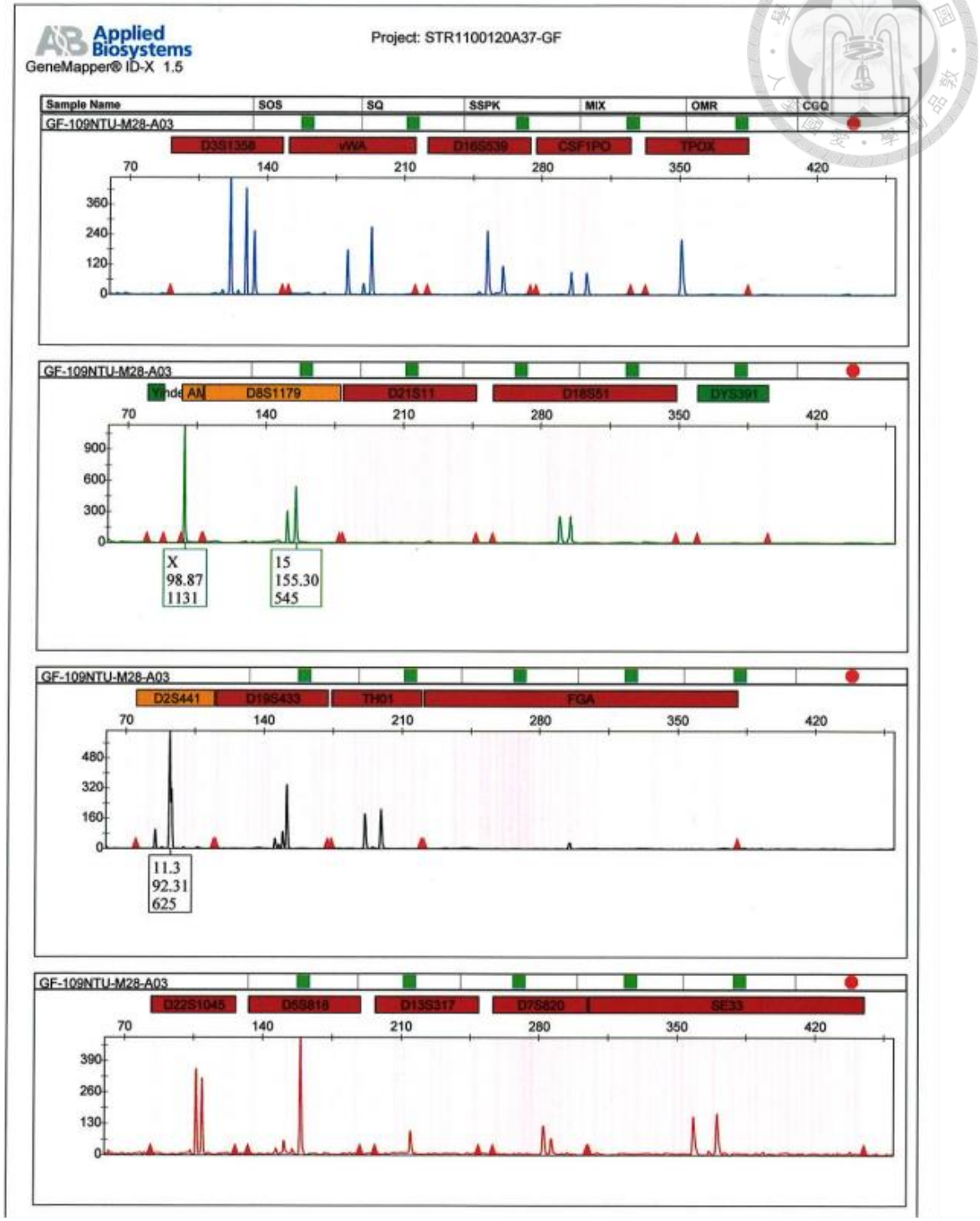

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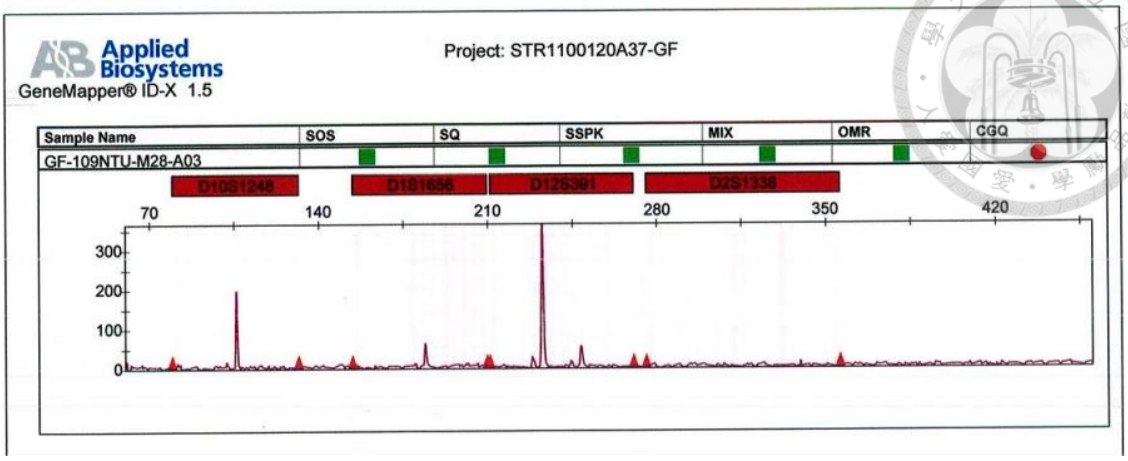

Fig. 27 GlobalFiler® electropherogram of STR genotyping from pulp sampling of sample E9 (NTU-M28)

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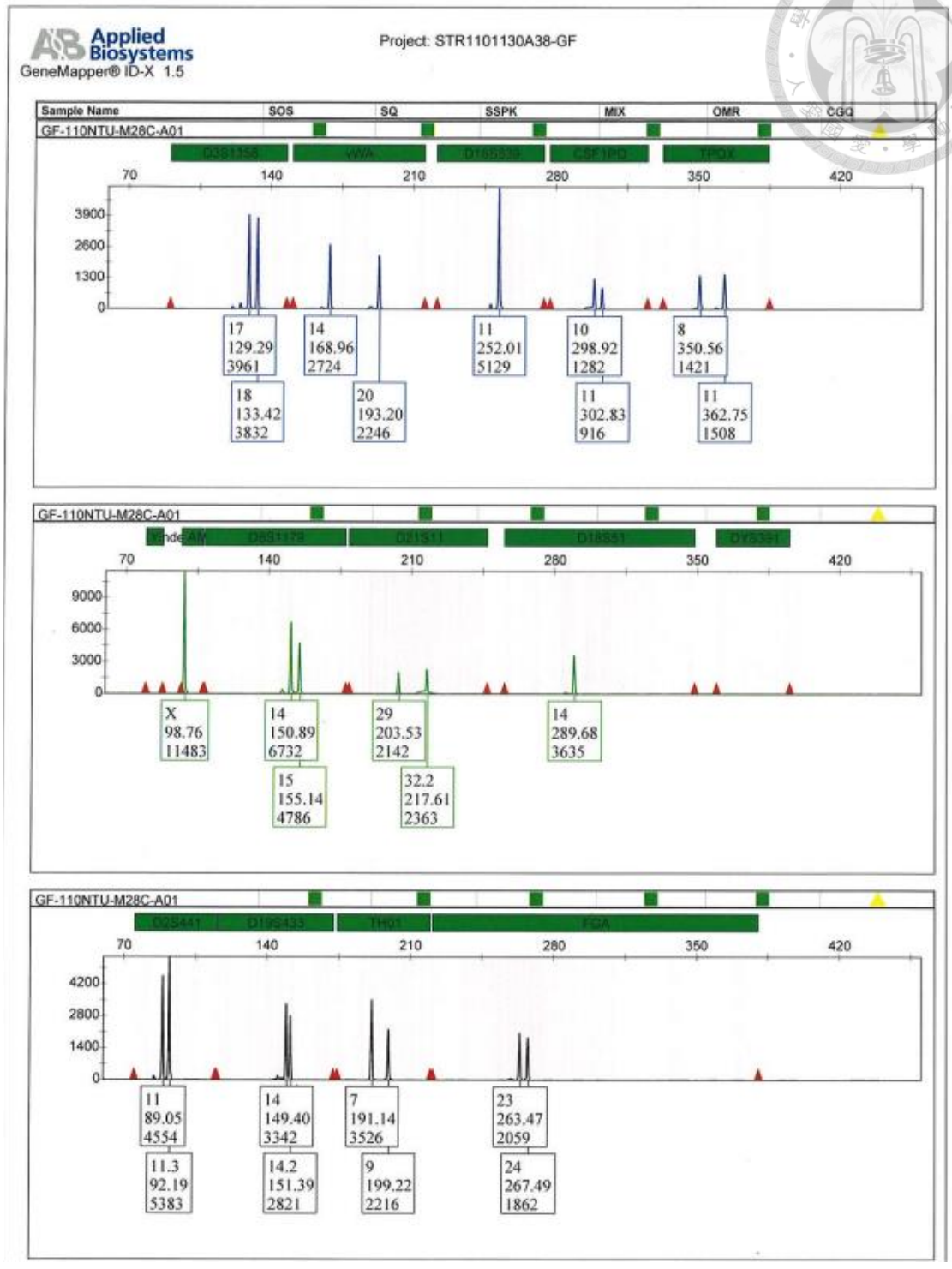

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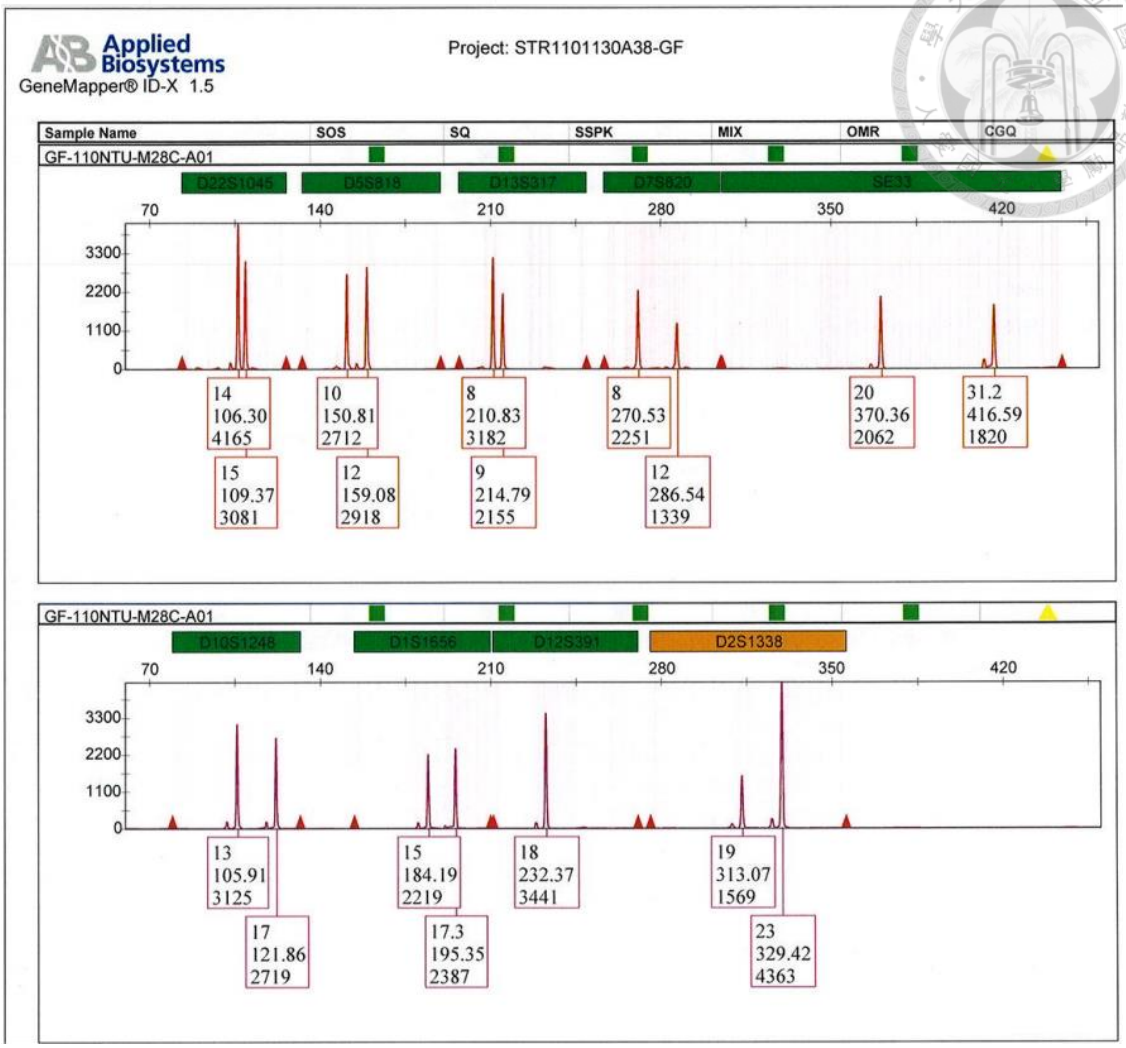

Fig. 28 GlobalFiler® electropherogram of STR genotyping from cementum sampling of sample E9 (NTU-M28)

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The content of DNA of the teeth buried in the soil was obviously less than those kept in the air. The influence of microbials on DNA degradation was obvious. It reduced DNA yields by nearly two orders of magnitude. During the period of 92 days the teeth were buried in the soil, it rained 10 days and the other days were sunny. The success rate for STR-typing in group E was 62% (14.9/24) and mean DNA yield quantity was 0.43 ng by pulp sampling. It indicated that pulp necrosis happened already because of large cavity and further degraded by microbes when buried in the soil, while cementocytes in the cementum were well protected and it showed 100% (24/24) success rate and 3.0ng DNA yield. The overall results of success rate of STR-typing and DNA yield quantity for pulp and cementum of the same tooth were in Table 8.

The results of four teeth of two forensic cases labeled TA and TB were shown in Table 9. Pulp sampling and cementum sampling of genotypes of TA1 tooth were shown on Fig. 29-30.

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Table 8 Average of DNA quantity and success rate of STR genotyping in pulp and cementum samplings among 5 groups.

Group	Average of DNA quantity in		Success rate of STR genotyping in 24 loci	
	Pulp (ng)	Cementum (ng)	Pulp (%)	Cementum (%)
A (mixed, air)	1,590	648	100	100
B (incisor, soil)	1.83	0.22	68	100
C (premolar, soil)	21.4	0.47	77	100
D (molar, soil)	11.9	1.6	87	98
E (cavity,mixed, soil)	0.42	3	62	100
B-E	7.2	1.65	72.4	99.5

Table 9 TA, TB two drown victims of real forensic cases, cementum sampling showed excellent results over pulp sampling.

sample	FDI tooth position notation	DNA quantity(ng) pulp	STR-typing (GlobalFiler® kit) pulp	DNA quantity(ng) cementum	STR-typing (GlobalFile®kit) cementum
TA1	#26	0.25	15/24	0.45	24/24
TA2*	#36	0.65	0/24	2.35	24/24
TA3*	#45	UD	21/24	0.05	24/24
TB	#24	0.15	0/24	0.3	24/24
mean		0.26	9/24	0.79	24/24

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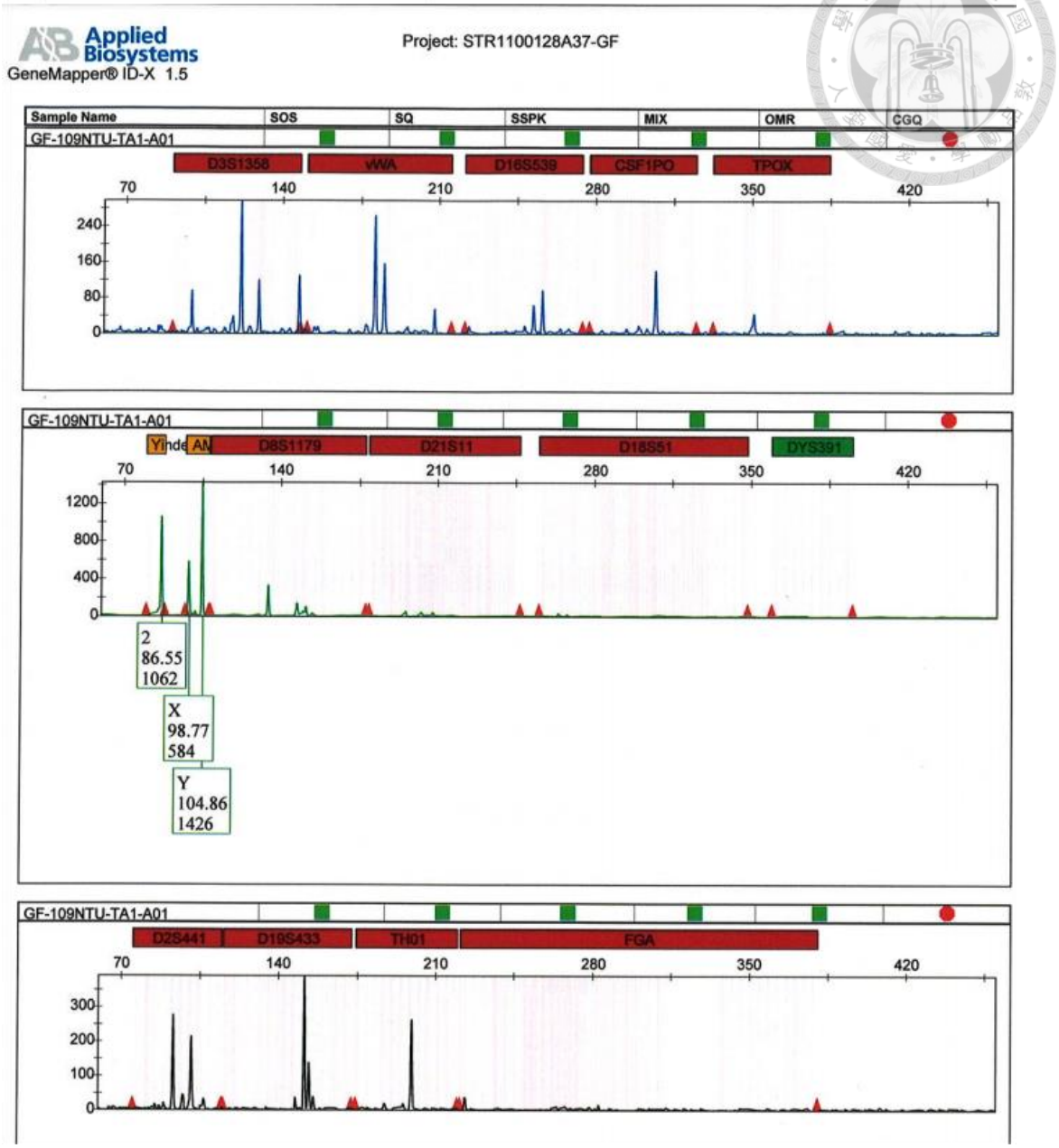

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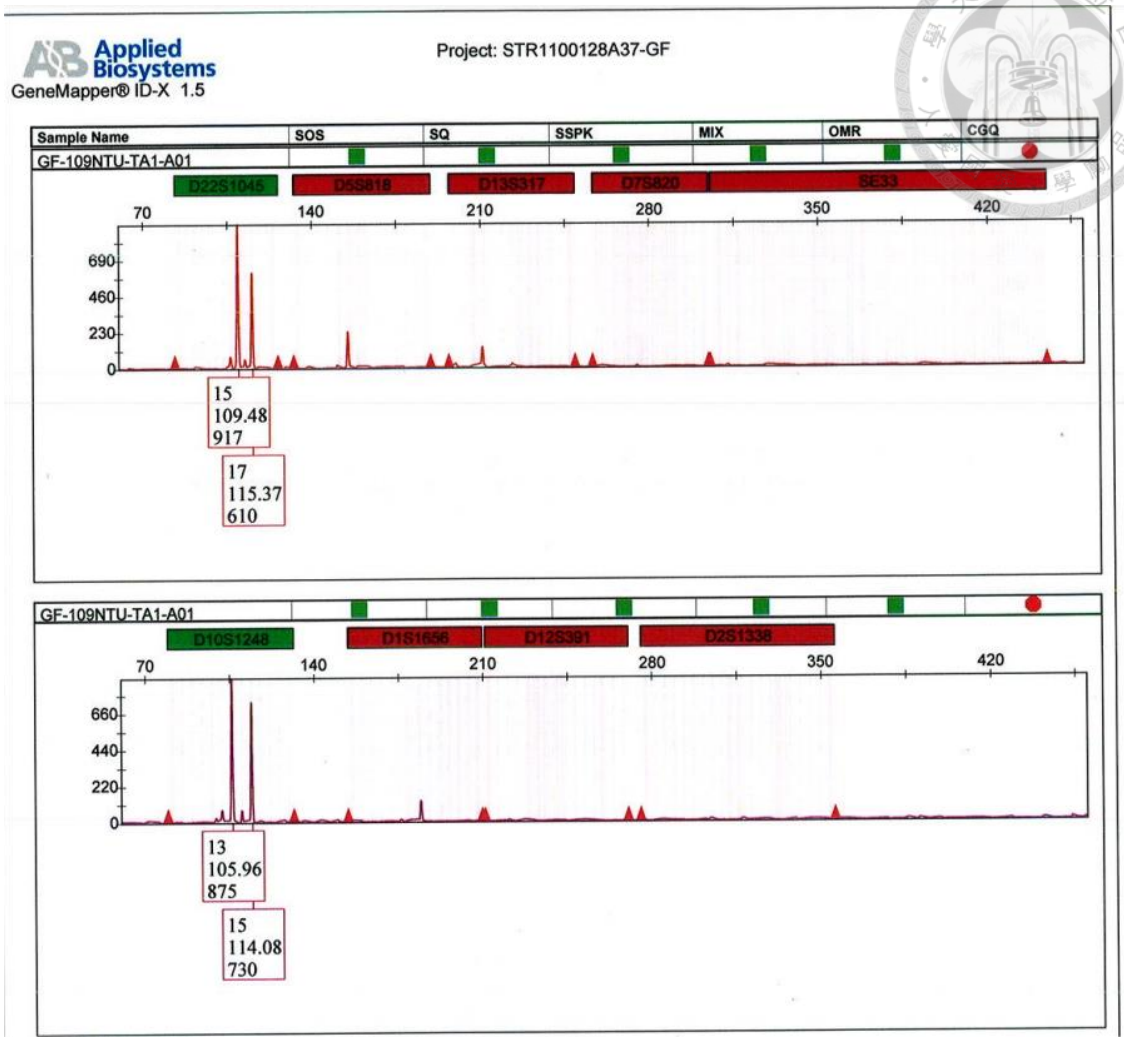

Fig. 29 GlobalFiler® electropherogram of STR genotyping from pulp sampling of sample TA1.

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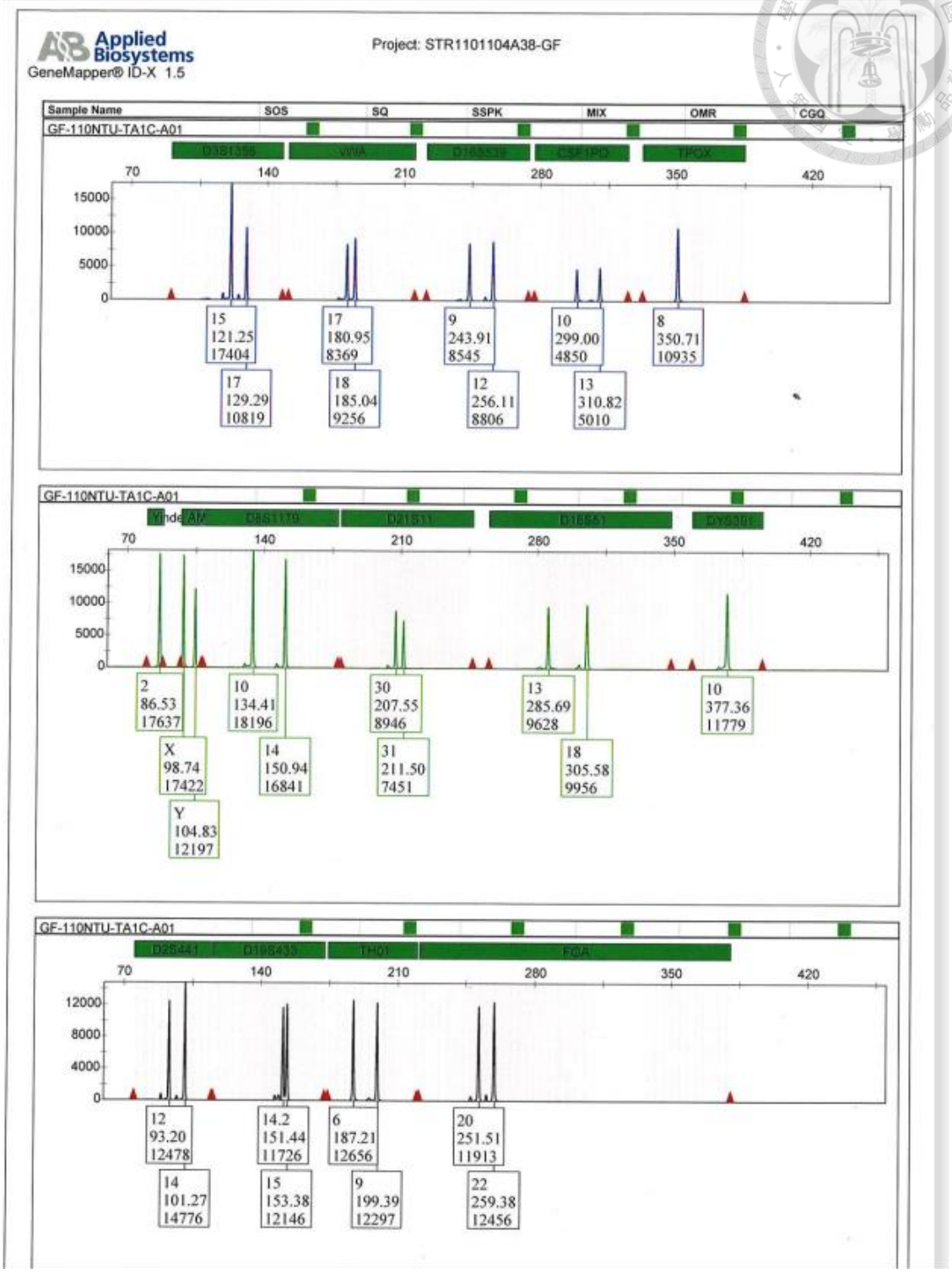

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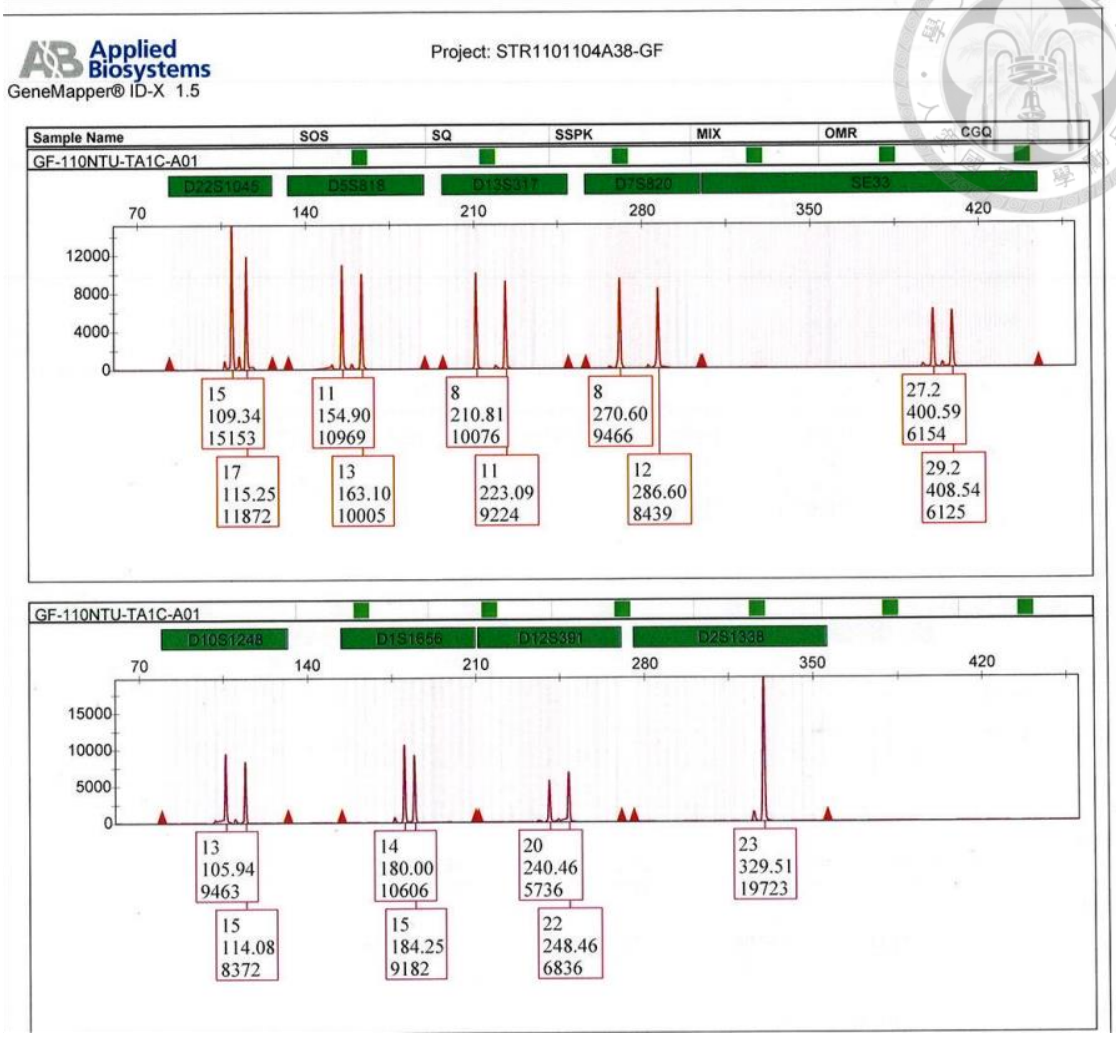

Fig. 30 GlobalFiler® electropherogram of STR genotyping from cementum sampling of sample TA1.

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Chapter 5 Discussion

5.1 DNA extraction

Teeth and bones are valuable samples in forensic and ancient DNA research because they are often the only samples available. In recent studies, they have shown that ancient DNA preservation in petrous bone outperformed tooth cementum [37], however, tooth sampling has advantages in number, simplicity and less destructive. Except for petrous bone, tooth sampling obtained better results than bones as DNA sources. The reason for that would be the fact that teeth are less porous and better prevented from degradation and contamination. The current or classical technique of DNA extraction from postmortem human teeth has involved grinding the whole tooth. It is practical, yet the tooth no longer exists. Morphological studies of teeth provide valuable information including races, chewing habits, lifestyles, and even social status that are very valuable to anthropologists [17]. Therefore, we want to preserve the tooth structure while tooth sampling for DNA extraction.

Through a comprehensive understanding of tooth structure, there are two sites in the tooth that has nucleated cells. They are pulp tissue including odontoblasts (lining in the inner part of root canal) and cementocytes (lining in the root surface). Enamel is

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acellular and dentine is abundant with mtDNA. If we only want mtDNA, dentine is a good source. But in most cases, we need nucleated cells. The other preserving tooth structure methods like horizontal cervical crown cut or vertical split needs glue to bring the separated parts together afterwards. They are not reliable methods. The two halves of the tooth may separate again. It must pay attention to avoid heat effect and contamination, and more steps bring more chances of contamination. As for the "reverse root canal" technique, its poor view from root access and one-way opening would make pulp tissue and powder collection difficult, making this "reverse root canal " method not practical. The "orthograde entrance" or " conventional endodontic occlusal access " technique would be the best choice for DNA collection from teeth if pulp was targeted. This sampling method, however, needs some techniques and better be done by dentists. As for cementum sampling, it can be easily done in forensic laboratory simply by blades with hands. After collecting powder, with the use of commercial QIAamp DNA Investigator Kit (Qiagen®), DNA profiles can be easily obtained without pre-extraction treatment.

Another concerned point was decontamination, there were two places to be concerned, one was sample itself, another was dental instruments. Currently used agents for decontamination are bleach, UV light, and ethanol. Since it was likely that

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sterilization agents might perpetrate into the root canal through apex and damage pulp or directly deteriorate cementum. It seemed not using these agents would be better. We targeted pulp and cementum which were vulnerably damaged by bleach or UV light. A large amount of distilled water with brush were used to remove exogenous DNA. Dental instruments including diamond burs, hi-speed dental handpiece, low-speed rotary handpiece, k-files were cleaned in an ultrasonic bath for 10 min and all were packed and put into the autoclave for 30 min at 121°C. There is no DNA contamination observed in the results of STR profiling.

There are rich DNA extracted both in pulp and cementum samplings as expected in this study in unburied teeth (group A). However, DNA quantity of samples varied surprisingly from 0.5 to 8690 ng. Samples A1 (43.5 ng), A4 (8.35 ng), A5 (0.5 ng) and A7 (1.95 ng) was found to be much less than the group average (1,590 ng) in pulp. It was also observed in samples A2 (15.0 ng) and A8 (12.0 ng) away from the group average (648 ng) in cementum. Interestingly, the less DNA quantity in pulp was not found less in cementum. For instance, there are 43.5 ng in pulp and 2,035 ng in cementum of sample A1, 0.5 ng and 556.5 ng of sample A5, and 929.5 ng and 12.0 ng of sample A8. It showed that DNA quantity in pulp and cementum was not correlated to each other. And it may vary among individuals and teeth health conditions.

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The DNA quantity of the teeth (groups from B to E) buried in soil was obviously much less than those unburied in both pulp and cementum observed in Table 3-6. It reduced almost 2 orders of magnitude. This showed that the influence of microbials on DNA degradation was evident. Dental pulp may be invaded by microbes through the apical foramen. It is the tooth's natural opening, found at root's every tip - that is, the root's apex- whereby an artery, vein, and nerve enter the tooth and commingle with pulp. Besides, the apical foramen is the point where the pulp meets the periodontal tissues, the connective tissues that surround and support the tooth. The average size of the orifice is 0.1-0.3mm in diameter. There can be two or more foramina separated by a portion of dentin and cementum or by cementum only. It is inevitable the invasion of microbes to degrade DNA through this channel for tooth deposited under microbe-rich environment after fallen or death.

DNA quantity among groups with buried samples (groups B, C, D and E) was still found great fluctuation among individuals and groups in pulp samplings. The variation from UD to near 100 ng was observed in groups C and D. In groups B and E, the variation was comparatively small, and the averages (1.83 ng and 0.42 ng) were far less than those of groups C and D (21.4 ng and 11.9 ng). It showed that molar and multirooted teeth provided more DNA than incisors (group B) in pulp samplings. This is consistent with

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the findings of De Leo et al. [23]. DNA quantity in group E was unsurprisingly low because they were caries. There are 7 undetected cases in 18 pulp samplings. The rest samples were found only from 0.2 to 1.7 ng of DNA. Although they were caries and undetected DNA, there may be pulp DNA for identification (samples E4, E6-E9, E11).

For DNA quantity of cementum in buried teeth, although it showed much less than those of unburied teeth, there are only 3 undetected out of 49 samples. The variation of DNA quantity of detected samples was pretty stable and ranged from 0.05 to 15.6 ng. And the averages among groups B, C D and E were found that groups D and E have more DNA than groups B and C. Obviously, molar teeth have more surface area that can provide more DNA than incisors in cementum scraping because groups D is the group of molar teeth and some molar teeth (E8:14.35 ng, E12:5.8 ng and E15:15.6 ng) in group E provided large DNA quantity. DNA was seriously affected by caries in pulp samplings, but it was not found in cementum samplings.

5.2 STR genotyping

Results of STR genotyping can be used to evaluate the quality and quantity of DNA extraction. From results of unburied teeth (group A), if the DNA quantities were above 0.5 ng, all STR loci were successfully genotyped. The buried teeth, however, can be

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extracted only a small amount of DNA. It showed that in some pulp sampling teeth had some degree of DNA degradation like B3 1.0ng, 18/24 and D8 0.5ng ,10/24 and D12 1.5ng, 21/24 and E13 1.2ng ,11/24 etc. Although their DNA quantity is above 0.5ng, their genotyping lost some alleles. Matthew J. Ludeman et al. in the previous study showed that the increasing loss of larger alleles was observed with increasing levels of degradation [39]. Some of them were even undetected. From Table 2, there are samples observed no DNA in quantification but success in STR genotyping, for instance, samples D11 and TA3 in pulp samplings, samples E11 in cementum. It seems that the failure of DNA quantitation may still have detectable DNA of STR loci in seriously degraded samples.

The success rates of STR genotyping of buried teeth in all groups from B to E were much higher in cementum than in pulp. It showed that DNA quality of cementum samplings was better than those of pulp samplings. It indicated that DNA in cementum sampling was not so seriously degraded by environmental microbes or dental caries bacteria and may be protected by cementum matrix from microbial invasion. It was particularly clear in group E (with cavity), the success rate in pulp sampling was 62 % while in cementum sampling was 100 %. Compared to groups B, C and D, group E low

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success rate indicated that pulp necrosis happened and degradation by microbes may have occurred already before buried in soil.

In the samples of B2 and B4 canals were calcified and obstructed, and the pulp and the odontoblastic powder were not available by using dental files, while using blades to scrape cementum can get the positive results (B2 -cementum, 1.5 ng, 24/24; B4-cementum, 0.15 ng, 24/24). It showed the “orthograde entrance” technique to retrieve pulp had some limitation and was not applicable to all cases. The other undetected DNA samples would probably be due to degradation of DNA or trace amount of DNA that Quantifiler® cannot detect [40].

Dental instruments used in pulp sampling like burs, handpieces, and files were reused and re-sterilized in autoclave. Blades used in cementum sampling were disposable. The manual process of removing pulp and odontoblasts with endodontic files was laborious and time consuming (50-60 min). We instead used low speed rotary machine (Analytic Quantec® ETM 18:1 at 340rpm, Taiwan), and the time of all processes for pulp sampling can be reduced down to 30 min. In this study we used the k-files from #10 to #80 in sequential order, which meant we expanded the orifice from 0.1mm to 0.8 mm. As for cementum sampling, it only took about 5-10 min to finish. In addition, we found some samples (24/58, 41%) root tips were fractured (Fig. 31-33). The

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loss of root length was about 2-3mm. This was partly because of fragility of tiny, curved root tip and partly because we enlarged root canals to 0.8mm (#80 dental file). The root tip of cementum is abundant of cementocytes, if we did cementum sampling first, the quantity of DNA in cementum sampling might be higher.

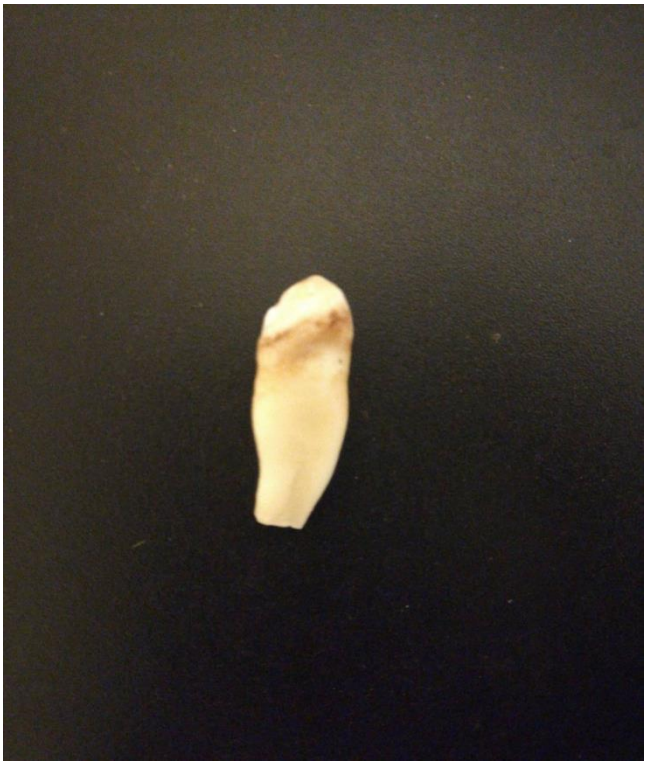

Fig. 31 It showed root tip broken of B7 tooth sample during pulp sampling.

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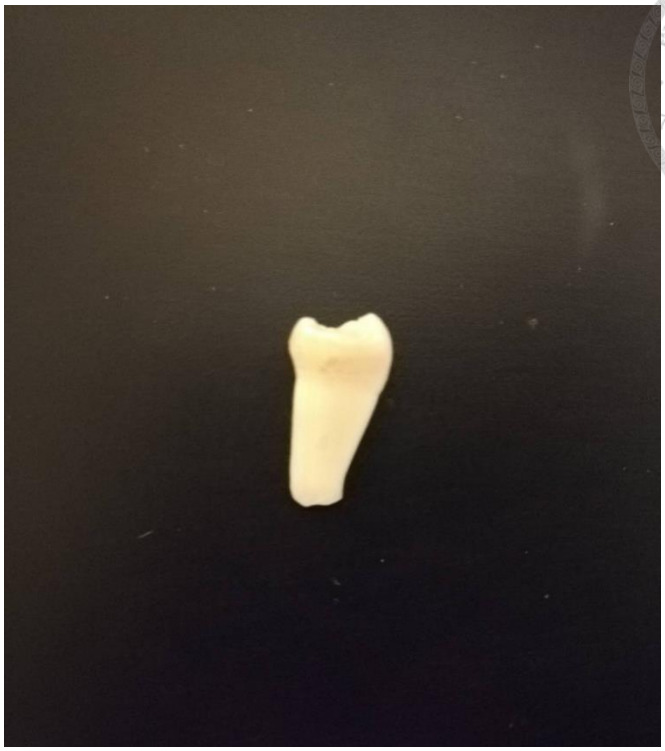

Fig. 32 It showed root tip broken of C1 tooth sample during pulp sampling.

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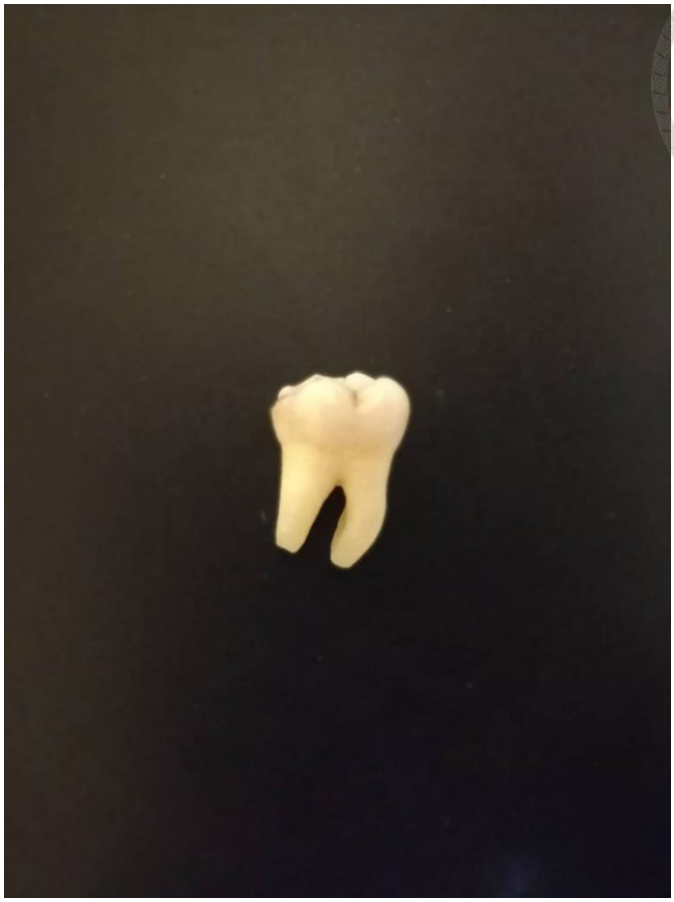

Fig. 33 It showed root tip broken of D12 tooth sample during pulp sampling.

As the hole in the crown of the tooth can be filled back by composite resin after “orthograde entrance” technique has been completed, tooth morphology is thus preserved. In fact, the tooth can be restored to its natural morphology.

The overall success rate of buried teeth in our study (group B+C+D+E) for pulp sampling was 72.4% and 99.5% for cementum sampling regardless of tooth types, or tooth condition. We recommended cementum sampling be first choice and molars be the first priority if chosen possibly. Tooth samples had better be selected free of cavity,

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attrition and periodontal disease. Pulp sampling can be viewed as back-up or special consideration issued, like pathogens were of interest. In this study bleach nor UV light was used to decontaminate the exogenous DNA, and the results showed that it might not be necessary using bleach or UV light, yet this still needs more research to be confirmed.

The teeth used in this study were the donors of the patients in a local dental clinic. The reasons for extraction of these teeth were either advanced periodontitis or large cavity. If teeth secured in jaw bones, and with no periodontitis or cavity the results would be much better than this study. Periodontitis is a kind of dental periodontal disease which was always described as a more or less rapidly but continuously progressing chronic disease. This belief was based on the findings of epidemiologic studies, which had demonstrated increasing attachment loss with age. During acute, active destruction, gram-negative anaerobic bacteria come to predominate in the pocket. Direct microbial invasion into the pocket may occur. This leads to a massive host-defense reaction with formation of necrosis or purulent abscesses. The mechanisms of collagen destruction and bone resorption are activated. The quantity and virulence of microorganisms (pathogens) on the one hand, and host resistance factors (immune status) on the other hand are primary determinants for the activity and progression of periodontal destruction. With destruction progressing, pronounced attachment loss occurs extending beyond the mid-

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point of the root, often in the form of vertical defects. Probing depths of 8 mm or more, usually infrabony, are detected. The relationship of the periodontium to the endodontium is indeed close. These two anatomic entities are quite literally connected to each other at the apical foramen and via lateral canals. Thus, pulp pathology may directly affect the periodontal tissues. On the other hand, advanced periodontitis may occasion pulpal inflammation or necrosis by way of the apex, lateral canals or the furcation [38]. The apical foramen is the tooth's natural opening, located at every root's tip - that is, the root's apex- whereby an artery, vein, and nerve enter the tooth and interconnect with pulp. The apical foramen is where the pulp meets the periodontal tissues, the connective tissues that surround and support the tooth. The average size of the orifice is 0.1-0.3mm in diameter. There can be two or more foramina in one root. An apical constriction is often present. Calcified root canals sometimes happen, and it's difficult to get the smallest file down to the apical. Aging is the main cause of tooth calcification. As the body ages, the supply of blood slows down, and teeth cannot get enough blood supply that leads to calcification. In immature teeth, the root is not completely formed and makes an appearance of wide-open apex [41]. Bacteria are typically 0.5-5.0 um in length. Bacteria inhabit soil, water, hot springs, radioactive waste, and the deep portion of Earth's crust. Bacteria are vital in the nutrient cycle by recycling nutrients including the

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decomposition of dead bodies and the fixation of nitrogen from the atmosphere. The soil is a rich source of bacteria, and a few grams contain about a thousand million of them. In an ideal dry postmortem environment pulp may mummify and persist intact for extended periods of time but in a moist environment putrefaction proceeds rapidly leading to a complete loss [39]. The elder's teeth might have greater chance to get DNA typing than if they were in the soil, but if they were kept in the air dried, the younger's teeth might have more chances to get genotyping than the elder's [Fig.34].

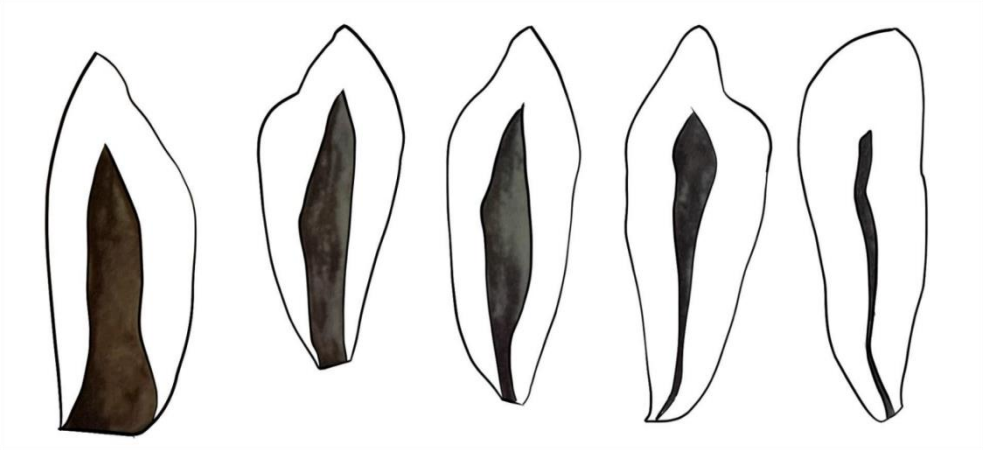

Fig. 34 from left to right, shows from immature younger tooth to mature older tooth.

A possible danger during root canal enlargement is the fracture of dental files within the canal. Dental files should be inspected before they are inserted into the root canal. If there is any sign of fracture, the file should be discarded. Never force a file into a canal. Dental files may be used with a reaming action. The reaming action consists of a quarter

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to a half turn and withdrawal. Filing action is carried out by inserting the instrument and withdrawing it while exerting even pressure on the wall of the canal. The entire wall of the canal is filed by gradually working circumferentially in a clockwise direction. There are two key points about access cavities. One is removal of the entire roof. Another one is direct line access (Fig.35). Removing the entire roof of the pulp chamber makes the view of the pulp chamber clear and its walls continuous with the access cavity. The shape of the access cavity should be made in the direct line with the apical third of the root canal so that the coronal walls do not deflect instruments and instruments can passively pass through the apex [39].

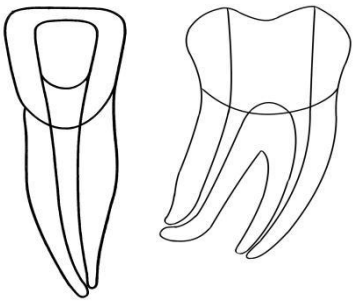

Fig. 35 Illustrate direct line access for incisor and molar in pulp sampling.

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5.3 Secondary samplings in cementum

Cementum accumulates in the root concavities and its average thickness varies between 0.1-0.3 mm [42]. Cementocytes mostly reside in the apical third of the root and the average size of them varies between 20-30 um^2 with a distribution more on the surface than in the deeper portion toward dentine [43]. The secondary sampling in cementum (shown in Table 10) was tested on one sample with previously undetected DNA (D9) and three samples with successful STR genotyping (D11, E1 and E18) to see the possibility of further DNA extraction. The results indicated that it is possible to find DNA in the secondary samplings of cementum. However, it cannot be assured of having better quality and quantity of DNA. Second time pulp sampling of the same tooth could not possibly be available.

Table 10 It showed D9, D11, E1 and E18 DNA yield and STR profiling of the first and secondary scrapings in cementum. UD indicates undetected.

Sample	DNA quantity (ng)		Success No. in 24 STR loci	
	1st Test	2nd Test	1st Test	2nd Test
D9	UD	UD	19/24	13/24
D11	3.95	UD	24/24	24/24
E1	0.7	1.0	24/24	24/24
E18	6.25	6.5	24/24	24/24

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5.4 Forensic samples

Four teeth of forensic teeth were found only very few and even undetected DNA were extracted in pulp sampling. The success rates of STR genotyping were very low. It is probably due to the drowned condition and seriously decomposed remains. However, in cementum sampling, all 4 samples can be extracted a few detectable DNA. All of them showed full genotypes of 24 STR loci.

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Chapter 6 Conclusion

In this study, we compared the quantity of DNA and quality of DNA (number of alleles) of the same tooth by two different sampling methods. One sampling method was to drill a hole through occlusal entrance to retrieve pulp and odontoblastic powder and another method was to scrape cementum with blades. Both methods preserved tooth morphology. The powder collected to extract DNA in both samplings was 5-10 mg. It spared decalcification processes. We used distilled water with brushes to remove exogenous DNA. We did not use bleach or UV light to decontaminate exogenous DNA in this study. Cementum sampling method outperformed pulp sampling method in DNA quality very obviously, while pulp sampling method got more DNA quantity. It showed that DNA was degraded by microbes through apical foramens or cavities. We recommended cementum sampling be first choice because it was easier and more chances (99.5%) to get full profiles of 24 loci for buried teeth. Although the pulp tissue was easily affected in poor conditions (large decay and in the soil), there is still 72.4% of success rate in 24 loci. Pulp sampling method could be considered as a backup, or for specific consideration like pathogens research. Both methods could get positive results and meanwhile preserve the integrity of the tooth structure.

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