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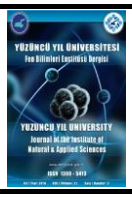

Research Article

Comparison of Rotary and Cryostat Microtomes in the Skeletochronology on Green Turtle Humerus Bones

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Abstract: Skeletochronology is a powerful tool that provides information on the age of sea turtles. Digital images of histologically prepared humerus bone sections are commonly used for age determination. The quality of digital images, which is critical for robust age estimation, varies depending on the type and histological technique associated with microtome. This study aimed to compare the quality of digital image sections of humerus bones prepared by rotary and cryostat microtomes. The humerus bones of 11 juvenile stranded (dead) green turtles (mean CCL 292.1 mm) on Samandağ beach during the 2021 nesting season were used for the study. Three readers scored the quality of histologic sections prepared with two different microtomes and associated protocols, as well as the readability of the lines of arrested growth (LAGs), which they saw from 0 to 5. The Wilcoxon-signed rank-paired test was used to ascertain the disparities between the quality scores of the two protocols. In addition, the compatibility of readers was evaluated by determining the proportion of specimens with at least two identical scores for each protocol. The scores obtained from the cryostat microtome protocol had a wider variation compared to the rotary microtome protocol. The rotary microtome protocol has higher median score values (4) than the cryostat microtome protocol (3) ($p<0,001$). The compatibility of three cryostat microtome readers was lower than that of the rotary microtome. Although the cryostat microtome is less time-consuming and less costly, the results of this study showed that the rotary microtome protocol was preferable to the cryostat microtome protocol. Future studies may test techniques like various staining protocols to gain valuable insights into skeletochronology.

Yeşil Kaplumbağa Humerus Kemiklerinde İskeletkronolojisi Yönteminde Döner ve Kriyostat Mikrotomun Karşılaştırılması

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Öz: İskeletkronolojisi, deniz kaplumbağalarının yaşı hakkında bilgi sağlamak için kullanılan güçlü bir araçtır. Histolojik olarak hazırlanmış humerus kemiklerinin enine kesitlerinin dijital görüntüleri yaş tayini için yaygın olarak kullanılmaktadır. Sağlıklı yaş tahmini için kritik öneme sahip olan dijital görüntülerin kalitesi, mikrotomla ilişkili histoloji tekniğine ve türüne bağlı olarak değişir. Bu çalışmanın amacı, döner ve kriyostat mikrotomlarla hazırlanan

Anahtar Kelimeler
Kriyostat,
Mikrotom,
Samandağ,
Yaş belirleme,
Yeşil kaplumbağa

humerus kemiklerinin kesitlerinin dijital görüntülerinin kalitesini karşılaştırmaktır. Çalışma için 2021 yuvalama sezonu Samandağ kumsalında karaya ölü olarak vurmuş 11 juvenil yeşil kaplumbağanın (ortalama CCL 292.1 mm) humerus kemikleri kullanıldı. Üç okuyucu, iki farklı mikrotom ve ilgili protokollerle yapılan histolojik kesitlerin kalitesini ve gördükleri LAG'lerin okunabilirliğini 0 ile 5 arasında puanladı. İki protokolün kalite puanları arasındaki farklılıkları belirlemek için Wilcoxon işaretli eşleştirilmiş sıralama testi kullanılmıştır. Buna ek olarak, okuyucuların uyumluluğu, her protokol için en az iki aynı puana sahip numunelerin oranı belirlenerek değerlendirilmiştir. Kriyostat miktotom protokolünden elde edilen skorlar döner mikrotom protokolüne kıyasla daha geniş bir varyasyona sahipti. Döner mikrotom protokolü kriyostat mikrotom protokolünden daha yüksek medyan skor değerlerine sahiptir ($p<0.001$). Üç kriyostat okuyucusunun uyumluluğu (0.73) döner mikrotomunkinden (0.91) daha düşüktür. Kriyostat mikrotom tekniği daha az zaman alıcı ve daha az maliyetli olmasına rağmen, bu çalışmanın sonuçları rotary mikrotom protokolünün kriyostat mikrotom protokolüne tercih edildiğini göstermiştir. Gelecekteki çalışmalar, iskelet kronolojisi hakkında değerli bilgiler edinmek için çeşitli boyama protokolleri gibi diğer teknikleri test edebilir.

1. Introduction

Skeletochronology relies on the yearly formation of bone growth marks, which provide evidence of growth cycles (Zug et al., 1986; Snover et al., 2007; Avens et al., 2009; Goshe et al., 2009). Castanet (1994) states that it is a very effective instrument for obtaining data on the age of both living and extinct reptiles, growth rates, the age at which sexual maturity is reached, sexual differences, and variations between different populations. Skeletochronology may also provide insights into an individual's life history by examining changes in bone structure over time. This approach is unsuitable for studying live individuals since it often necessitates obtaining samples from the humerus, femur, or phalanges. However, it is extensively used to determine the age of sea turtles.

The skeletochronology method requires laboratory processes, including cutting sections from intact bone, fixation and decalcification, thin sectioning, and staining (Goshe et al., 2020). Among these laboratory processes, some authors use a cryostat microtome in the cross-sectioned stage, while others use a rotary microtome. For example, Guarino et al. (2004) estimated the age of phalanx in Mediterranean loggerhead turtle populations using a cryostat microtome. Similarly, Lenz et al. (2016) used a cryostat microtome to estimate the age and growth of loggerhead turtles in the South Atlantic Ocean. Usategui-Martín et al. (2023) estimated the growth rates of loggerhead turtles in the Canary Islands using the same method. In addition, many studies have used rotary microtome to determine the age and growth rates of various sea turtles (Zug et al., 1986; Zug et al., 2002; Snover and Hohn, 2004; Goshe et al., 2010; Şirin & Başkale, 2021).

A study that compared the rotary microtome and cryostat microtome methods for preparing dolphin teeth for age determination discovered that the rotary microtome method is both possible and inexpensive, making it an excellent alternative to the cryostat microtome (Luque et al., 2009). Although studies have been conducted on using both procedures (i.e., cryostat and rotary microtome) for determining the age of sea turtles, there is currently no research comparing both procedures to estimate the age and growth rates. The reasons for the choice between the two procedures are still unknown. Hence, this research aimed to investigate the potential of the cryostat microtome as a feasible substitute for the rotary microtome approach in assessing the age and development rate of the green turtle (*Chelonia mydas*).

2. Material and Methods

The investigation was conducted during the 2021 nesting season as a component of the sea turtle conservation and monitoring research on Samandağ Beach in the eastern Mediterranean. As part of this conservation and monitoring program, stranded dead green turtles (*C. mydas*) were gathered. The dead

green turtles that were stranded were categorized by their species, and their curved carapace length and width (CCL and CCW) were determined using a flexible tape measure with an accuracy of one mm (Sönmez, 2019). During the conservation and monitoring investigations, researchers encountered green turtles at various life cycle phases. The CCL ≤31.5 cm was considered to be the oceanic stage, and the CCL ≥85 cm was considered to be the adult stage (Türkozan et al., 2013). As a result of potential variations in growth rates across adult, juvenile, and oceanic stage green turtles, only humerus samples were obtained from the oceanic stage and juvenile individuals. A total of 11 stranded green turtles were necropsied in situ, and the humerus was dissected and collected (Zug et al., 1986; Lenz et al., 2016). The humeri were macerated in water to remove the soft tissues, then dried and stored in the collection (Zug et al., 1986; Lenz et al., 2016).

A bandsaw was used to cut the humerus across the middle, just below the deltopectoral ridge, and at the diaphysis's thinnest point (about 5–7 mm diameter) (Zug et al., 1986; Şirin & Başkale, 2021). The section was stored in at least 10% neutral buffered formalin for 48 hours prior to decalcification (Snover & Hohn, 2004). The fixed specimens were washed in distilled water and placed in a decalcification solution (Bio-Optica, Biodec R, 05-03009Q).

The decalcifying solution was later removed by several rinses with distilled water. Bone sections were cut through a SLEE mnt cryostat at minus 25 °C. The sections (7 um diameter) were stained with Harris haematoxylin and eosin (Zug et al., 1986; Usategui-Martín et al., 2023) using the free-floating technique (120 second haematoxylin-water wash and then 30 second eosin-water wash). The same bone sections were placed on a microscope slide and observed under a Stereo Binocular microscope (Olympus SZ51).

The remaining portion of the same sample was washed with distilled water and used for preparation. Each bone was prepared using the methods described in Zug et al. (1986) and Goshe et al. (2020). Briefly, the tissue was subjected to a series of alcohol rinses (dehydration), transparency with xylene (transparency), paraffin embedding with Leica HistoCore Arcadia H (paraffinization), sectioning with the microtome (Leica RM 2145 RTS) (sectioning), and routine haematoxylin and eosin staining (staining) and observed under a microscopic examination with the stereo microscope (Olympus SZ51).

Three independent readers assessed the location and number of lines of arrested growth (LAGs), which define the outer edges of skeletal growth marks in each humeral section (Goshe et al., 2010). Each reader assigned a quality score between 0 and 5 to the cryostat and rotary microtome sections taken from the same sample based on the LAGs' tissue integrity and readability. The Wilcoxon-signed rank-paired test was used to determine the factors contributing to the discrepancies in quality scores between the two protocols. In addition, the compatibility of readers was evaluated by determining the proportion of specimens with at least two identical scores for each protocol. Statistics were analyzed using R Studio 2023 (R Core Team, 2023).

3. Results

The mean CCL of these turtles was measured at 292.1 mm ±57.8 (150 mm–360 mm), and the CCW was 275 mm ±58.1 (140 mm–345 mm). The CCL frequency distribution and boxplot graph of stranded green turtle individuals are shown in Figure 1.

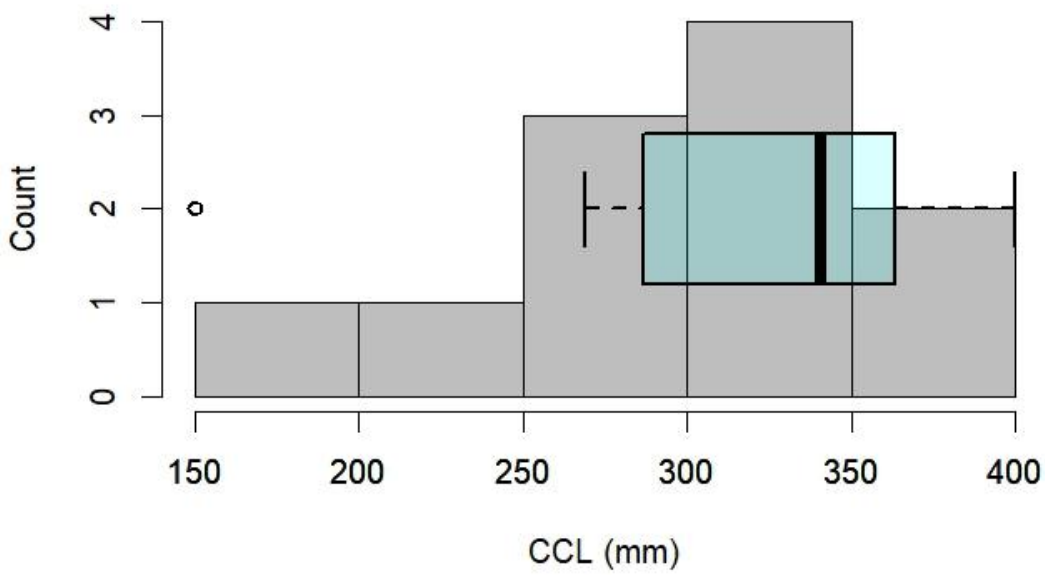

Figure 1. The CCL frequency distribution and boxplot graph of stranded green turtle.

The distribution of the quality scores given by each of the readers to each of the samples for the two different methods is shown in Figure 2. None of the readers rated any preparation a five. For four out of eleven samples, all three readers awarded the same and high marks to preparations generated using both techniques (Figure 2). The first reader's cryostat scores varied greatly, as Figure 2 illustrates. The quality of preparations produced by rotary and cryostat procedures differs depending on the sample (Figure 3).

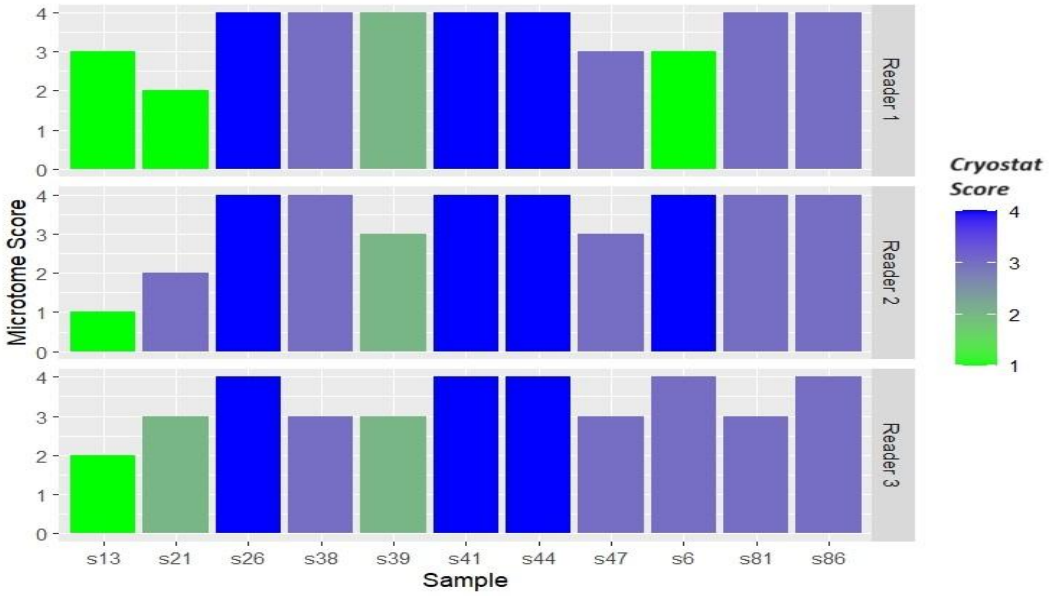

Figure 2. Distribution of quality scores given by readers to each sample for both methods (Cryostat microtome quality scores are indicated in the graph by color assignment from lowest (green) to highest (blue)).

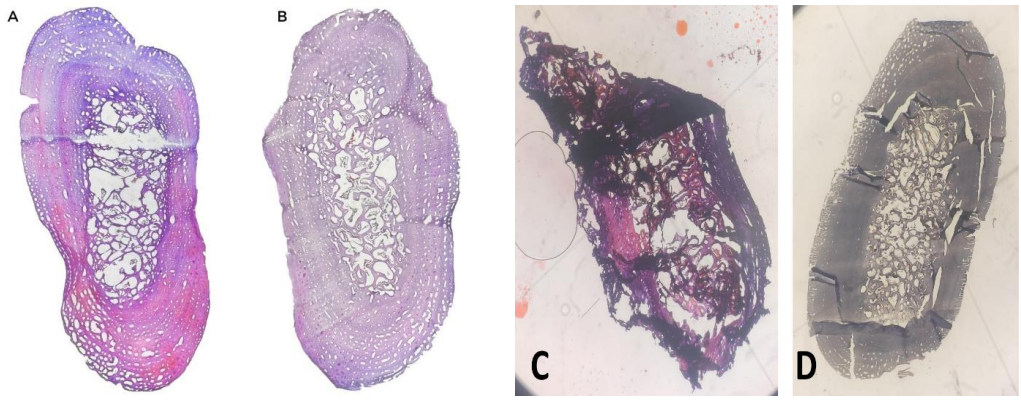

Figure 3. Three readers gave identical scores to preparations (S44: A and B) or different scores (S39: C and D). Preparations A and C were made using the cryostat microtome protocol, and preparations B and D were made using the rotary microtome protocol.

Moreover, the mean quality scores given by three different readers according to tissue integrity and readability of LAGs are also given in Figure 4. The median values of cryostat and rotary microtome preparation were 3 and 4, respectively. There were statistically significant differences favoring rotary preparation between the score values of the two preparations (Wilcoxon = 7.5, p<0.001).

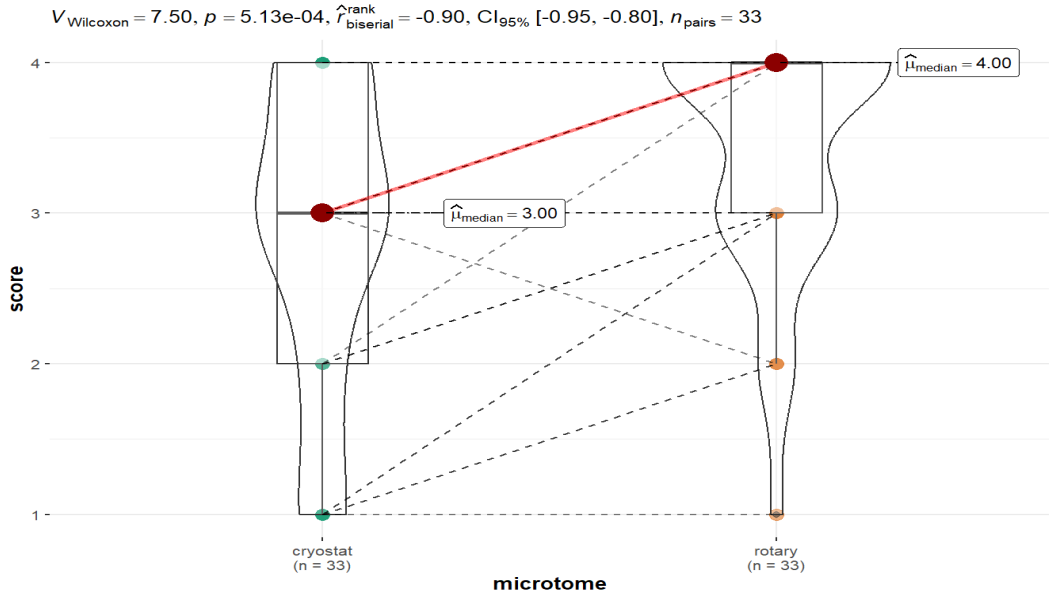

Figure 4. The boxplot (median, range and whiskers) of scores given for cryostat and rotary microtome preparations. The Wilcoxon test results are also given at the top of the plot.

4. Discussion and Conclusion

Various authors have done research to assess age and growth rates, and they have found both techniques to be valuable (Zug et al., 1986; Snover & Hohn, 2004; Guarino et al., 2004; Goshe et al., 2010; Lenz et al., 2016; Şirin & Başkale, 2021; Usategui-Martín et al., 2023). Nevertheless, these investigations are unable to provide a comparison between the two methodologies. This research observed a statistically significant disparity in the quality of preparations when comparing the two methods. Our results align with the discoveries made by Luque et al. (2009). It is important to note that the quality of both age determination approaches might be affected by the processes used after the sectioning process, such as staining. Similarly, the sectioning methods themselves can also affect the quality.

Cryostat and rotary microtomes have both benefits and drawbacks. Cryostat microtome enable the utilization of tissues from living organisms for enzyme histo-chemistry research. By freezing the tissues, cryostat microtome prevents the influence of ambient temperature, leading to reduced chemical expenses (Lojda et al., 2012). On the other hand, only a microtome can analyze tissue samples from deceased organisms, which requires a more intricate and expensive process (Read et al., 2018). Alternatively, preserving the collected samples for an extended period is possible by embedding them in paraffin and then cutting them into sections using microtomes (Grillo et al., 2017). After evaluating the pros and cons of both procedures, it can be concluded that the rotary microtome approach is appropriate for preparing cross-sections of bone tissue from stranded turtles.

According to Luque et al. (2009), Mayer's hematoxylin-stained sections yielded more "excellent quality" preparations when using the conventional microtome method. The purpose of this study was not to investigate the differences in staining. Nevertheless, the staining technique will unavoidably influence the determination of the position and quantity of LAGs. In future sea turtle investigations, comparing the two procedures using various staining methods is advisable.

The appearance of growth layers on histological preparation is contingent upon the manner of sample preparation (Goshe et al., 2009). Prior research on the visibility of growth layers in preparation has emphasized the significance of staining (Sinsch et al., 2007; Klevezal, 1996; Rossell et al., 1998) and the thickness of the cross-section (Woodward et al., 2014; Sinsch, 2015).

In conclusion, in our research, we primarily focused on the types of microtomes rather than characteristics like staining and thickness, which might affect the quality of the preparation, and we concluded that the visibility of growth layers is also sensitive to the type of microtome.

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