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An evaluation of age estimation using teeth from South Asian River dolphins (Platanistidae)

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ABSTRACT

The South Asian river dolphins (*Platanista gangetica minor* and *P. g. gangetica*) are endangered, geographically isolated, freshwater cetaceans. Accurate age estimation of individuals is an important aspect of population biology as it is used for calculating parameters such as age at maturity and reproduction, longevity, and growth and survival rates. However this has never been comprehensively studied for this endangered cetacean family. A sample of 41 teeth from 29 skulls stored in museum collections was available. We compared two different aging methods to select the most appropriate. This involved decalcification and freeze-sectioning of teeth at variable thicknesses (10–25 micron), and staining with 1) Toluidine Blue, or 2) Ehrlichs Acid Haematoxylin. Stains were then compared for readability of Growth Layer Groups (GLG). The optimum section was found at 20 micron using Erlhichs Acid Haematoxylin. Both dentinal and cemental GLG were readable and comparable, but cemental GLG were generally easier to interpret because they were better defined. Ages varied from newborn / young of year (with none or only a neonatal line present) to a maximum age of 30 GLG. There is currently no validation available for GLG deposition rate, but it is likely annual because of the extreme seasonal changes in the river habitat.

BACKGROUND

The South Asian river dolphins are endangered, geographically isolated, freshwater cetaceans, currently classified as subspecies in a monotypic family (Platanistidae) (Smith and Braulik, 2012; Braulik *et al.* 2014). They are not closely related to present day marine dolphins, and are one of the most basal cetacean families, most closely related to Kogiidae (dwarf and pygmy sperm whales) and Physeteridae (sperm whales) (McGowen *et al.* 2009; Zhou *et al.* 2011). The Indus River dolphin (*Platanista gangetica minor*, Owen, 1853) (Figs. 1a,b) is endemic to the Indus River system primarily in Pakistan but with a small population in Western India. The Ganges River dolphin (*Platanista gangetica gangetica*, Lebeck 1801) occurs in Bangladesh, Nepal and India, in the Ganges–Brahmaputra–Meghna, and Karnaphuli–Sangu river systems.

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Accurate age estimation of individuals is an important aspect of population biology as it is used for calculating parameters such as age at maturity and reproduction, longevity, and growth and survival rates. Although Kasuya (1972) conducted a preliminary age estimation study from teeth in this genus, the most appropriate method has never been comprehensively studied for this endangered cetacean family and is therefore examined in detail here.

MATERIAL AND METHODS

A sample of 41 teeth from 29 individuals stored in museum collections was available for this study. The teeth of *P.gangetica* vary greatly in size and shape both with age, and along the jaw (Figs. 2a,b) of an individual (Home, 1818). Longer and thinner teeth are situated forward in the jaw whilst teeth far back in the jaw are shorter and wider. As individuals age the teeth become broader, increasingly flattened, and the root of the tooth becomes fan-shaped and flattened in the bucco-lingual plane. The museum specimens had all been collected at least 40 years ago. The teeth were dry, often with cracks in the surface and in the dentine. It is not known whether or not these specimens had been boiled or cleaned with chemicals but it is likely. For 11 of the specimens examined, duplicate teeth were available, and often of different sizes and positions in the jaw. For these specimens, all teeth were sampled (Table 1). In addition, different staining techniques were applied and compared, allowing comparison of staining methods and also inter-individual comparison of tooth GLG from the same animal.

The preparation method involved decalcification of the teeth using RDO Rapid Decalcifier (Apex Engineering Products, Illinois). The teeth, depending on size, were exposed to RDO for between 3–5 hr for very small teeth, to 15–20 hr for intact whole teeth (could be up to 2 cm in length and variable width), and even 36 hr for the largest teeth from older animals with a widest diameter in one plane of approximately 8 mm, even if the 90° plane diameter might only be 1–2mm. The decalcified teeth were then rinsed thoroughly in water for several hours before freeze-sectioning on a freezing microtome at thicknesses of 10–25 micron. Before sectioning, the teeth were assessed as to which plane would be most suitable for demonstrating the cementum. Where more than one tooth was available from a single animal, the teeth were sectioned in both the bucco-lingual and transverse planes relative to the jaw to allow comparison. Sections for examining cementum were at 10 micron whereas those for dentine were at 20–25 micron. The staining was tried with two stains: 1) 0.3% Toluidine Blue in a solution of 1% sodium bicarbonate in water for 20 sec, and/or 2) Ehrlichs Acid Haematoxylin (ripened) for 30 min.

Fig. 1a. *Platanista gangetica* showing the body shape and especially the head with a long beak.

Fig. 1b. *Platanista gangetica* in natural habitat.

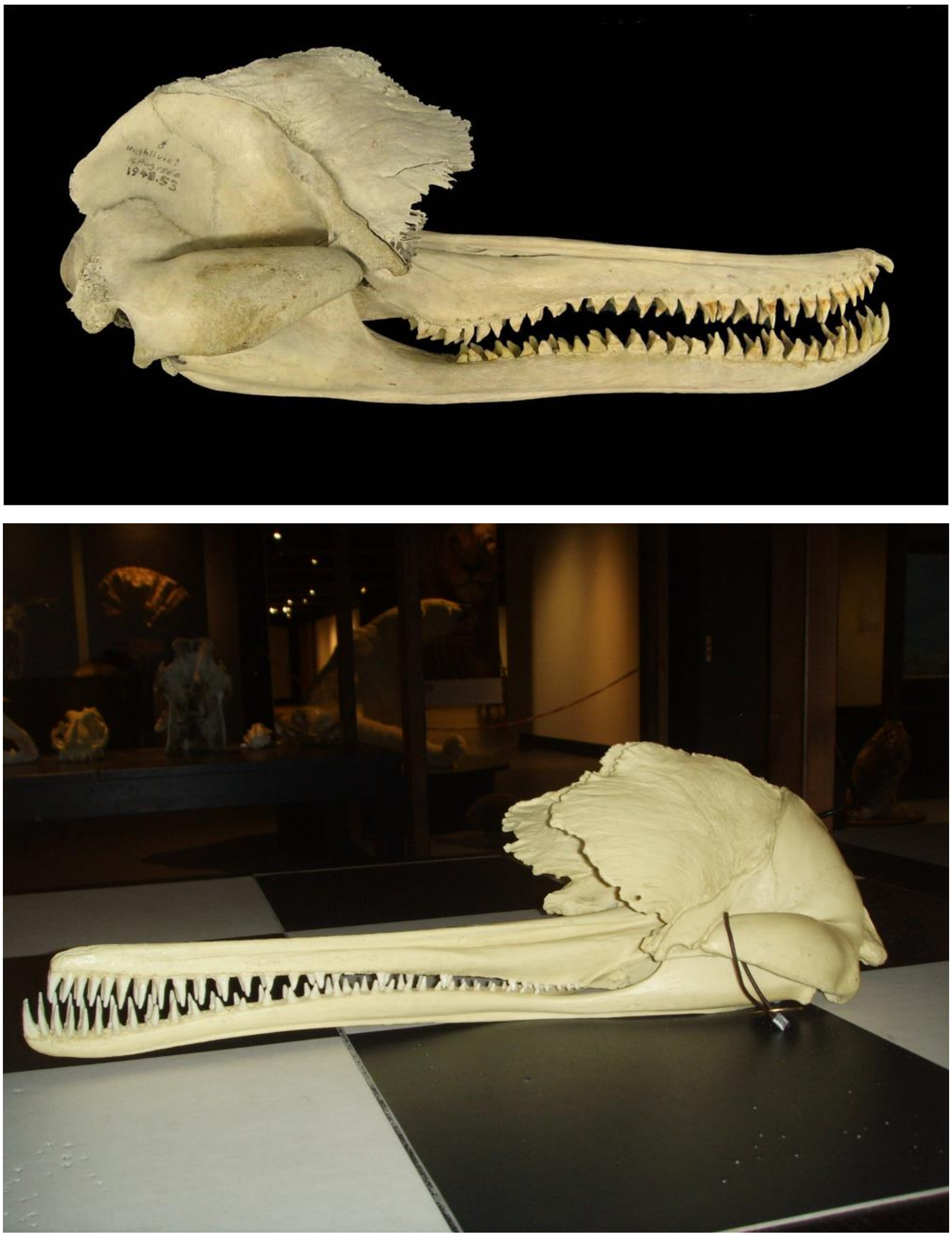

Fig. 2a. Skulls of *Platanista* showing the jaw and teeth insertion and variability in tooth shape and size. Longer and thinner teeth are situated forward in the jaw whilst teeth far back in the jaw are shorter and wider. As individuals age the teeth become broader, flattened, and the root of the tooth becomes fan-shaped and also flattened in the bucco-lingual plane. Top image is specimen Museum Code: SMNH45637, Length: not known, Sex: not known, Origin: Chak, Sindh, Indus River. Lower image – specimen from Indus River but no other details.

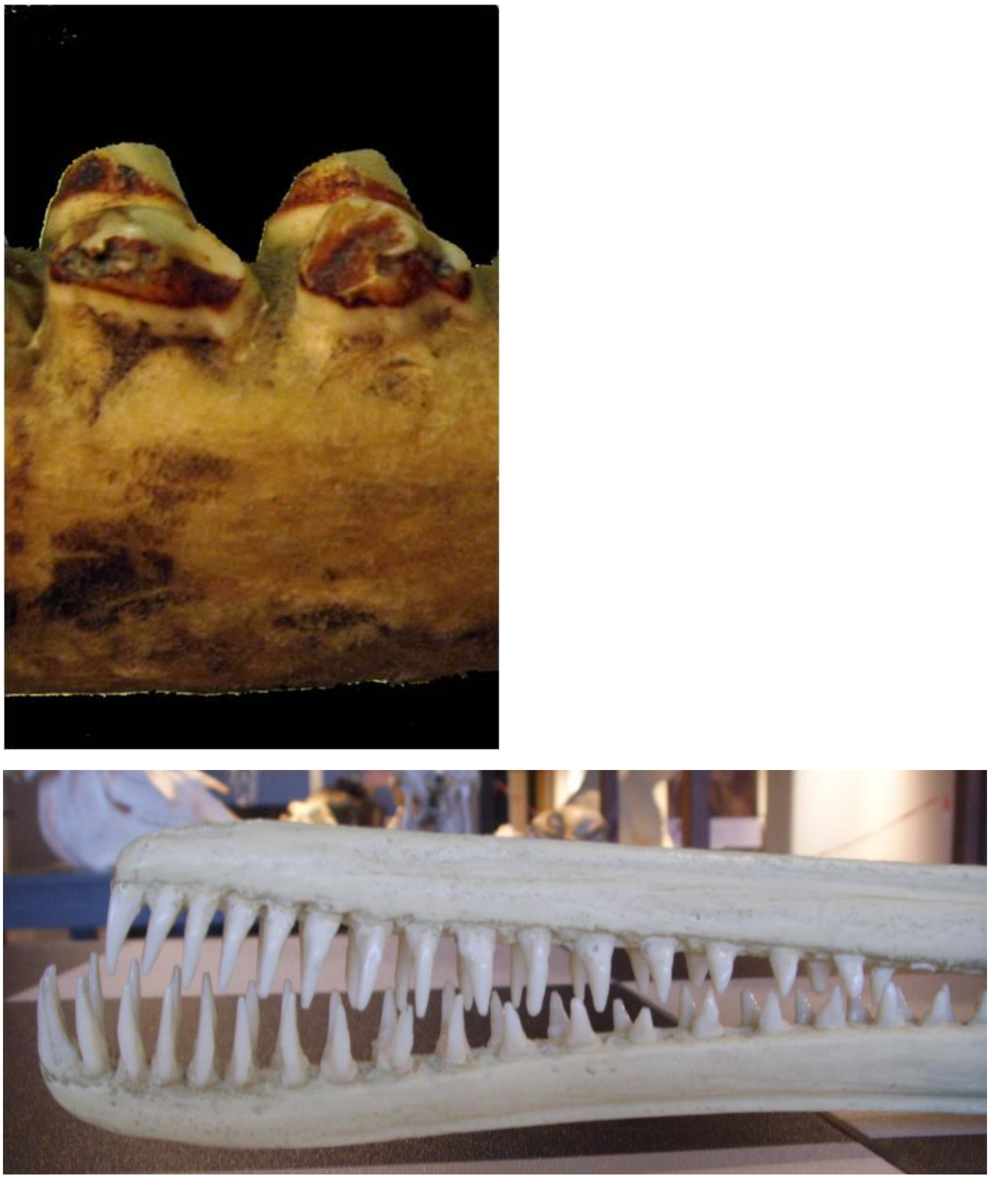

Fig. 2b. Close up of teeth of *Platanista*. Top image from specimen Museum Code: SMNH45644, Length: not known, Sex: not known, Origin: Guddu to Sukkur, Indus River. Lower image – specimen from Indus River but no other details.

Table 1. Teeth from 11 Platanista specimens where two or three teeth were available from the same animal. Age estimations from duplicate teeth indicated close agreement in all instances, both in dentinal and cemental GLGs, with a range maximum of ±1 only in older specimens.

Dolphin ID	**No. of teeth**	**Tooth size**	**Age in GLGs >= more than; + = neonatal line only; *ca* = approx. ; just = on the boundary layer**		
			Dentine	**Cement**	**Final agreed[1]**
SMNH 45633	2	small	1	1	1
SMNH 45633_2		medium	1	1	1
SMNH 45636	2	medium	*ca* 12	*ca* 12	12
SMNH 45636_2		large	13	12	12
SMNH 45637	2	very small	0.5	none seen	0.5
SMNH 45637_2		very small	0.5	none seen	0.5
SMNH 45639	2	small	1 to 2	>1	1.5
SMNH 45639_2		small	>1	1	1.5
SMNH 45640	2	small	1 just	1	1
SMNH 45640_2		large	1 just	1	1
SMNH 45641	2	small	1.5	1	1
SMNH 45641_2		small	1.5	?	1.5
SMNH 45643	2	medium	13 to 14	13	13
SMNH 45643_2		medium	14	ca 13	13
SMNH 45644	2	small	2 or 3	>2	2
SMNH 45644_2		large	2	2	2
SMNH 45645	2	small	1.5	none seen	1.5
SMNH 45645_2		small	1.5	none seen	1.5
SMNH 45646	3	small	0+	none seen	0
SMNH 45646_2		small	0+	none seen	0
SMNH 45646_3		small	0+	none seen	0
SMNH 45649	2	medium	18 to 20	*ca* 20 in root	20
SMNH 45649_2		large	19	19	19

[1]Based on a consideration of GLG counts in both dentine and cementum

The 10 micron sections were floated off the microtome blade to water where they were selected for mounting on 5% gelatin-coated slides under water and then blotted carefully with a paper towel and allowed to air-dry for a few minutes. These mounted sections were immersed in and stained using the 0.3% Toluidine Blue stain mixture. They were then rinsed off in running water before drying for about 10 min. The 20–25 micron sections destined for Haematoxylin were left free-floating in a histocasette enclosed in fine nylon mesh, such as from stockings, secured with an elastic band, and then immersed in stain, and subsequently rinsed clear in water and blued in ammonia vapour for 5 sec. before mounting and drying on gel-coated slides. All sections, once dried, were permanently mounted under glass using

DPX (a proprietary brand xylene-based resin) in the fume cupboard and allowed to dry for at least a couple of days before handling.

The Growth Layer Groups (GLG) were then counted in each type of the stained sections (Table 1). Examination, including image capture, was accomplished using a Meiji trinocular microscope at x 40 to x 100 magnification. Unfortunately it was not possible to have cross-reading with other researchers.

RESULTS AND CONCLUSIONS

Staining technique

The optimum section was found at 20 micron using Erhlichs Acid Haematoxylin stain. The Toluidine Blue stain, while quick and efficient to use, did not provide good definition of GLG in the dentine compared to the Ehrlich’s Acid Haematoxylin. In sections with the latter stain, both dentinal and cemental GLG were readable and comparable, but cemental GLG were generally easier to interpret because the boundary layers were well defined. This method of treatment differed from that of Kasuya (1972) who made thin untreated sections for examination of GLG.

Tooth ages varied from newborn/young of year (with none or only the neonatal line present) to a maximum age of 30 GLGs. Figures 3a–n show an age series with GLG marked both in dentine and cementum. Because many of the teeth had been stored dry, the treatment resulted in teeth splitting along cracks (see Figs. 3f, h and l). It is interesting to observe that cemental GLGs are often easier to interpret in the root, perhaps because of the wider spacing here (Fig. 3i).

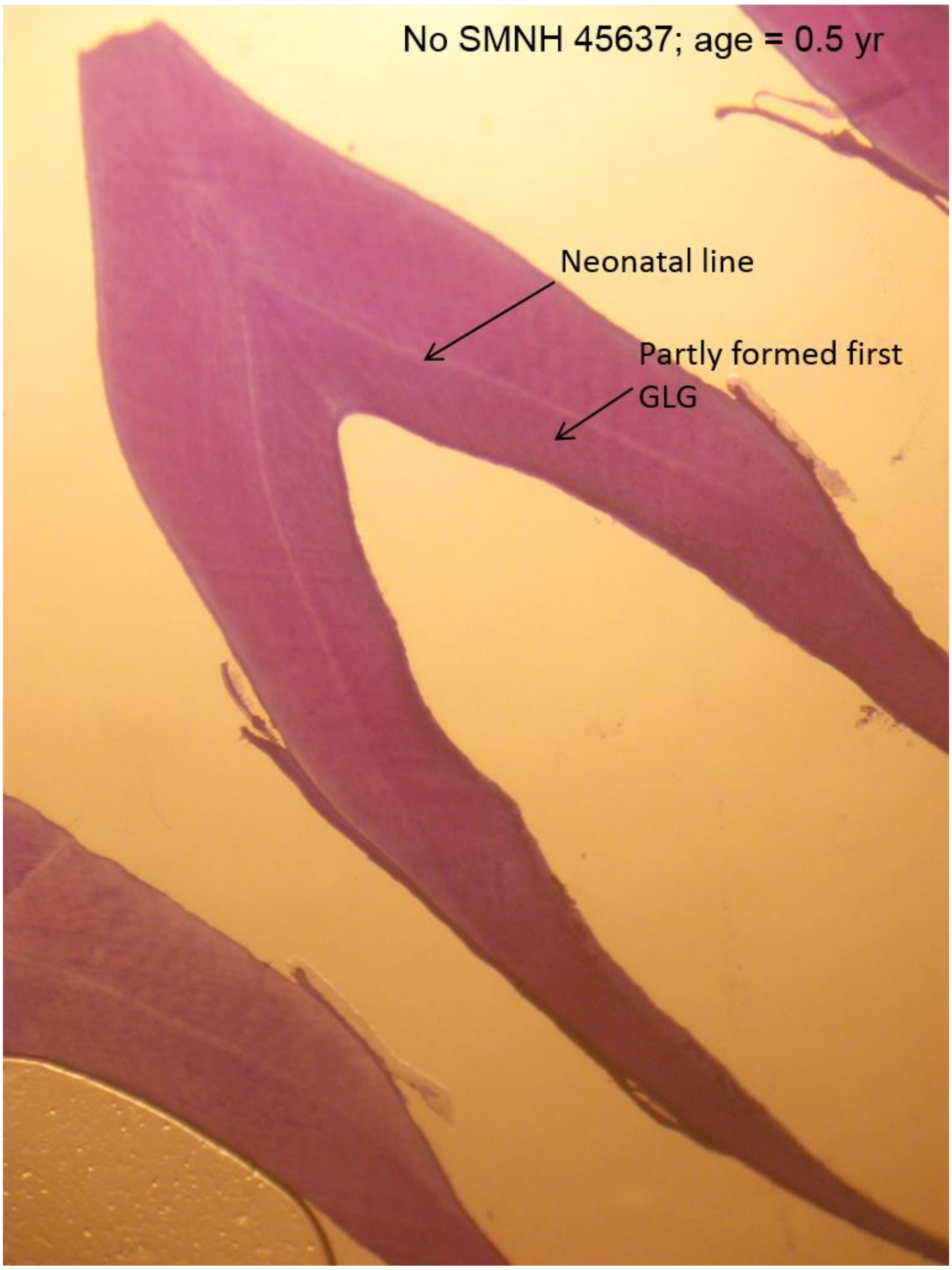

Fig. 3a. Dentinal GLG – age less than 1 yr

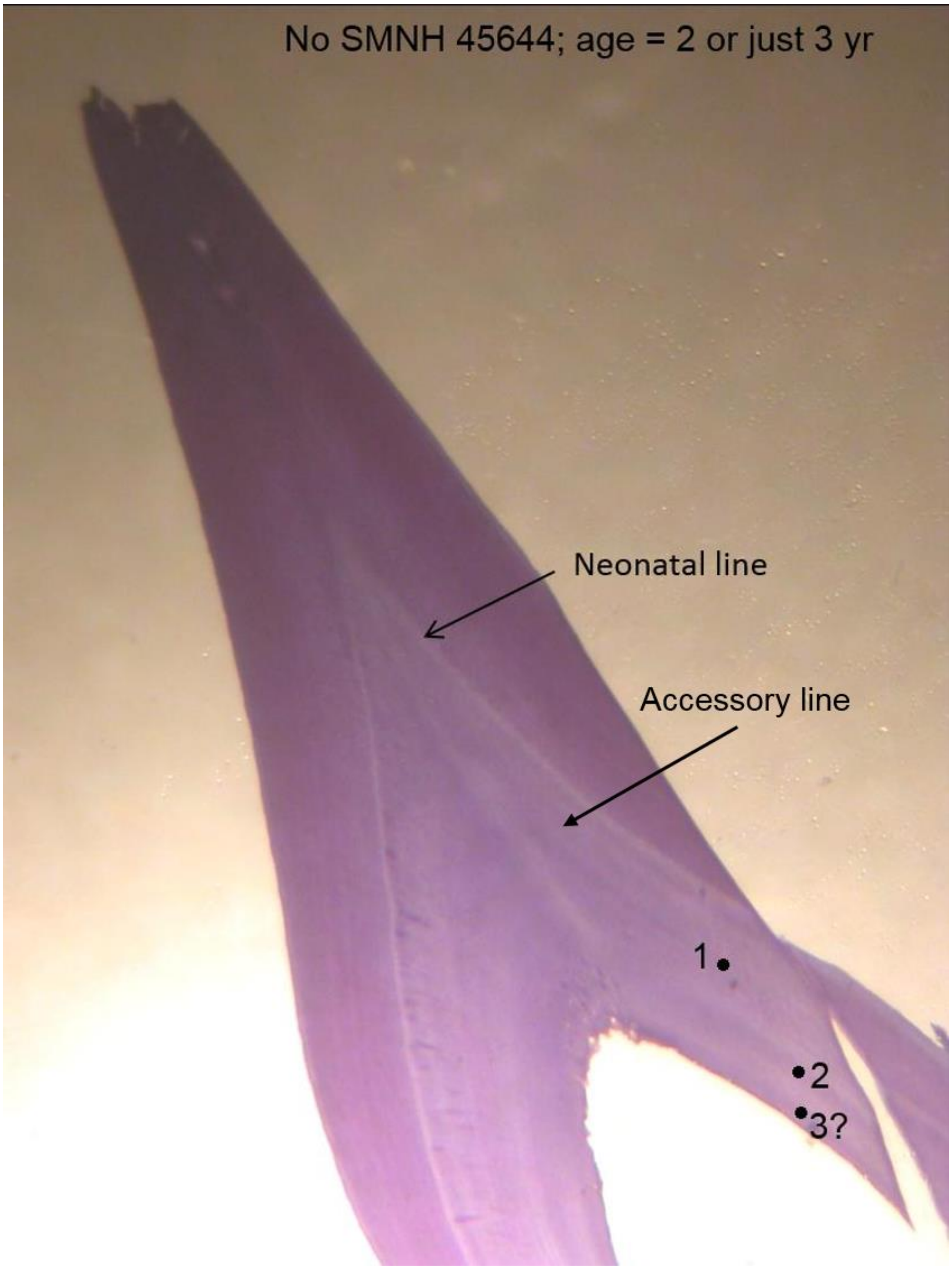

Fig. 3b. Dentinal GLG – age just 3 yr

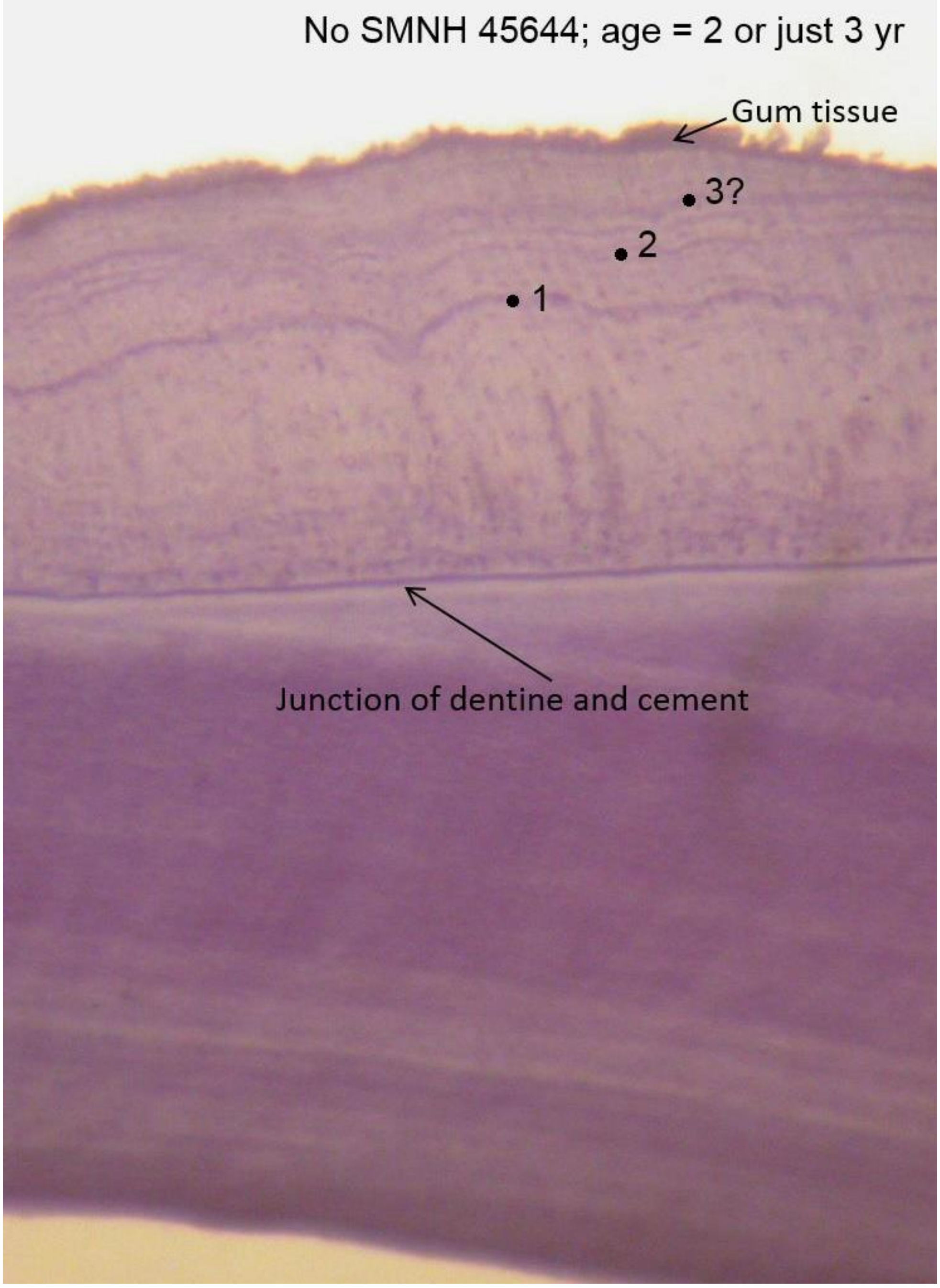

Fig. 3c. Cemental GLG – age 3 yr

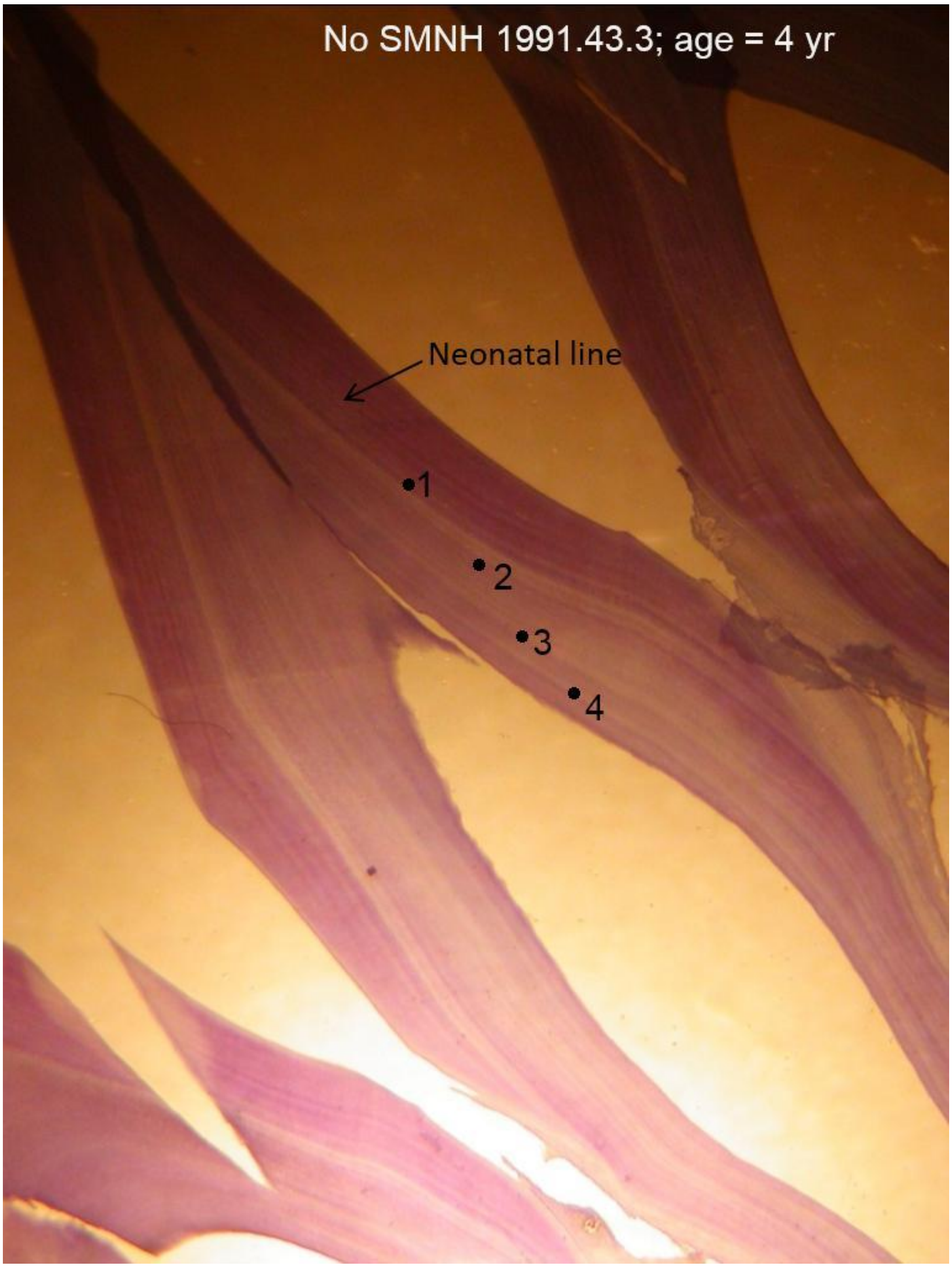

Fig. 3d. Dentinal GLG – age 4 yr

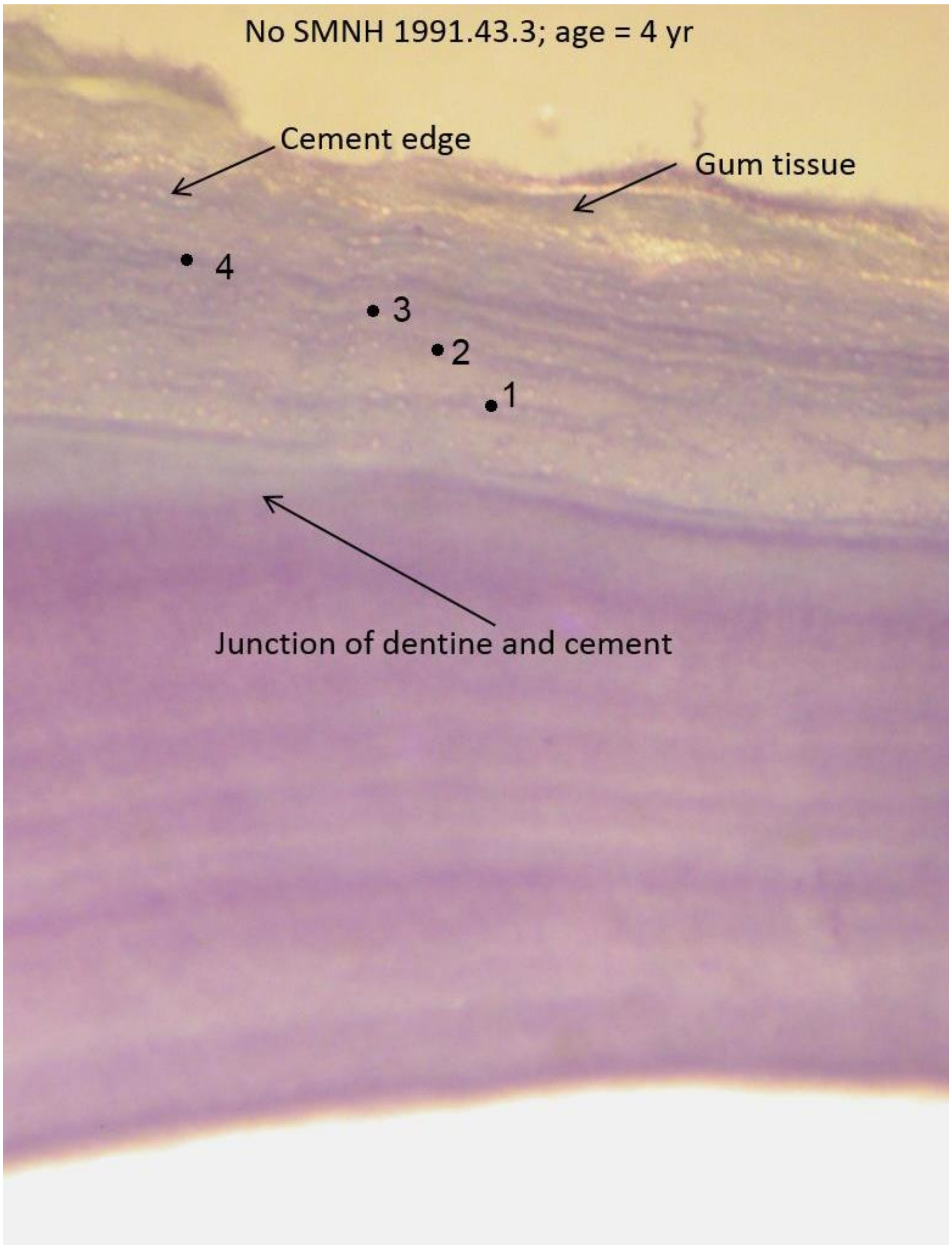

Fig. 3e. Cemental GLG – age 4 yr

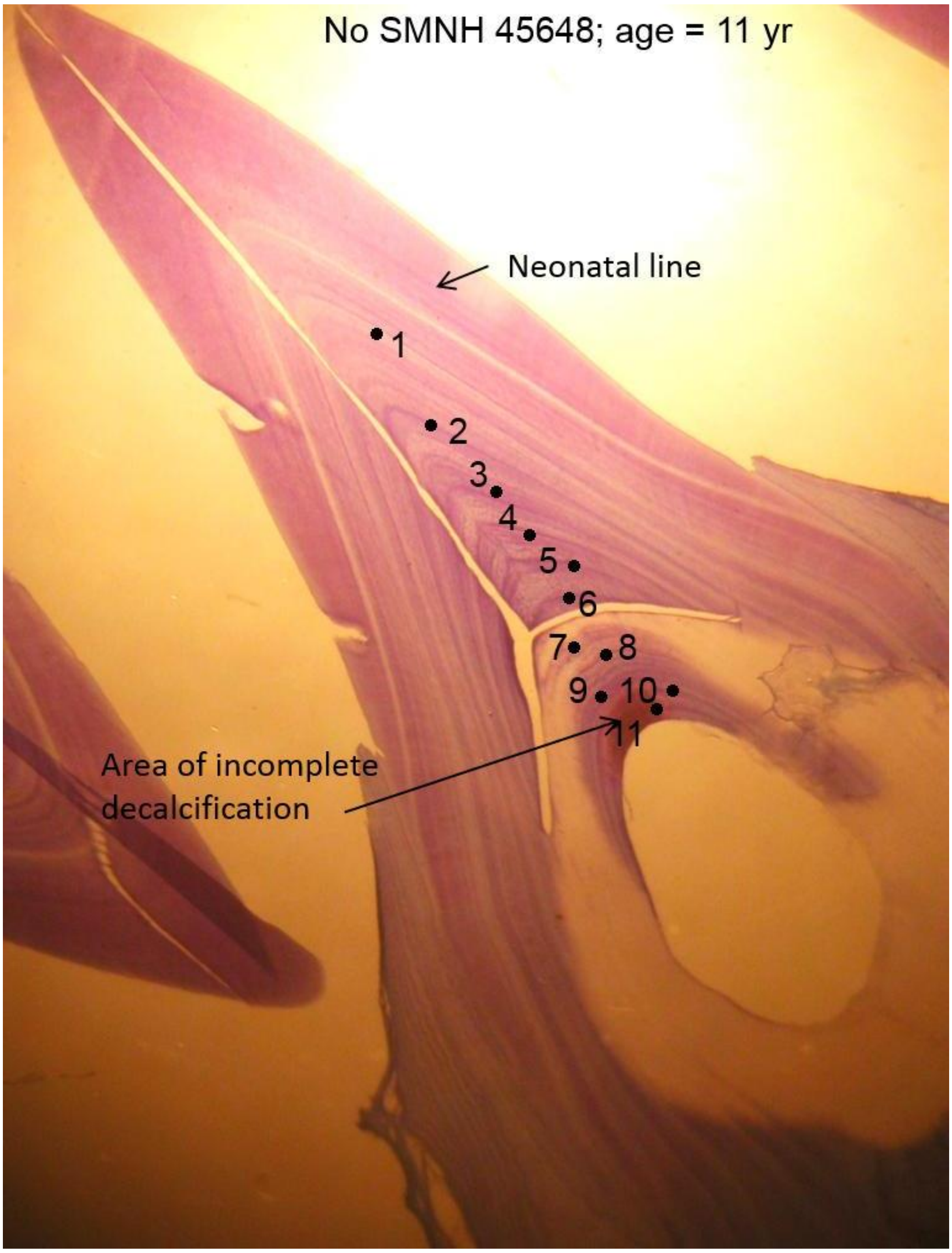

Fig. 3f. Dentinal GLG – age 11 yr; note the cracks in the dentine

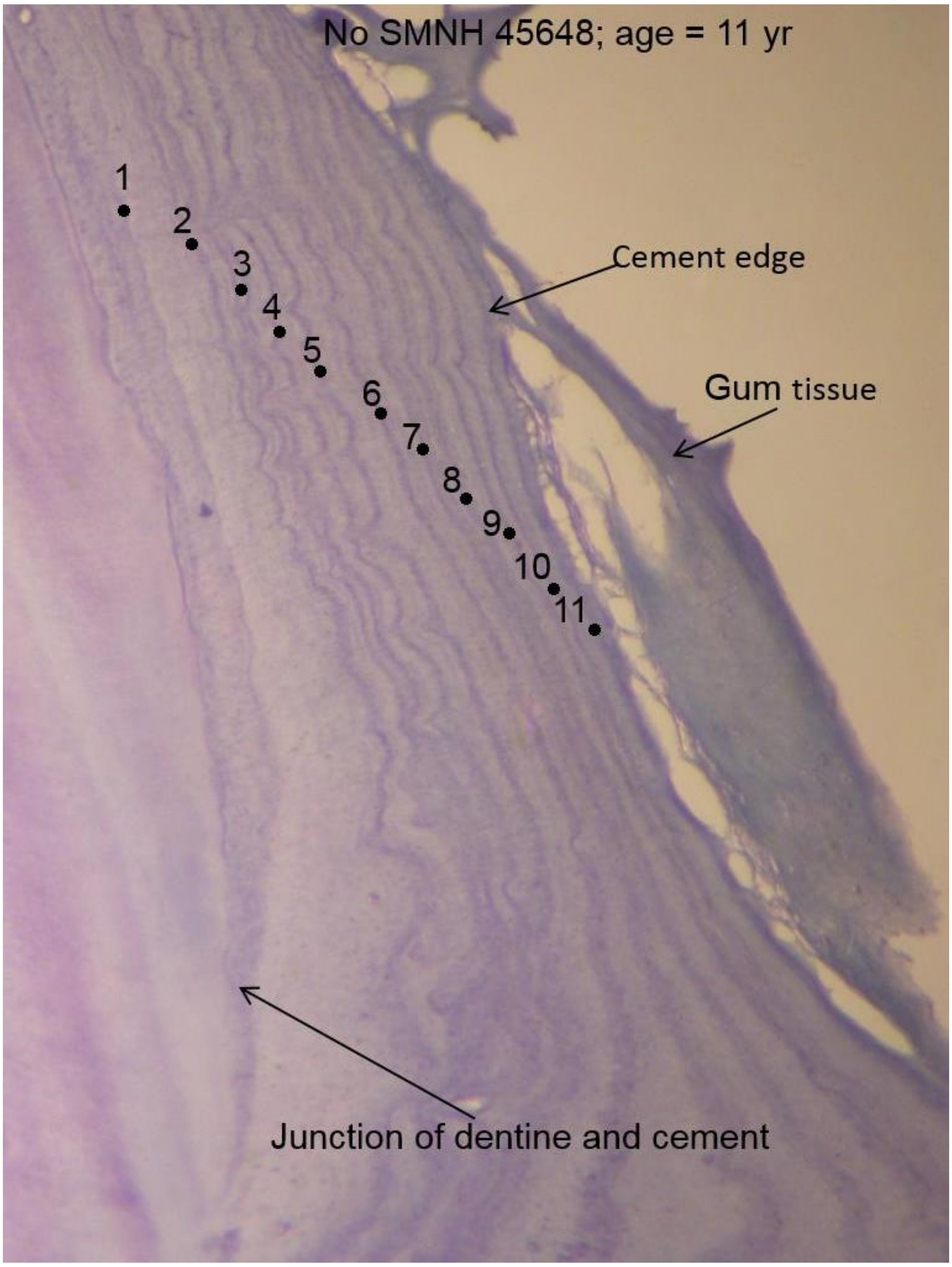

Fig. 3g. Cemental GLG – age 11 yr; note the boundary line 3 is double in places

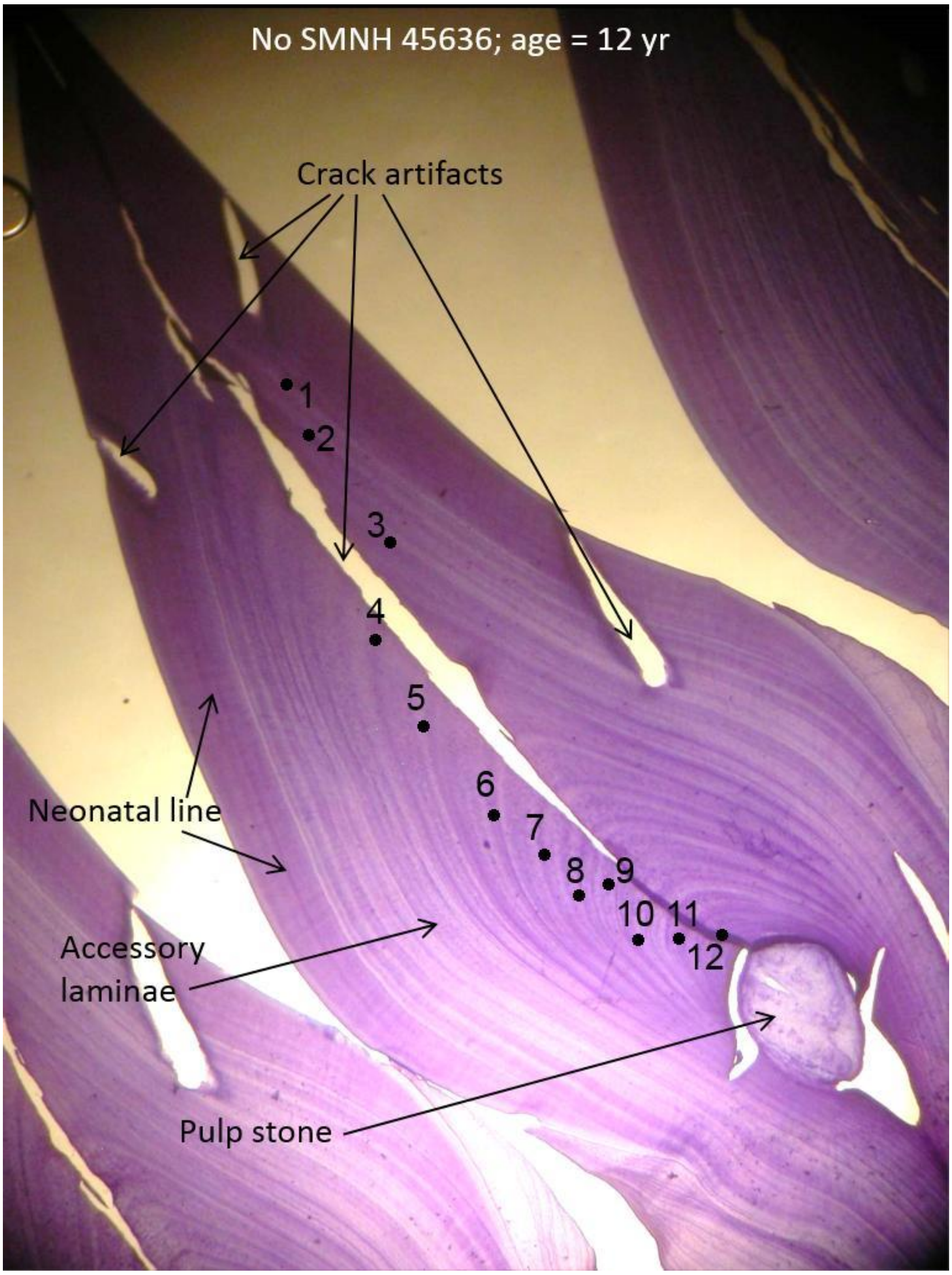

Fig. 3h. Dentinal GLG – age 12 yr; note the many cracks in the dentine and also the large pulp stone occupying part of the pulp cavity

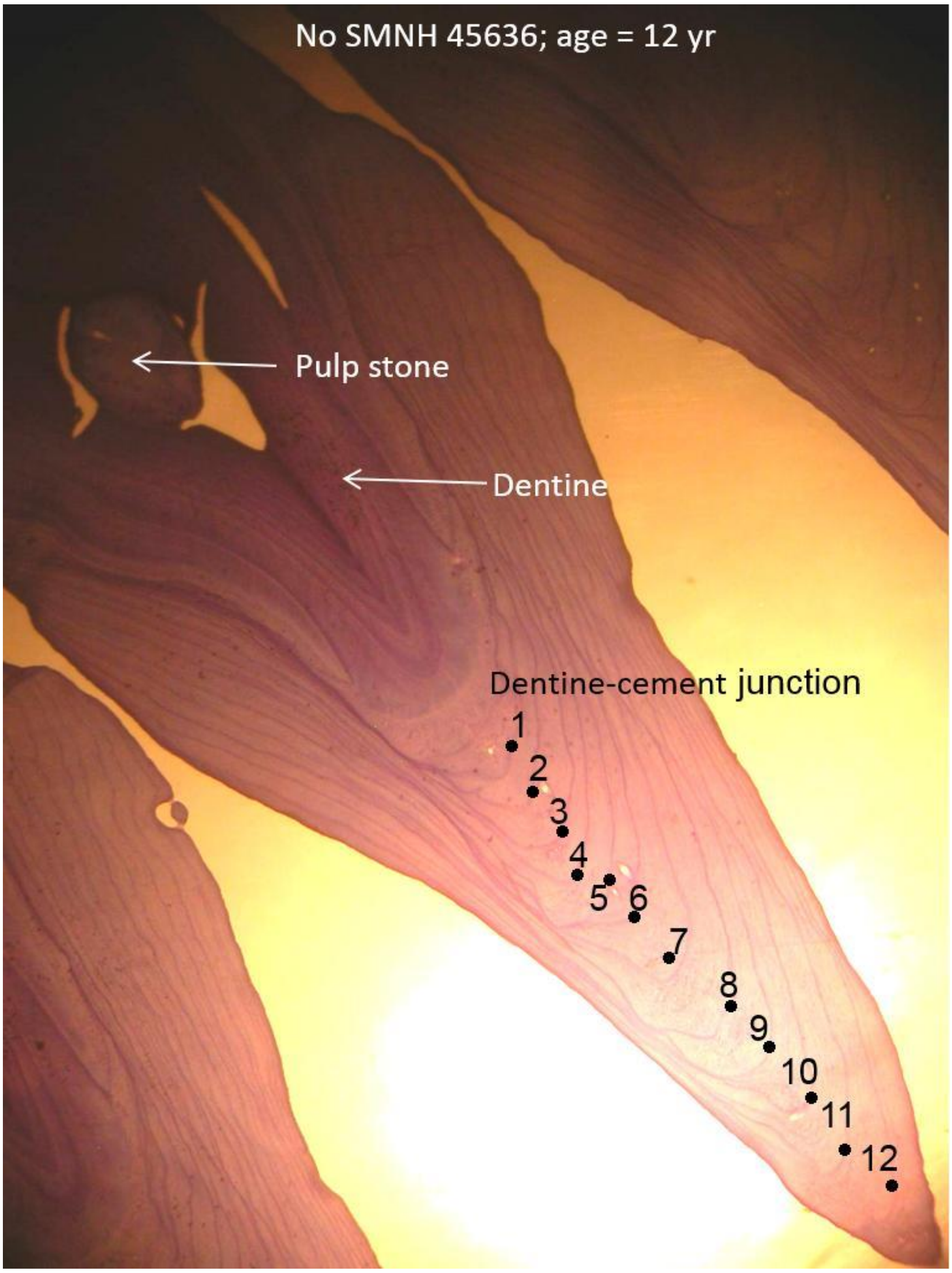

Fig. 3i. Cemental GLG – age 12yr

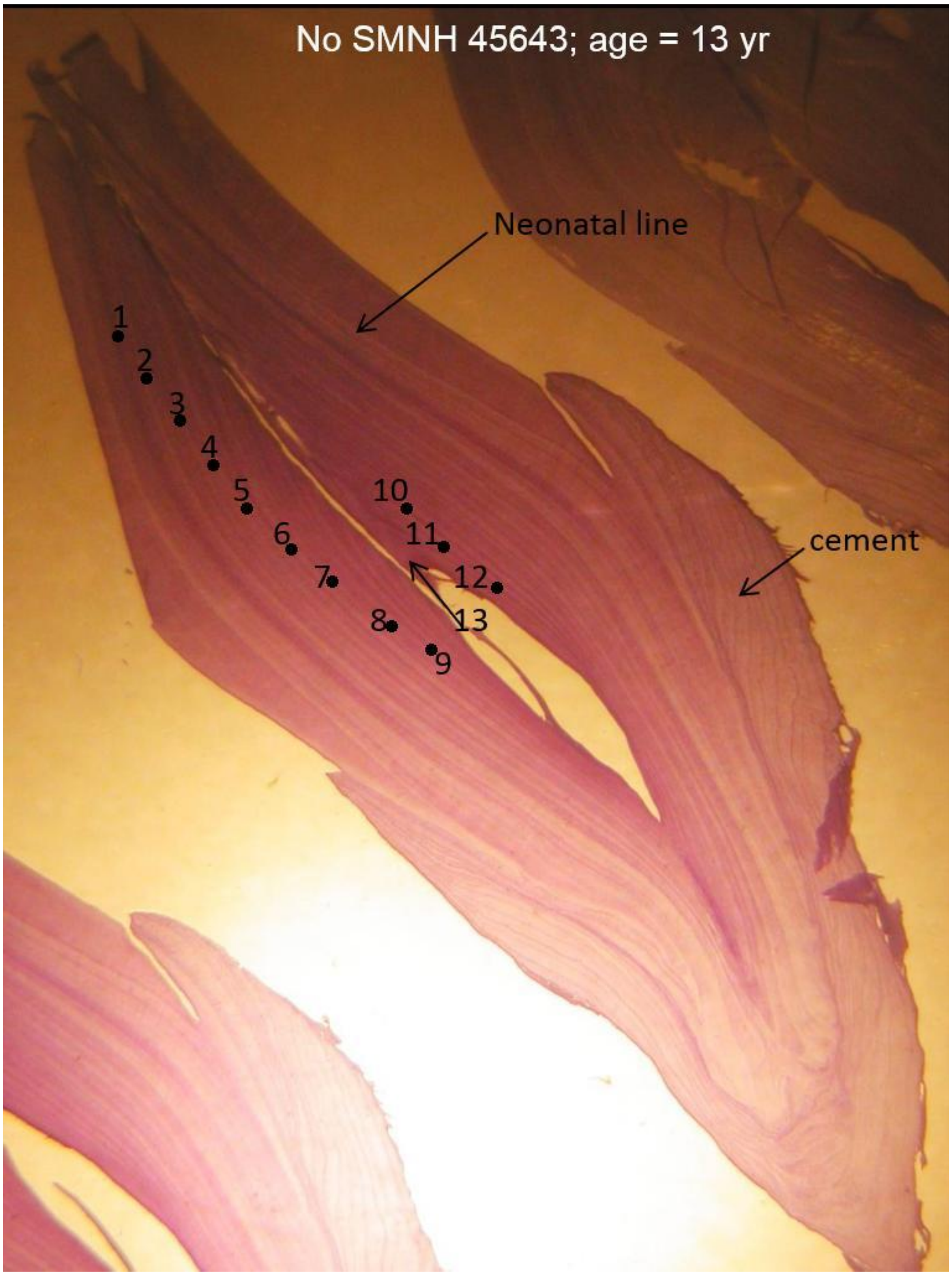

Fig. 3j. Dentinal GLG – age 13 yr

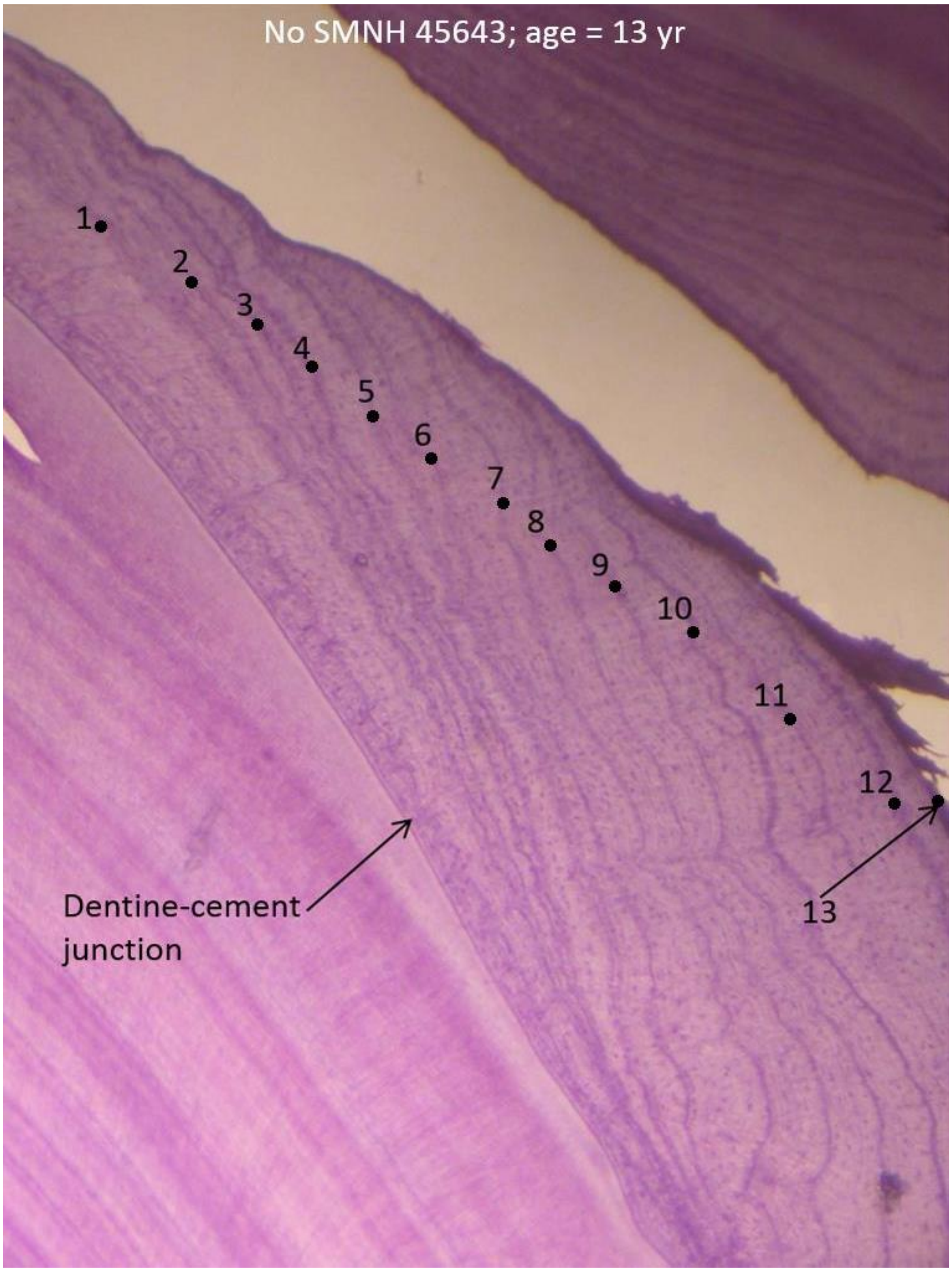

Fig. 3k. Cemental GLG – age 13 yr

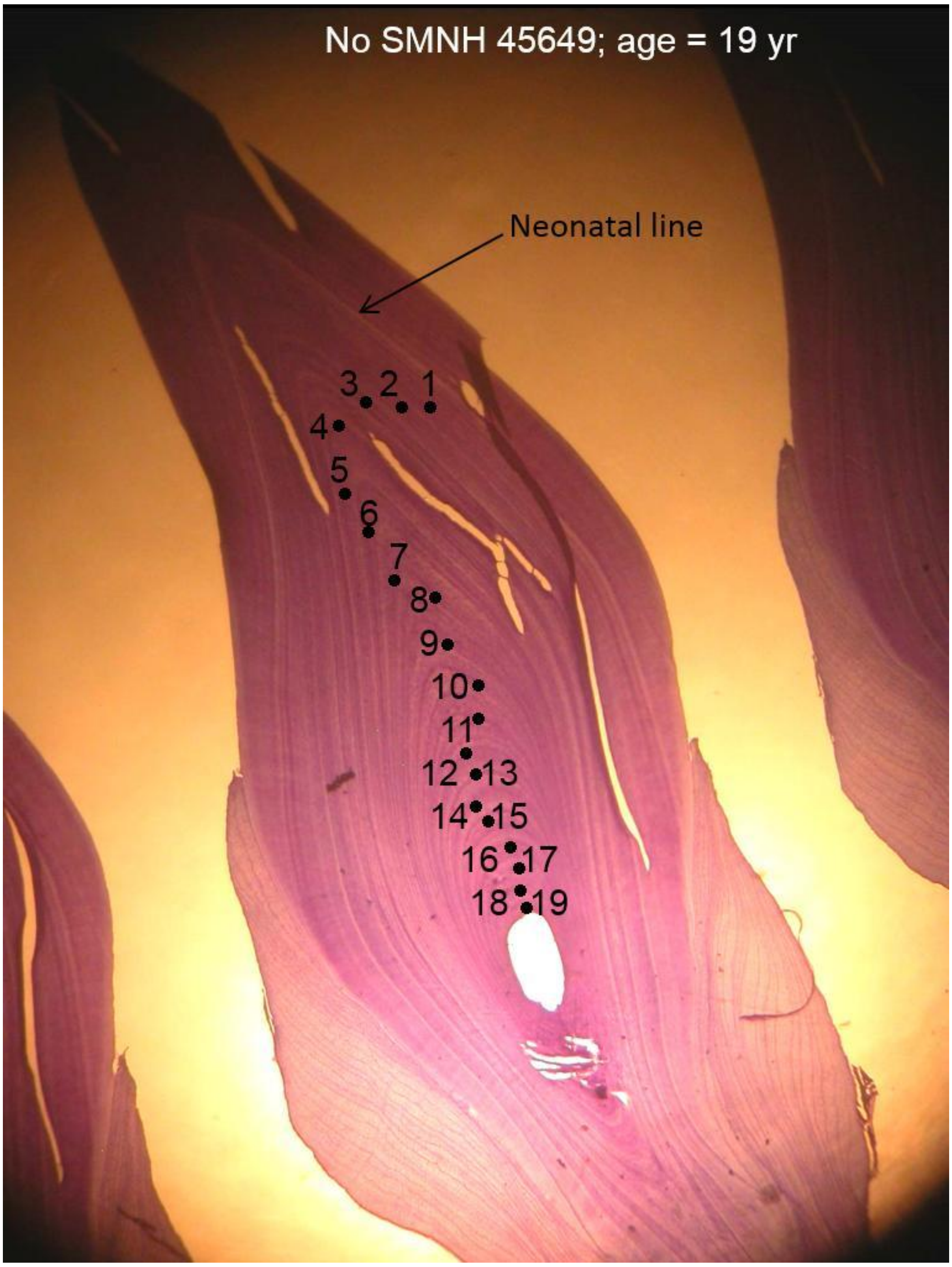

Fig. 3l. Dentinal GLG – age 19 yr; several cracks in the dentine

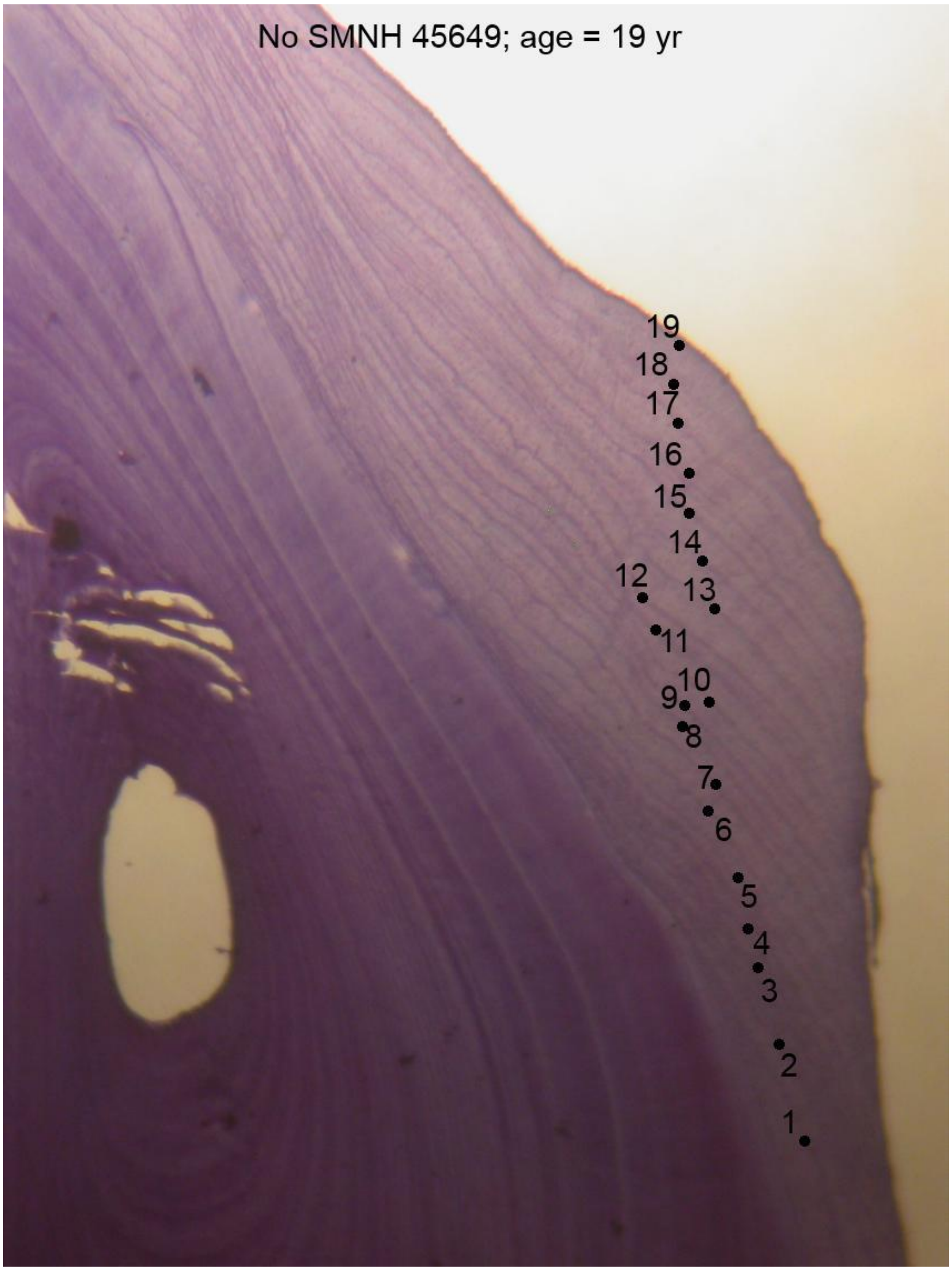

Fig. 3m. Cemental GLG – age 19 yr

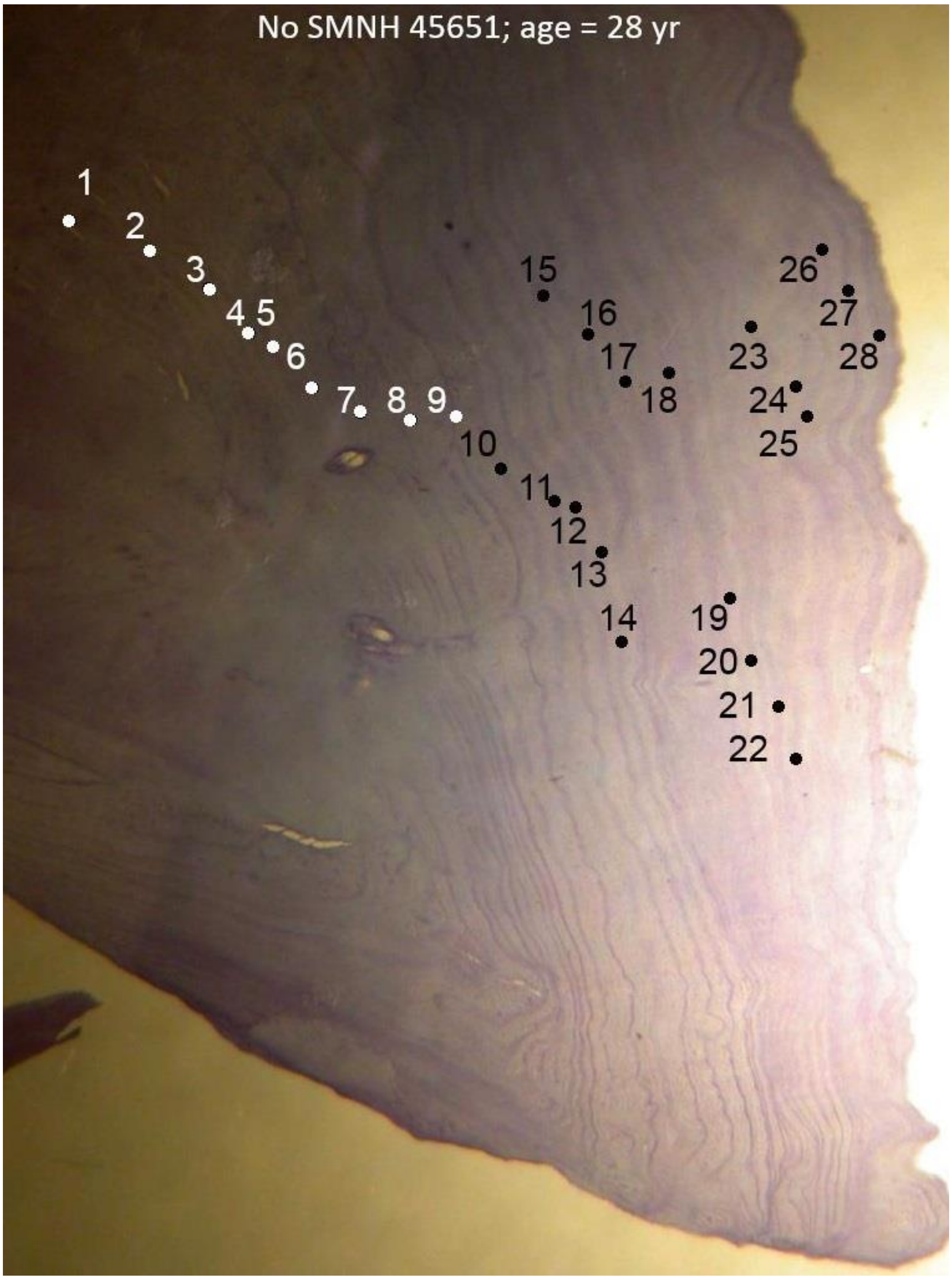

Fig. 3n. Cemental GLG – age 28 yr

Fig. 3a–n. Ontogenetic age series of decalcified, sectioned and stained *Platanista* teeth showing Growth Layer Groups in both dentine and cementum. (Digital images were captured with a Nikon Coolpix 450S mounted on the third ocular of a Meiji dissecting scope.)

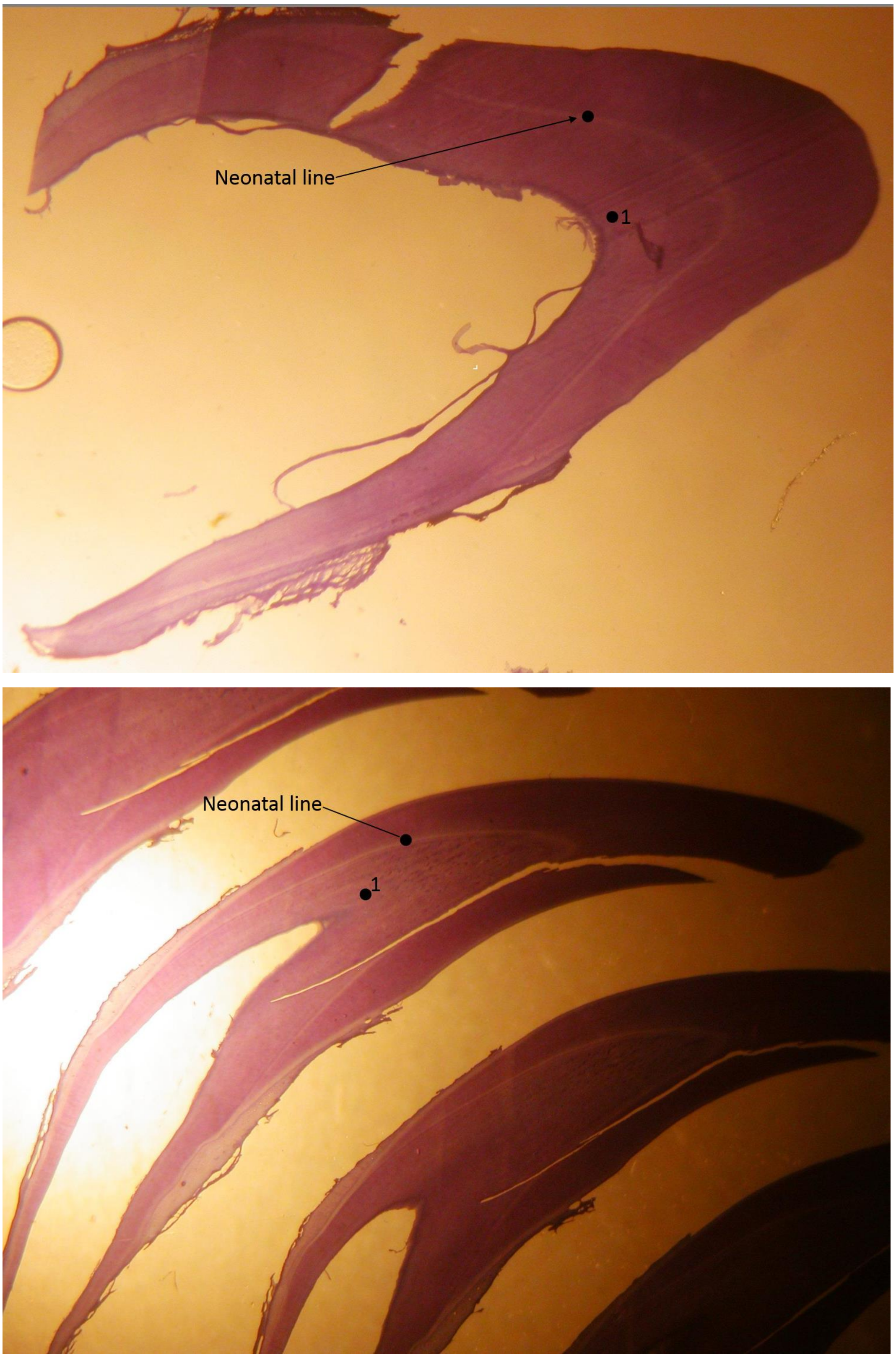

Fig. 4a and b. Sections from two different teeth from a young animal SMNH 45640 aged 1 yr. The section above is from a small squat tooth and the bottom section is from a long thin tooth. Both provide the same age.

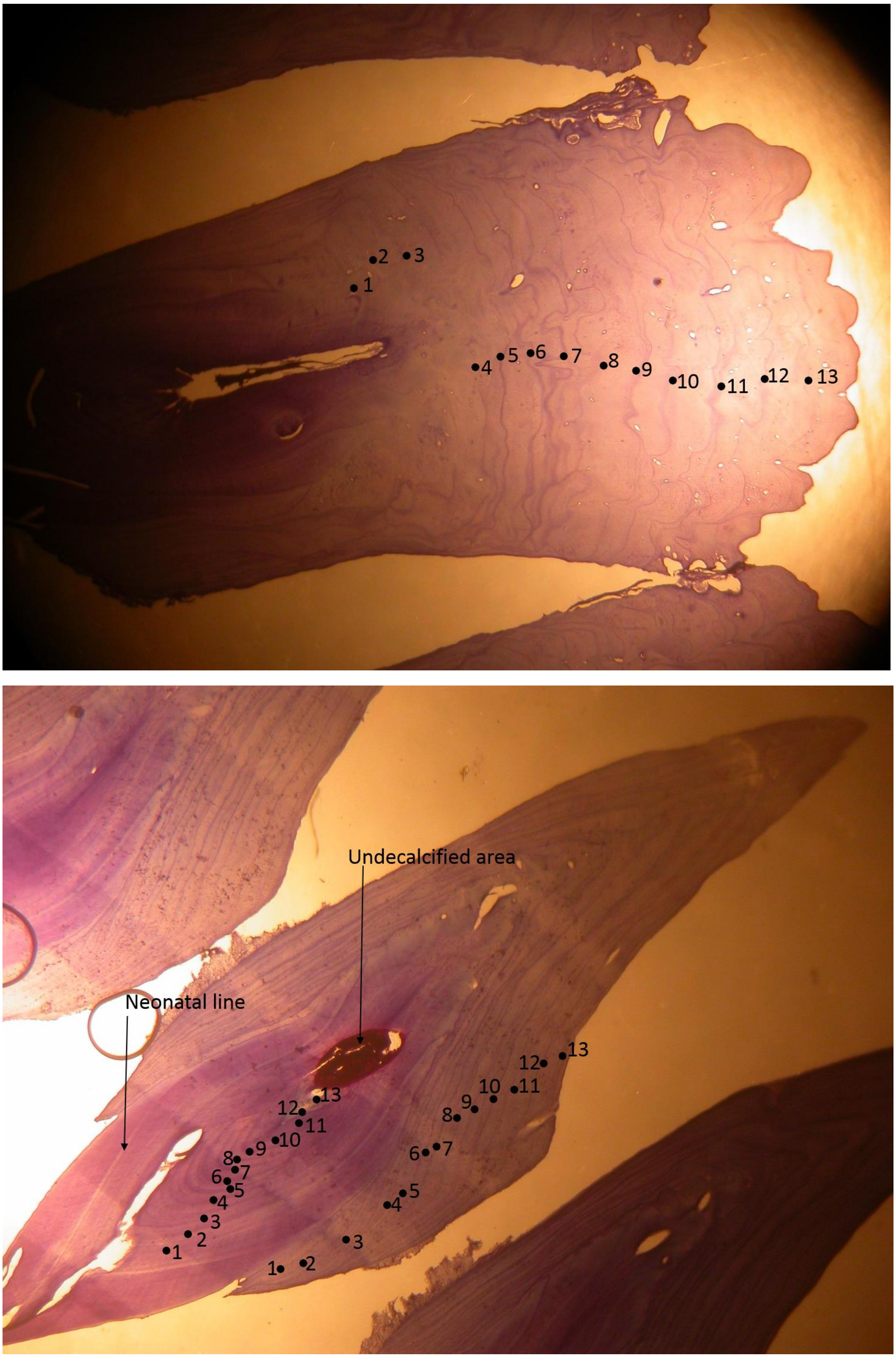

Fig. 5a and b. SMNH 45643 – age 13 yr (see also above Fig. 3j), showing the cemental GLG in sections of two teeth from the same jaw cut in two different planes (90° to each other) through crown and root. The lower section is easier to cut and align on the freezing stage for a central cut, although before doing this, the sides of the root "fan" need to be trimmed down so that the tooth will sit on the stage in a stable manner. The GLG are also less crenellated in the lower section.

Inter-comparison of GLG in dentine and cement, and from duplicate teeth
Dentinal and cemental GLG counts were in agreement for all teeth examined. Teeth from very young animals sometimes varied greatly in shape with both a small squat shape which originated from the rear of the jaw to a very long elongated and thin shape from the jaw tip (Figs. 4a,b) but both teeth, irrespective of their position in the jaw showed the same dentinal age (see below). In large older teeth, the root had become compressed in one plane and splayed out like a fan in the other, and sections were made in two planes to examine the difference in appearance of the cemental GLG. Figures 5a,b show a comparison; GLG were readable in both sections.

Comparison of duplicate teeth from the same animal for 11 specimens showed that there was close agreement in GLG counts (Table 1). The maximum observed variation was ±1 GLG in older animals. We are therefore inclined to conclude that age can likely be estimated accurately from teeth throughout the jaws.

Validation of GLG
There is currently no validation available for GLG deposition rate, but it is likely annual because of the extreme seasonal changes in the river habitat (Braulik, 2012). An endogenously regulated rate of deposition of tooth layers appears normal for all mammals (Grue and Jensen, 1979; Langvatn, 1995), and where the rate has been validated, is generally annual. This has been well demonstrated for *Tursiops truncatus*, for example, from free-ranging animals of known-age and -history (Hohn *et al.* 1989).

Conclusion
The advantage of using the decalcification and freeze-sectioning method is that the teeth which are frequently highly curved and even twisted along the axis, can be manipulated once softened, so enabling a better approximation to a central cut through the crown and pulp cavity. A central section is important for displaying all structures within the dentine, and without off-centre distortion of the GLG. Such sections are good for providing ages from both dentinal and cemental GLG which are both in agreement.

ACKNOWLEDGEMENTS

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