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Short communication

X-ray microcomputed tomography reveals putative trematode metacercaria in a 100 million year-old lizard (Squamata: Agamidae)

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ABSTRACT

X-ray microcomputed tomography was used to reveal putative trematode metacercariae (Platyhelminthes: Digenea) located in cysts positioned at the base of the femora in a 100 myr agamid lizard preserved in Myanmar amber. The cysts are characterized and compared with encysted metacercariae recovered from a similar location in an extant *Anolis* lizard. This discovery provides evidence that lizards were serving as intermediate hosts of trematodes some 100 Ma.

1. Introduction

Cretaceous amber from Myanmar is well known for its invertebrate fossils and has provided fossil records of over 250 families of arthropods (Rasnitsyn et al., 2016). This amber source also contains vertebrate remains, including various lizards, such as the gecko, *Cretaceogekko burmae* Arnold and Poinar (2008) and undescribed taxa of Lacertoidea, Chamaeleonidae and Agamidae (Daza et al., 2016).

An X-ray microcomputed tomography examination of a fossil agamid lizard in Myanmar amber revealed two sac-like bodies at the base of the femora. Based on their size, shape and position, these structures are interpreted to represent cysts of trematode metacercariae. These putative metacercariae are characterized and compared with trematode metacercariae infecting an extant anolid lizard. This discovery provides evidence that lizards were serving as intermediate hosts of trematodes some 100 Ma.

2. Materials and methods

2.1. Origin of amber specimen

The fossil lizard is in a block of amber measuring 60 mm long by 23 mm wide and 13 mm deep. The amber originated from the Noije Bum 2001 Summit Site mine located in the Hukawng Valley southwest of Maingkhwan in Kachin State (26°20′N, 96°36′E) in Myanmar. Based on paleontological evidence, this site was dated to the late Albian, of the Early Cretaceous (Cruickshank and Ko, 2003), placing the age at 97 to 110 Ma. A more recent study using U–Pb zircon dating determined the age to be 98.79 ± 0.62 Ma (Shi et al., 2012). Nuclear magnetic resonance (NMR) spectra and the presence of araucaroid wood fibers in amber samples from the Noije Bum 2001 Summit Site indicate an araucarian tree source for the amber (Poinar et al., 2007). Also present in the same piece of amber is a scarab beetle (Scarabaeidae: Coleoptera) approximately 6 mm in length, a planthopper (Achilidae: Hemiptera) approximately 7 mm in length, and several small flies. It is likely that the beetle was attracted to the lizard remains as an oviposition site (See Supplementary Fig. 1). The fossil lizard will be deposited in the Senckenberg Museum and Research Institute in Frankfurt-am-Main, Germany under accession number F28461BUICJW.

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2.2. *Extant specimen of* Anolis sagrei *Duméril and Bibron, 1837*

A specimen of the Brown Anolis, *Anolis sagrei*, which is native to Cuba and the Bahamas but now widespread and sold in pet stores, was stained in 11% Lugol's iodine solution (12 Kl) for 4 days, then rinsed for 5 min in 50% EtOH and mounted for microCT scanning. Metacercarial cysts were observed at the caudal end of the trunk on the dorsal side and in the tail posterior to the distal tip of the hemipenis. Verification that these were trematode cysts was made by Dr. Edward Stanley at the Florida Museum of Natural History.

2.3. *Methods of examination*

Features of the fossil lizard and metacercariae are based on visual assessment and microcomputed tomography (μCT) imaging. The light micrograph photograph was taken with a Nikon SMZ-10 R stereoscopic microscope. A Scanco μCT40 scanner (Scanco Medical AG, Basserdorf, Switzerland) at a voxel size of 15 × 15 × 15 μm (55 kV_p x-ray voltage, 145 μA intensity, and 200 ms integration time) was used to scan the amber-encased specimen to obtain a nondestructive 3-dimensional evaluation. The filtering parameters, sigma and support, were set to 0.8 and 1, respectively. Segmentation was conducted at an empirically determined threshold of 180 (scale, 0–1000). The resulting volume was manually programmed to separate the specimen from other objects embedded within the amber. Length measurements were conducted on the segmented volume by determining the distance between points positioned to define the boundary of the respective segments of the long axis. Visualizations of the μCT images were generated using Scanco imaging software and Adobe Photoshop (Adobe, San Jose, USA). False colored images illustrate scales in green, bone in gray and trematode cysts in brown (Figs. 1 and 2A). Microcomputed tomography (μCT) imaging was also used to examine trematode cysts in an extant male *Anolis sagrei* Duméril and Bibron.

3. Results

3.1. *Fossil lizard (Fig. 1)*

The lizard apparently had been attacked by a predator and most of its body, including the head, was missing and flesh was even stripped from its legs. Essentially all that is left, aside from the cysts, is a portion of the skeleton and some scales covering the limbs and tail. The remains can be detected much clearer in the CT scan than in a color photograph of the specimen (Fig. 1, Supplementary Fig. 1). Based on characters presented by Daza et al. (2016), Witten (1989) and the analysis of Hutchinson et al. (2012) who combined morphology and molecular data sets employing parsimony and Bayesian inference, the fossil lizard is assigned to the family Agamidae (Squamata; Lacertilia). The projected length of the complete lizard, including the missing head and tail tip, is calculated to range between 85 and 92 mm, a size range falling within the dimensions of adult agamids (Manthey and Schuster, 1996). The long tail, reduced claws and long 4th digits of both the manus and pes and

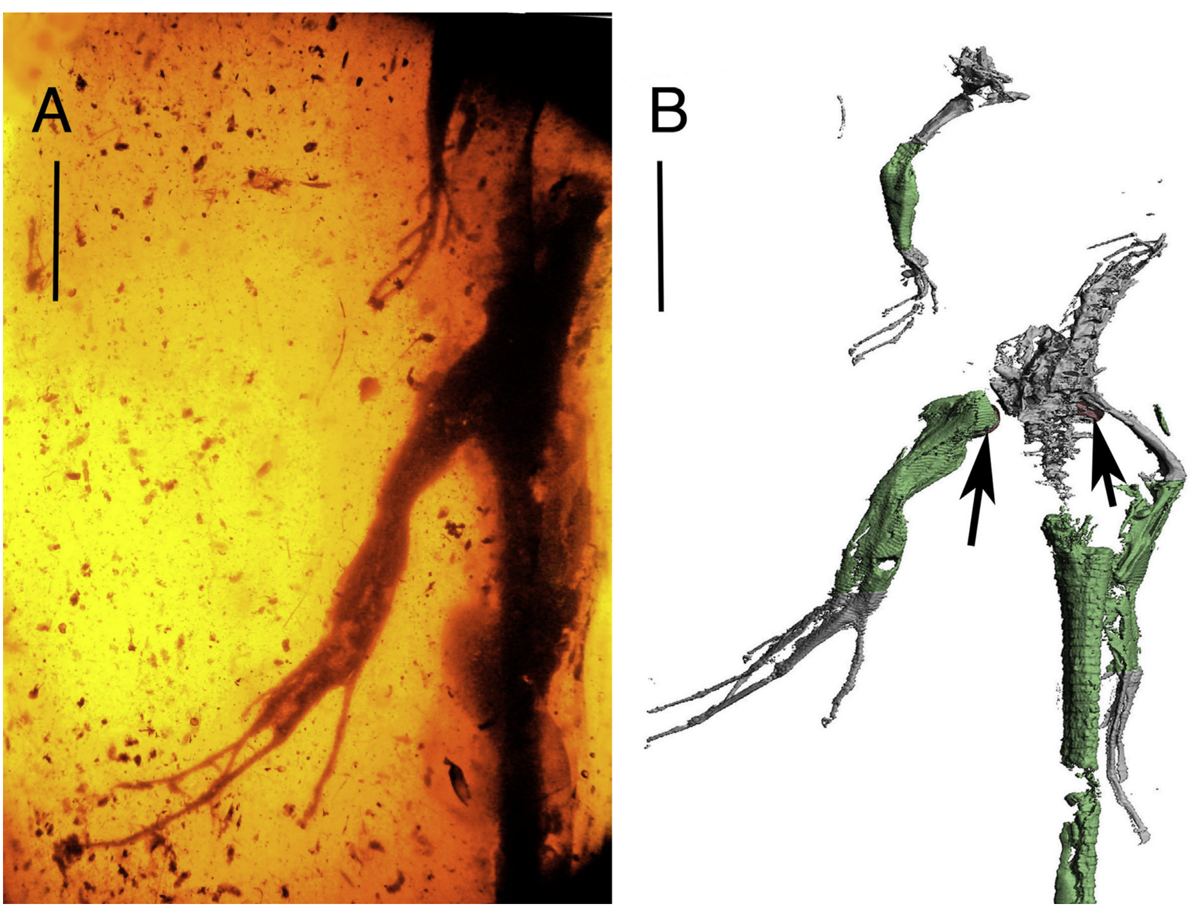

Fig. 1. Remains of agamid lizard in Myanmar amber. A. Lizard viewed with normal lighting (terminal tail portion not shown). Scale bar = 7 mm. B. Lizard viewed with X-ray microcomputed tomography colored to facilitate tissue identification. Bones = gray; scales and skin = green. Arrows show position of putative trematode cysts. Scale bar = 7 mm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

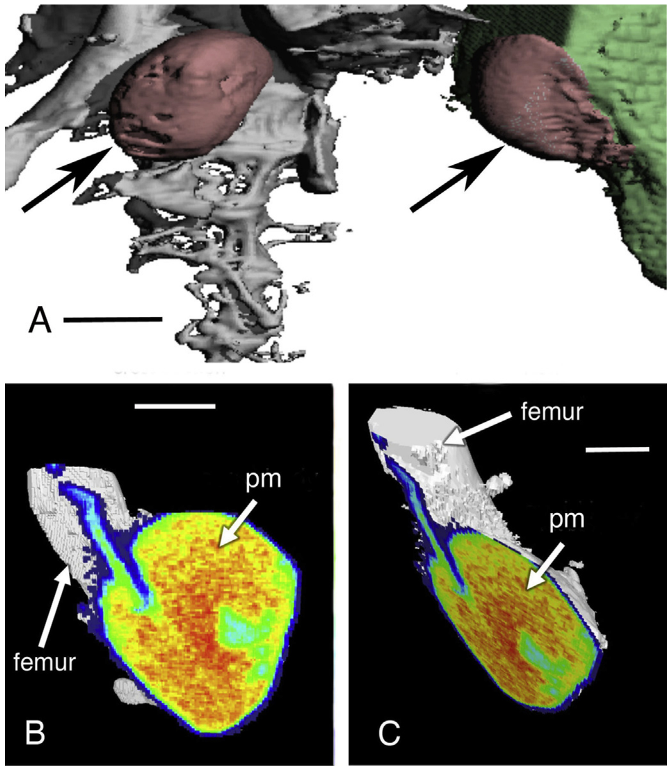

Fig. 2. CT visualization of the putative trematode cysts positioned at the base of the femora of the agamid lizard in Myanmar amber. (A) Ventral view of cloacal region with fossil trematode cysts (arrows). Scale bar = 1.4 mm. Cross-section (B) and transverse view (C) of one of the trematode cysts showing a ventrally curved metacercariae (pm) inside the cyst. Scale bars = 0.8 mm.

configuration of the lateral scales also support its assignment to this family. However, because the head and neck are diagnostic but missing, the lizard could not be described (Witten, 1989; Manthey and Schuster, 1996; Diong and Lim, 1998; Gauthier et al., 2012; Daza et al., 2016).

3.2. Fossil cysts (Fig. 2)

The two fossil cysts that are positioned at the base of the femora measure 2.2–2.4 mm in greatest dimension. They show a variation in x-ray density with the densest area (Fig. 2B, C) forming a C-shape that is considered to represent the curved body of the metacercaria within the cyst. The outer less dense area is thought to represent fluid surrounded by the cyst wall.

3.3. Extant trematode metacercariae (Fig. 3)

The two metacercariae in the femora region of an extant male *Anolis sagrei* using Microcomputed tomography (μCT) imaging measured 2.0 mm in greatest dimension.

4. Discussion

The size and shape of the fossil cysts fall within the range of extant trematode metacercariae (Hunter and Hamilton, 1941; Hyman, 1951; Frye, 1991) and resemble the extant encysted metacercaria observed in *Anolis sagrei*. Not only are the shapes (round in cross section and oval in transverse section) of the fossil and extant cysts similar, but they are all positioned near the base of the femora. It is possible that the parasites search out locations to

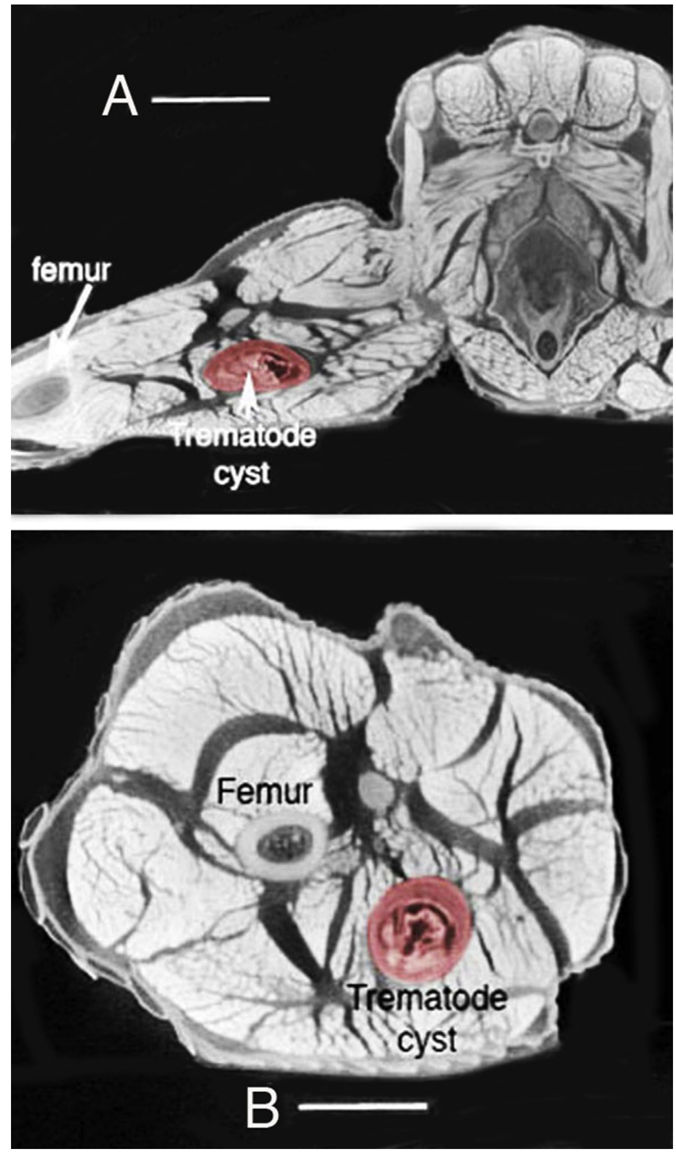

Fig. 3. Extant trematode metacercariae encysted adjacent to the base of the femora of *Anolis sagrei*. A. Transverse CT section of the right leg with the trematode cyst adjacent to the femur. Scale bar = 2.0 mm. B. Cross CT section through the base of the left leg of *Anolis sagrei* showing a trematode cyst adjacent to the femur. Scale bar = 2.0 mm. These cysts are roughly in the same position in relation to the femur in *Anolis sagrei* as they are in the fossil agamid lizard.

encyst based on features in the host's tissues. They may be attracted to discrete fat bodies that exist in *Anolis* species (Greenberg and Gist, 1985) or glandular deposits that occur in some lizards (Mouton et al., 2014). It is possible that the cysts remained with the skeleton because tissue at the articulation of the femur with the pelvic girdle is somewhat protected. It is also possible that metacercaria positioned at this site in the host may hinder mobility, making the lizard more easily captured and giving the trematodes an opportunity to complete their development. In the life cycle of digenetic trematodes, eggs from adults are passed out of the host's body with feces, urine or oral mucus. Each egg develops into a miracidium, which in most species enters a snail and continues through one or more developmental stages in various intermediate hosts before infecting a vertebrate definitive host. Many species of Digenea parasitize reptiles and some 75 species are known to have lizards as definitive hosts (Frank, 1984; Brooks, 1984). Lizards also serve as intermediate hosts to trematodes such as *Platynosomum* spp. that utilize birds and mammals as definitive hosts (Heidebegger and Mendheim, 1938). *Platynosomum fastosum* Kossak, a parasite of cats and dogs in Puerto Rico, uses lizards as second intermediate hosts and up to 300 trematode cysts have been recovered from a single anole with an average mean intensity of 16 cysts per lizard (Maldonado, 1945). Pinto et al. (2014) also noted high numbers of *Platynosomum illiciens* (= *P. fastosum*) cysts in gecko intermediate hosts in Brazil. It is not possible to determine if the fossil cysts belong to the genus *Platynosomum*, since 100 Ma, other trematode lineages certainly existed.

While external features of metacercarae can sometimes be observed through the cyst wall, as noted in *Anolis sagrei* (Fig. 3), intermediate hosts often form a thick outer wall around the encysted parasite that masks external features (Hunter and Hamilton, 1941). Also, host-secreted walls can become melanized in both invertebrates and vertebrates, thus reducing further visibility (Chapman and Hunter, 1954). However the CT scans of the fossil cysts show an inner curved structure (Fig. 2B, C). Based on the position of some extant metacercariae, many of which are doubled over ventrally within their cysts, this curved structure is interpreted to be the bent body of the parasite (Hunter and Hamilton, 1941; Hyman, 1951; Sohn et al., 2015).

Consideration was given to the possibility that the fossil cysts could represent other structures, such as fat bodies, femoral glands, or hemipenes. However, fat bodies are not found in this location in extant lizards, femoral glands are multiple, superficial and much smaller than the present cysts and hemipenes are positioned at the base of and within the tail and not in the hind legs.

5. Concluding remarks

The fossil record of trematodes is quite sparse and most refer to recent (subfossil) records, such as *Schistosoma* spp. remains from Egyptian mummies dated at 5000 years (Ruffer, 1910; Deelder et al., 1990) and dicrocoelid eggs from 5500 year old bear coprolites in France (Jouy-Avantin et al., 1999). Apparently the Early Cretaceous record of *Digenites proterus* Poinar and Boucot (2006), represented by a fossil trematode egg recovered from a dinosaur coprolite found at the Lower Cretaceous Bernissart Iguanodon shaft in Belgium, is the oldest record of a trematode. The present study indicates that lizards were serving as intermediate hosts to trematodes in the mid-Cretaceous.

It is highly probable that the lineages of both the trematode and agamid are extinct today. The agamid could have disappeared at the Cretaceous-Paleogene boundary, when species-level extinctions of Squamata (lizards and snakes) were calculated to have reached 83% (Longrich et al., 2012).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cretres.2017.07.017.