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Analysis of the skull of *Proteus anguinus anguinus* by high-resolution X-ray computed microtomography

Analiza lobanje *Proteus anguinus anguinus* z uporabo visokoločljivostne rentgenske računalniške mikrotomografije

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Proteus anguinus (Laurenti, 1768) is a cave amphibian of the order Caudata. Nearly all proteus' populations have very similar morphology with reduced pigmentation and vestigial eyes. This stygobiont amphibian lives in underground waters of the karst along the East Adriatic coast, from the Isonzo (Soča) river in the Northeast Italy to the Trebišnjica river in the Southeast Bosnia and Herzegovina (Sket 1997).

The skull morphology of proteus and its cartilaginous parts are interesting to study because they could be related to life in darkness. The first detailed description of the proteus skull was performed by Dolivo-Dobrovolsky (1926) and later by Sket & Arntzen (1994) through a comparative analysis of skeleton of both white and black proteus subspecies. A few years later, Ivanović et al. (2013) conducted a detailed analysis of the skull by using X-ray computed microtomography (micro-CT) technique. This approach allows to investigate the three-dimensional (3D) microstructure of a biological sample and to visualize regions with different density and/or chemical composition, using virtual slicing or volume rendering procedures.

In the Erwin Pichl Speleovivarium (Società Adriatica di Speleologia) of Trieste (Italy), operating under the supervision of the Natural History Museum of Trieste, the proteus individuals have been investigated applying micro-CT techniques. We aim to obtain high spatial and contrast resolution images, which allow high accuracy in measuring the anatomical details of the skull. Here we present results of the skull measurements of one adult proteus individual. The individual was collected in Postojnska jama (Slovenia) in 1989, and kept in in Speleovivarium laboratory until 1999 when preserved in alcohol. The first micro-CT measurements were performed in 2012 at the TomoLab station of Elettra Sincrotrone Trieste facility (situated in Basovizza near Trieste in Italy; https://www.elettra.trieste.it/). The TomoLab station was conceived as a micro-CT instrument complementary to the SYRMEP beamline (Tromba et al. 2010) and devoted to synchrotron-based micro-CT (Zandomeneghi et al. 2010). At TomoLab, it is possible to achieve a spatial resolution close to the minimum focal spot size (~5 µm) working in a voltage range of 40-130 kV and with a cone-beam geometry. Moreover, a micro-CT system based on a hard X-ray microfocus source shows limited (but clearly detectable) phase-contrast effects (Wilkins et al. 1996) also if with a limited potential with respect to a synchrotron X-ray micro-CT set up. This property could be particularly beneficial for the visualization and subsequent image segmentation of the soft tissues in biological samples.

The examined proteus had a total body length of 276 mm. The individual was placed in an empty plastic cylindrical tube sealed with Parafilm® for the micro-CT scan. The use of a non-destructive method allowed us to visualize bones, cartilages and soft tissues in their original position, without damaging the individual. Volume rendering (obtained with the commercial software VGStudio Max 2.0®, Volume Graphics) and morphological measurements were based on an X-ray microtomographic (micro-CT) scan. The micro-CT scan was performed with the following experimental parameters: Voltage: 65 kV, current: 123 µA, filter = 0.75 mm Al, 2400 projections recorded over a scan angle of 360°, exposure time/projection: 5.5 s, source-to-sample distance: 220 mm; source-to-detector distance: 320 mm, isotropic voxel size: 17.2 µm. A 3D image segmentation, based on a manual thresholding, was performed to visualize only the hard tissues of the animal.

The morphological analysis of the skull was performed using the same landmarks as in Ivanović et al. (2013) (Tab. 1, Fig. 1). Our results (Tab. 1) are in agreement with the range of values reported by Ivanović et al. (2013). We observed 6 teeth on the right premaxilla, with two missing teeth and 7 teeth on the left premaxilla and only one missing tooth. We counted 27 teeth on the right vomer and 26 teeth on the left one (with a probably missing tooth). In this individual, we did not detect teeth on palatopterigoide bones.

Table 1. The list of measurements done on proteus' skull, with distances measured between the landmarks as presented in Fig. 1.
Tabela 1. Seznam meritev, opravljenih na lobanji močerila, z razdaljami med merskimi točkami, ki so označene na Sl. 1.

Distance measured	Landmarks, as shown in Fig. 1	Size (μm)
Premaxilla (Pmax)	1 ↔ 2	937
	5 ↔ 6	3389
	3 ↔ 4	1311
Vomer (Vom)	7 ↔ 8	4759
Palato-pterigoid (Pal-pt)	9 ↔ 10	4295
	11 ↔ 12	6310
Quadrate (Quad)	13 ↔ 14	10349
	15 ↔ 16	10389
Cranium width	15 ↔ 16	10389
Parietal (Par)	17 ↔ 18	4667
Exoccipital (Exoc)	19 ↔ 20	6484
Cranium length	1,2 ↔ 21	22163
	1,2 ↔ 22	19550
	1,2 ↔ 19 or 20	25784

Using phase-contrast X-ray micro-CT, we were able to visualize not only the hard tissues composing the proteus' skeleton but also some of the well-preserved soft tissues (Fig. 2). This revealed that the skeleton and the gill arches were embedded in the muscles constituting a robust and elastic structure characterized by a rather ample cartilaginous joint. In the highlighted detail the joint between the dentary, quadrate and prearticular or gonial bones was visible; the front and posterior depressors of the mandible were inserted in the gonial bone, similarly to what was described for *Necturus maculosus* (Bauer 1997). The muscular structures and their functional organization, together with the shape of the skull, are probably related to predatory activities, the type of prey and other the ecological variables (Herrel et al. 2001).

Investigation techniques are continuously improving and offering new important opportunities for research. The morphological characterization of the proteus skull will allow a 3D reconstruction and modelling of parts of the cranial bones. Furthermore, in 2016, the analyses with synchrotron micro-CT were performed on various sexually mature proteus individuals from different localities at the SYRMEP beamline (Elettra Sincrotrone Trieste). The analyses were performed in collaboration with several national and international institutions, and present the work in progress.

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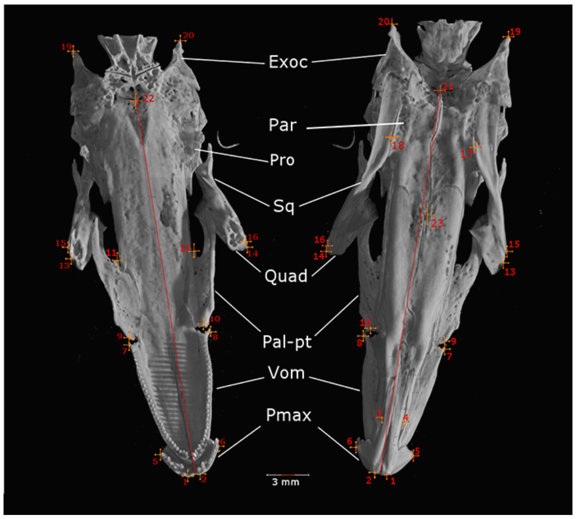

Figure 1. The images show the ventral (on the left) and dorsal (on the right) views of the upper bones of the skull of *Proteus anguinus anguinus*. The parameters of micro CT scans are given in the text. The distances, measured with landmarks 1 to 23, are explained in Tab. 1. Abbreviations on the photo relate to: Exoc – exoccipital, Par – parietal, Pro – prootic, Sq – squamosal, Quad – quadrate, Pal-pt – palato-pterigoid, Vom – vomer, Pmax – premaxilla.

Slika 1. Prikaz ventralnega (levo) in dorzalnega (desno) pogleda na zgornje kosti lobanje *Proteus anguinus anguinus*. Parametri mikro CT skenov so podani v tekstu. Razdalje, ki so bile merjene z oznakami od 1 do 23, so prikazane v Tab. 1. Oznake na fotografiji pomenijo: Exoc – eksokcipitalna kost, Par – parietalna kost, Pro – prootična kost, Sq – skvamozna kost, Quad – kvadratum, Pal-pt – palato-pterigoid, Vom – vomer, Pmax – premaksila.

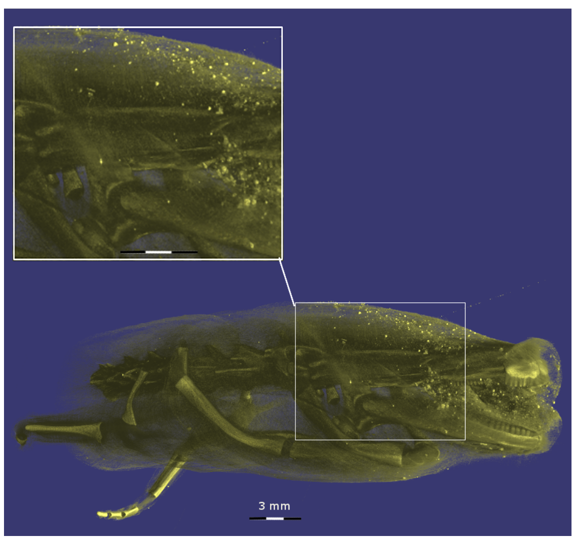

Figure 2. Volume rendering based on micro CT scans acquired with the parameters as given in the text. This picture shows both the hard and soft tissues; in the enlargement (on the left top) the joint between the dentary, quadrate and the prearticular (or gonial) can be observed. The selected level of transparency of the soft tissues allows seeing the hard tissues behind as well.

Slika 2. Volumska predstavitev, ki izhaja iz micro CT skenov s parametri, kot navedeno v tekstu. Ta slika prikazuje tako trda kot mehka tkiva; v povečavi (levo zgoraj) je prikazan sklep med dentarno, kvadratno in preartikularno (ali gonialno) kostjo. Nastavitev prozornosti prikaza mehkih tkiv omogoča tudi prikaz trdih tkiv zadaj.