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# High quality 3D imaging of vertebrate microremains using X-ray synchrotron phase contrast microtomography

## Imagerie 3D haute qualité de microrestes de vertébrés par microtomographie à rayonnement X synchrotron en contraste de phase

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## ABSTRACT

Vertebrate microremains, particularly teeth, represent a substantial part of known vertebrate biodiversity. Many groups, such as Mesozoic mammals, are known mostly through isolated teeth. Classical imaging techniques of such complex millimetric to inframillimetric objects are most often limited by problems of manipulation, depth of focus or limited orientation. The methods generally used are stereomicroscopy (including in-focus z-series reconstruction) and Scanning Electron Microscopy (SEM), which provide good images. Nevertheless, both provide 2D static images or partial and directional 3D data, making complete observation difficult. Propagation phase contrast synchrotron X-ray microtomography is a powerful technique alleviating these limitations. Thanks to submicron resolution and to the edge detection effect, it rapidly provides 3D data from minute samples with levels of quality and detail unattainable using conventional microtomographs. Complex morphology of small specimens can be studied with unlimited orientation possibilities and, when coupled with 3D printing, it allows enlarged 3D reproductions of such small and fragile fossils.

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## RÉSUMÉ

L'étude des microrestes de vertébrés, particulièrement des dents, devient de plus en plus fréquente, car ils représentent une part importante de la diversité d'un assemblage fossile, certains groupes comme les mammifères mésozoïques n'étant principalement connus que par des dents isolées. Les techniques classiques d'imagerie d'objets millimétriques ou inframillimétriques aussi complexes sont le plus souvent limitées par des problèmes de manipulation, de profondeur de champ et de possibilités d'orientation. Ces méthodes habituelles sont la stéréomicroscopie (incluant les reconstructions de séries de mise au point en z) et le microscope électronique à balayage (MEB), qui fournissent de très bonnes images. Cependant, ces deux techniques aboutissent à des images statiques en 2D, rendant difficile l'observation complète de l'objet. La microtomographie à rayonnement X synchrotron en contraste de phase de propagation

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est une technique puissante permettant de dépasser ces limitations. Grâce à la résolution submicronique et l'effet de renforcement des interfaces, elle permet d'obtenir très rapidement des données en 3D de petits échantillons, avec un niveau de qualité et de détails inégalable par des microtomographes conventionnels. La morphologie complexe de petits spécimens peut être étudiée avec des possibilités d'orientation illimitées et, couplée à une impression 3D, cette technique permet de matérialiser des reproductions agrandies de fossiles si petits et fragiles.

## 1. Introduction

Vertebrate microremains are classically defined as small isolated mineralized parts of a vertebrate body. Their size generally ranges from a half millimetre to a few millimeters. Their extraction requires screen washing of sediment through sieves of various meshes to isolate these minute fossils. They are mainly represented by isolated teeth and bones of small forms (adults of small-sized taxa or juveniles of larger taxa), but other body parts can be represented, such as fish scales, otoliths, etc.

The interest of microremains in vertebrate palaeontology has been known for a long time, notably for the investigation of Mesozoic mammals or Cenozoic rodents, for which microremains are by far the main sources of information. More than half of the *ca.* 500 known Mesozoic mammal species have been described from isolated teeth (Kielan-Jaworowska et al., 2004). The same situation occurs for other rare and small taxa of fishes, sharks, amphibians and reptiles. Besides the taxonomic knowledge, vertebrate microremains provide original data on faunal assemblages, sometimes more diversified than macroremains, and in some sites, they can even represent the unique source of information. The small size of these particles involves also particular sedimentological and taphonomical characteristics which can be used as a palaeoenvironmental indicator.

Unfortunately, vertebrate microremains are not systematically studied in many palaeontological works. This is mainly due to two reasons, one linked to field work, the other to laboratory observation and imaging. In the field, extraction of vertebrate microremains requires particular processes, mainly based on sediment washing and screening. While the extraction of microfossils (i.e. ostracodes, foraminifers) needs relatively small quantities of sediment, extraction of vertebrate microremains often requires treatment of big amounts of sediment, due to the relative rarity of specimens in the matrix. This is particularly true for Mesozoic mammals, for which big amounts of sediment have to be washed and screened in the field, in order to concentrate the samples before sorting at the laboratory.

However, the main difficulty to study microvertebrate is due to the small size of the collected specimens. They are too small to be easily manipulated and to be observed without a lens. Furthermore, precise study of microteeth needs fully isolated and cleaned specimens, observable under at least the six anatomical views (occlusal, basal, mesial, distal, buccal and lingual). If basic observation of such tiny specimens under a stereomicroscope does not involve any particular problem, several difficulties come up with imaging. Three main imaging processes are currently used to study vertebrate microremains: (1) drawing and photograph through stereomicroscope; (2) Scanning Electron Microscopy (SEM) micrographs, and (3) X-ray microtomographic 3-dimensional reconstruction. These three imaging approaches are not exclusive and can be used synergistically. The review of their specific advantages, constraints and limits are presented and discussed hereafter in the purpose of performing accurate morphological observations, descriptions, illustrations and measurements (Fig. 1).

## 2. Classical observation methods

### 2.1. Stereomicroscope - Optical system

The stereomicroscope, or binocular lens, is a system of successive lens which allows one to observe small objects while keeping the possibility of the direct use of our stereoscopic vision.It constitutes an unavoidable instrument for the basic observations of microremains, thanks to its accessibility and ease of implementation. Modern stereomicroscopes offer great optical capacity and complementary tools, such as light systems, measurements and drawing mirrors, which can lead to impressive results. Image capture through stereomicroscope uses extension systems, most often a digital camera on a third column ("trinocular system"), which provides true-colour magnified pictures of the specimens, but without the stereoscopic aspect.

However, as easy as this process could be, it shows some technical limits such as distortions due to the perspective effect, which does not allow most of the measurements on the resulting image. The main limitation is linked to the weak focus depth (the "thickness" on which the focus is adjusted) nearly always thinner than the whole observed object (Fig. 1A). Furthermore, as the magnification increases the depth of focus decreases. Nevertheless, some modern in-z-focus systems provide a correction for this focus depth limitation by combining a succession of images focused at several height steps, and then reconstructing a sharp image (Optimas system, for example, or numeric microscope, Fig. 2), but even with in-z-focus correction, images obtained through stereomicroscope still are 2D static restitutions, and the sample has to be manipulated for each view. It has to be noticed that one of the great advantage of this technique is that it allows keeping the original sample colours and contrasts in the pictures. Also, recent data processing algorithm make possible to extract 3D partial and directional data from in-z-series that can be used for topological analysis or 3D renderings as long as the viewing angle stays quite close to the original z-series direction.
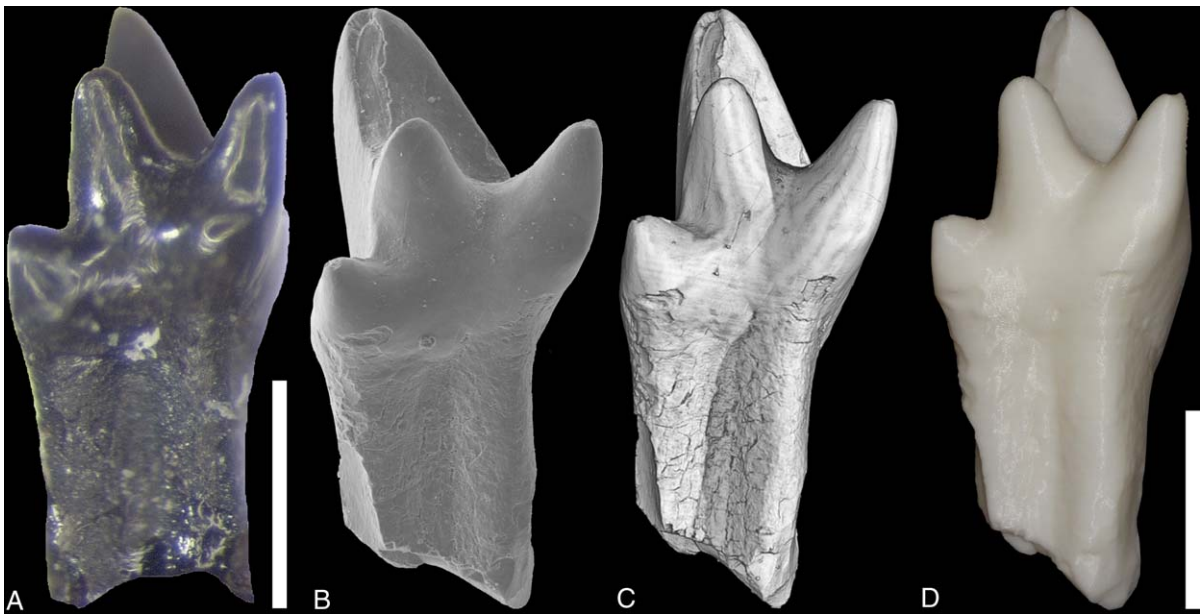
**Fig. 1.** Comparison of imaging results obtained using different techniques of a Dryolestidae mammalian tooth (CHEm03.546, lingual view) from the Berriasian of Cherves-de-Cognac, Charente, France. **A**: Image obtained on a stereomicroscope Leica MZ 7.5 with a camera Canon Powershot G6 adapted to a trinocular output. **B**: Image obtained from a Scanning Electron Microscopy (SEM) Hitachi S-570. **C**: 3D reconstruction from Propagation Phase Contrast Microtomography (PPC–SR–μCT) performed at the ESRF in Grenoble (ID19 beamline, voxel size: 1.4 μm, propagation distance: 50 mm, energy: 20 KeV). **D**: Enlarged 3D printing of the specimen using data tomography, performed on a Dimension Elite (Stratasys) 3D printer in ABS-plus plastic with layers of 170 μm. Scale bars: **A**, **B**, **C**: 0.5 mm, **D**: 5 cm.

**Fig. 1.** Imagerie par différentes méthodes d'acquisition d'une dent de mammifère Dryolestidae (CHEm03.546, vue linguale) provenant du gisement berriasien de Cherves-de-Cognac, Charente, France. **A**: Image obtenue sur une loupe Leica MZ 7.5, avec un appareil photo Canon G6 Powershot adapté sur une sortie trinoculaire. **B**: Image obtenue à partir d'un MEB Hitachi S-570. **C**: Reconstruction 3D à partir d'une microtomographie synchrotron en contraste de phase (PPC–SR–μCT) réalisée à l'ESRF de Grenoble (ligne ID19, taille de voxel: 1,4 μm, distance de propagation: 50 mm, énergie: 20 KeV). **D**: Impression 3D agrandie du spécimen à partir des données tomographiques, réalisée sur une imprimante 3D Dimension Elite (Stratasys) en plastique ABS-plus avec des couches de 170 μm d'épaisseur. Barres d'échelle: **A**, **B**, **C**: 0,5 mm; **D**: 5 cm.

## 2.2. SEM - electronic system

The SEM consists in bombarding the object with electrons which are reoriented according to the nature and topography of the sample. The original colours and contrasts of the sample are not visible in the resulting images. It is an efficient imaging tool, particularly well adapted to small samples. The quality of pictures, as well as the high magnification available without any focus depth problem (Fig. 1B), has placed the SEM in a prominent place in the modern study of vertebrate microremains. Most of the figured specimens of Mesozoic mammal teeth have been illustrated using SEM micrographs.

However, SEM imaging has important drawbacks, some of them related to sample preparation, generally involving a metal coating (even though environmental SEM offers the possibility of observation without such a metallization). Observation and picture acquisition are made in most of the cases in a environment under vacuum, which can lead to the break-up of microfractured fossil samples. However, the main problem with this technique is related to the manipulation of the specimen which is inaccessible during the acquisition. Sample mounting and limited mobility of the system make necessary five to six manipulations, fixations and dismantling of the specimen to generate the six different views for complete illustration of microteeth.

However, SEM imaging is nowadays the best compromise for high quality illustrations of vertebrate microremains, as it is an easily accessible way to obtain good images, and this kind of microscopes is quite widespread in laboratories. Despite its intrinsic qualities, that technique provides only, as for the stereomicroscopy, 2D images of the studied specimens.

## 2.3. Alternative methods with partial 3D information

Even if there are many techniques to image small samples in 2D or 3D, only two of them have been applied so far to vertebrate microremains: confocal microscopy, and laser or white light surface scanners. Despite the fact that these techniques can produce high quality pictures with partial or full 3D surface information, it has to be noticed that they only have been used so far on microremains for topological quantitative studies, but not for high quality illustrative purposes.

Confocal microscopy uses a complex optical design coupled with a scanning laser beam to extract optical sections through the samples. When coupled with a computer system driving a z-stage system, it allows retrieving 3D stack through the sample with limited penetration in the subsurface (typically a few microns to ∼100 μm). In most of the cases, it is based on the autofluorescence of the sample. It is a powerful technique for many biological materials, or

**Fig. 2.** Illustration of in-focus z-series reconstruction system which corrects the problems of depth of focus on stereomicroscope. Dryolestidae mammalian tooth (CHEm03.587, mesiolingual view, Berriasian, Cherves-de-Cognac, Charente, France) performed on a digital microscope Keyence VHX-600. **A**: Focus for a single image (classical stereomicroscope picture). The problem of depth of focus results in a blurring at the background outline of the tooth. **B**: Result of the compilation of in-focus planes on the whole depth of the tooth.

**Fig. 2.** Illustration du système de compilation d'images mises au point en z, permettant de corriger les problèmes de profondeur de champ sur une loupe binoculaire. Images d'une dent de mammifère Dryolestidae (CHEm03.587, vue mésilinguale, Berriasien, Cherves-de-Cognac, Charente, France) réalisées sur un microscope numérique Keyence VHX-600. **A**: Mise au point pour une seule image. Le problème de profondeur de champ se traduit par un flou sur les contours de la dent en arrière-plan. **B**: Résultat de la compilation des zones de mise au point sur toute l'épaisseur de la dent.

even for the imaging of small microfossils (Birkmann and Lundin, 1996, Feist-Burkhardt and Pross, 1999), including some microteeth for 3D topological analysis (Jernvall and Selänne, 1999). Confocal microscopy can easily reach sub-micron resolution, and is not really limited by focal depth problems. Nevertheless, the real vertical limitation in the z direction is given by the working distance of the objective that can be quite short, and the 3D data produced are only partial and directional. This technique does not produce a surface image, because it penetrates slightly in the sample. Nevertheless, as it is based on a vertical z-series of acquisition, the high quality of the 3D rendering is lost when the viewing angle becomes larger than 20–30 degrees from the original direction. The resolution in the z direction is far lower than in the focal plans used to produce the 3D stack, leading to a high anisotropy of the data quality in z, explaining the poor quality of off-axis pictures. It is then nearly impossible to produce good pictures of the lateral sides of a microremain, and therefore it is impossible to produce a basal view. To obtain all the necessary views, it is necessary to perform many manipulations, and regarding the long acquisition time for each z-stack, this technique cannot be used for the imaging of large amount of samples. Also, the z-series implies that complex shape can create shadow effects. Structures in a superior plane will hide the ones below. These limitations due to the z-axis acquisition approach are basically the same as those with the z-axis stereomicroscopic or epifluorescence microscope 3D reconstructions. Confocal microscopy is then widely used

to image microwear patterns on teeth at high resolution, as wear facets are topologically simple structures, but does not appear really adapted for full high quality imaging of microremains.

Laser and white light scanning are a variety of techniques able to provide real 3D data of nearly the entire surface of a sample, but not of the internal structures or hidden structures. They are generally linked to a motorized rotation and tilt system used to be able to image most part of the possible angles to reduce the shadows effect and produce accurate 3D models. Some of these systems can even map on the 3D surface the original colours of the sample. Despite the quite impressive results with macrofossils and some quite small teeth (Evans et al., 2007), their application on microremains is not really pertinent as it is very difficult or even nearly impossible in practice to reach the enough resolution level. The surface smoothing effect is then too important and precludes observations of fine structures that are essential for accurate description and determination of microremains.

## 3. Propagation phase contrast X-ray synchrotron microtomography

The main limitation of the two most frequently used techniques presented above (stereomicroscope and SEM) is the fact that they allow only the acquisition of 2D images, or of partial 3D data. They produce good illustrations of specimens, but do not allow more complex observations or duplication of the fossils using rapid prototyping techniques. Microtomographic scanning of specimens allows acquisition of real volumic 3D data that can be then used for 3D rendering and virtual manipulation of the sample in dedicated software. Use of tomography technologies for morphology imaging of fossils was firstly applied on quite large fossils using basically medical scanners (Conroy and Vannier, 1987) and later using industrial tomographs. Nevertheless these machines do not provide high enough resolution for small specimens, a single slice of a medical CT being typically the size of a complete microtooth. Recent microtomographs partially surpassed these limitations, but in many cases the quality level, as well as the acquisition speed in the micron range resolution, remain too low to be really compared with SEM rendering. Performance of Propagation Phase Contrast Microtomography (PPC-SR-μCT) (Tafforeau et al., 2006) is far better than the ones of conventional machines. It allows very fast acquisition (typically 2 to 10 min per tooth) up to submicron resolution, with very fine contrast, sensitivity and small structures detections thanks to the phase contrast effect. It then gives a high quality rendering on microvertebrates fossils (Fig. 3), with expanded possibilities for observation, manipulation or duplication of the specimen.

Application of synchrotron tomography on fossil objects is quite recent (Chaimanee et al., 2003) and its transposition to millimetric fossil specimens even more recent (Chen et al., 2006, Donoghue et al., 2006, Feist et al., 2005, Tafforeau, 2004, Tafforeau et al., 2006). Its use on microdental remains of vertebrates has been applied only in the latest years (Lazzari et al., 2008a, 2008b; Pouech, 2008; Tafforeau et al., 2006). The major interest of synchrotron
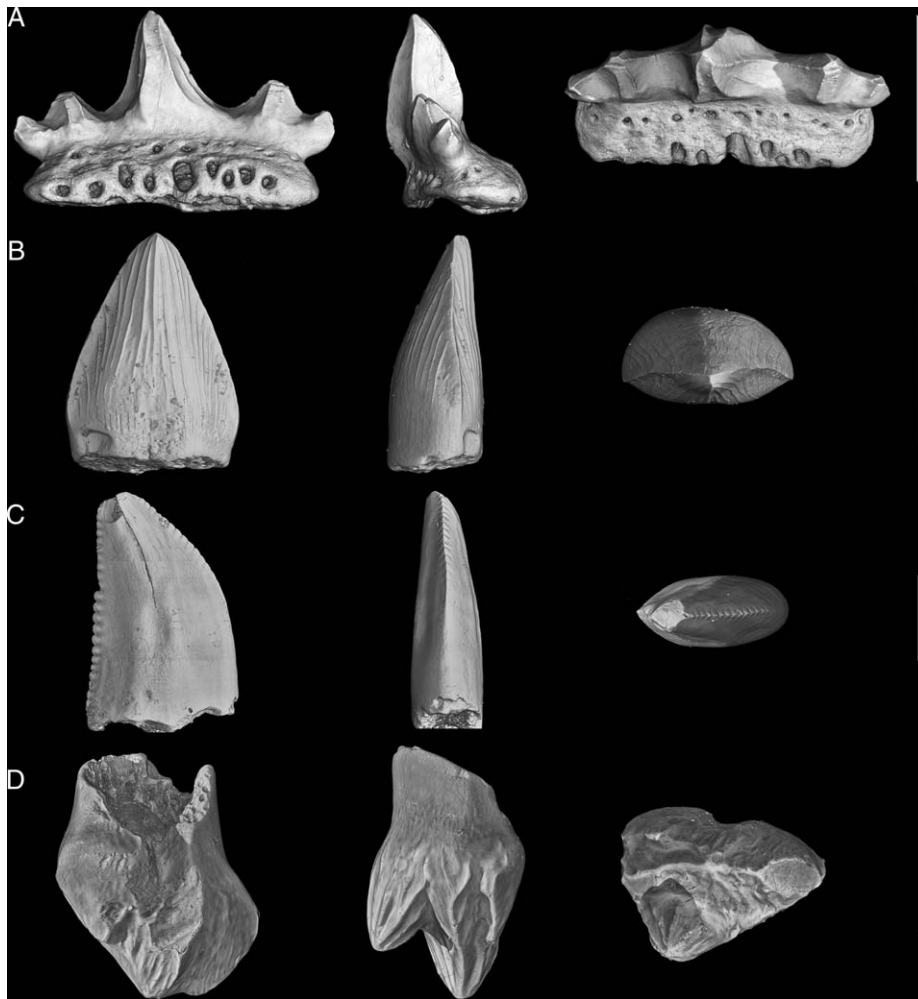
**Fig. 3.** Examples of 3D rendering of Propagation Phase Contrast Microtomography (PPC-SR-µCT) of microvertebrate remains. The specimens come from the deposit of Cherves-de-Cognac (Berriasian, Charente, France) and are presented respectively in lingual, mesial and apical views. PPC-SR-µCT were performed on the beamlines ID19 (**A**, **B**, **C**) and BM05 (**D**) of the ESRF. **A**: Tooth of shark Lonchidiidae (CHEm03.330). Features: voxel size: 2.8 µm, propagation distance: 50 mm, energy: 20 KeV. **B**: Tooth of crocodilian Atoposauridae (CHEm03.506). Features: voxel size: 1.4 µm, propagation distance: 50 mm, energy: 20 KeV. **C**: Tooth of theropod Dromaeosauridae (CHEm03.537). Features: voxel size: 2.8 µm, propagation distance: 50 mm, energy: 20 KeV. **D**: Tooth of mammal Pinheirodontidae (CHEm03.612). Features: voxel size: 0.7 µm, propagation distance: 30 mm, energy: 20 KeV. Scale bars: **A**, **B**, **C**: 1 mm; **D**: 0.5 mm.

**Fig. 3.** Exemples de rendu 3D par tomographie synchrotron de microrestes de vertébrés. Les spécimens proviennent du gisement de Cherves-de-Cognac (Berriasien, Charente, France) et sont présentés respectivement en vues linguale, mésiale et apicale. Les tomographies ont été réalisées à l'ESRF sur les lignes ID19 (**A**, **B**, **C**) et BM05 (**D**). **A** : Dent de requin Lonchidiidae (CHEm03.330). Caractéristiques : taille de voxel : 2,8 µm, distance de propagation : 50 mm, énergie : 20 KeV. **B** : Dent de crocodilien Atoposauridae (CHEm03.506). Caractéristiques : taille de voxel : 1,4 µm, distance de propagation : 50 mm, énergie : 20 KeV. **C** : Dent de théropode Dromaeosauridae (CHEm03.537). Caractéristiques : taille de voxel : 2,8 µm, distance de propagation : 50 mm, énergie : 20 KeV. **D** : Dent de mammifère Pinheirodontidae (CHEm03.612). Caractéristiques : taille de voxel : 0,7 µm, distance de propagation : 30 mm, énergie : 20 KeV. Barres d'échelle : **A**, **B**, **C** : 1 mm ; **D** : 0,5 mm.

imaging of fossils is evidently the possibilities of nondestructive investigations of internal structures (Fig. 4) with resolution, quality and sensitivity levels not achievable with any other techniques (see Tafforeau et al. (2006) for a more complete presentation of these aspects). Nevertheless, since it is not the topic of the present paper, it will then not be discussed further.

Accessing to a full 3D high-resolution dataset for microvertebrate fossils solves in one operation the main problems of observation, handling, imaging and duplication. These small objects usually have to be manipulated with pliers. Consequently, observing or fixing such tiny

complex and fragile specimen in a given view is often hard and risky. The X-ray microtomographic approach in general, and the PPC-SRµCT in the case of small fossils allow observing and rotating the virtual reconstruction of the specimen in dedicated software (in the present case VGStudioMax 2.0, Volume Graphics, Heidelberg, Germany). It is then easy to orient it and to generate any interesting viewing angle such as the commonly illustrated six faces of the tooth, possibly using stereoscopic rendering. The original colours are, nevertheless, not accessible to these techniques, but adapted false tuning with the 3D software can often bring very impressive results. The observation of
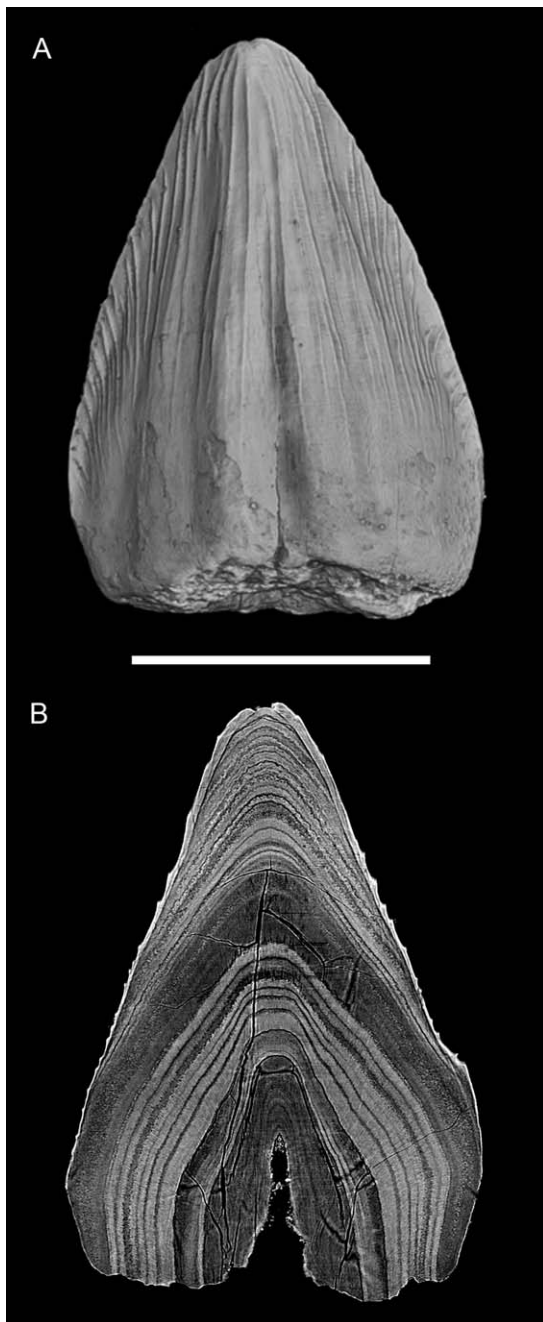
**Fig. 4.** Propagation Phase Contrast Microtomography (PPC-SR-µCT) provides access to both external morphology and internal structures. Example of a tooth of crocodilian Atoposauridae (CHEm03.503, Berriasian, Cherves-de-Cognac, Charente, France). **A**: 3D rendering of the surface of the tooth. **B**: Sagittal virtual slice. Tomography performed at the ESRF. Features: beamline ID19, voxel size: 2.8 µm, propagation distance: 50 mm, energy: 20 KeV. Scale bar: 1 mm.
**Fig. 4.** La microtomographie en contraste de phase à rayonnement X synchrotron permet d'accéder aussi bien à la morphologie externe qu'aux structures internes. Exemple d'une dent de crocodilien Atoposauridae (CHEm03.503, Berriasien, Cherves-de-Cognac, Charente, France,). **A** : Rendu 3D de la surface de la dent. **B** : Coupe virtuelle sagittale. Tomographie réalisée à l'ESRF. Caractéristiques : ligne ID19, taille de voxel : 2,8 µm, distance de propagation : 50 mm, énergie : 20 KeV. Barre d'échelle : 1 mm.

tiny details is easier and each part of the specimen is easily accessible and observable, which is particularly interesting for complex morphology specimens (for example for Mesozoic mammal crowns, Fig. 3D). This data acquisition scheme implies far less risks for the specimen, which has to be manipulated only once, without requirement of a precise orientation for the scanning (the precise orientation being done later during the 3D processing). In the case of scanning at the European Synchrotron Radiation Facility (ESRF, Grenoble, France), the fossil teeth are just placed in a glass cone fixed only by gravity. There is no need for glue, or the use of any other mounting material potentially risky for such a kind of specimen. After the scan, the fossil can be just safely housed, since all the work on morphology can be done using 3D rendering. Microtomographic data can also be easily shared or even deposited on public databases to become accessible to the whole community. The ESRF is developing such a kind of public database to host published specimens data (http://paleo.esrf.eu). Making these data available for important specimens render manipulations and new observations of the original sample not necessary in many cases, reducing significantly the risks of damage or of loss of the sample (quite common in very small fossils).

As for all projection-based tomographic techniques, there is no problem of depth of focus: the image is sharp in the whole outline of the most complex tooth (Fig. 1C). The quality of the rendering is directly linked to system resolution (between 0.4 and 3 µm for microremains imaging at the ESRF), to the overall data signal to noise ratio (generally very high with a synchrotron), and to the use of propagation phase contrast, or even of phase retrieval approach (Pradel et al., 2009).

Last, but not least, when using full 3D data, it becomes possible to create enlarged 3D printings of the samples (Fig. 1D) using different kinds of rapid prototyping technologies (using plastic, plaster, paper or resin). Such kind of materialisation of small samples allows direct manipulation ("seeing with the fingers") and is very useful to replicate at large scale very precious and important tiny fossils for collections, students or exhibitions.

The main limitation for these synchrotron approaches is the accessibility of this technique. Indeed, despite the fact that conventional machines cannot reach such a high level of quality, especially with short acquisition time, it is often quite difficult to obtain significant amount of beamtime on synchrotrons to image small fossils. The use of synchrotrons has then to be kept for important and rare specimens only, or when access to the internal structures with high quality is needed. Moreover, the acquisition of microtomographic data requires a heavy postprocessing after scanning, requiring powerful computing resources, regarding the amount of produced data. This situation is nevertheless evolving rapidly thanks to the constant increase in acquisition speed and data quality, automation systems, computer capabilities, and to the rising of new low energy third generation synchrotron sources that already have, or will have in the near future, the capabilities of such kind of studies. PPC-SRµCT is clearly one of the best imaging techniques for vertebrate microremains research and has many advantages when compared with other classical techniques. The increasing availability of synchrotron

microtomographic beamlines all around the world, as well as the constant increase in quality, speed and imaging possibilities of conventional microtomographs will probably lead to major evolution of the study of microvertebrates remains, especially for the rarest ones such as Mesozoic mammals.

## 4. Conclusion

The stereomicroscope and SEM are still the natural routine equipments for imaging of small fossils. The first is mainly dedicated to observation and it is used as a working instrument. The second is a widely used technique for acquiring good images that illustrate specific specimens. However, access to 3D is improving microremains treatment: it is simplifying observation and description, facilitating manipulation and providing access to a rendering quality unmatched by other equipments. It also has the double advantage of allowing access to both external morphology and internal structures of the object. However, the synchrotron imaging is presently not intended for imaging all vertebrate microremains. Indeed, it cannot be used as routine methodology since being too complicated to implement and difficult to access. But, according to the quality of the rendering and the new perspectives of studies, X-ray synchrotron imaging is becoming a powerful investigative and inescapable process for the study of scientifically important specimens.

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