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ORIGINAL PAPER

The first record of a soft-shelled turtle (Testudines: Pan-Trionychidae) from southern Balkans (Pliocene, Gefira, N. Greece) and new information from bone histology

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Abstract Soft-shelled turtles (Pan-Trionychidae) are not included in the present-day chelonian fauna of Greece and have been unknown in the Greek fossil record up to now. Here, we report the first fossil occurrence of a soft-shelled turtle from Greece, originating from the Pliocene Gefira Member (Angelochori Formation), in the lower Axios valley. The corresponding specimens were discovered with several mammalian remains, most of them attributable to the mastodon of Auvergne, *Anancus arvernensis*. The chelonian material includes five carapacial fragments that belong to the same individual and can be attributed to Pan-Trionychidae based on the typical sculpturing on the dorsal side of the carapace. Most of these bony plates were histologically sampled and thereby provide evidence for the "plywood" structure, another characteristic of pan-trionychids. They represent the first extended sampling of trionychid plates that belong to the same individual, allowing the documentation of the variation of the relevant trionychid morphologies in the carapace. These findings expand the paleobiogeographic range of this taxon to the southern Balkans and Greece and allow a better estimation of the chelonian paleo-fauna of the area. They are also important for the temporal distribution of this clade in the Paleoarctic, as they join specimens from Italy as being the last trionychids in Europe.

Keywords Pan-Trionychidae · Thessaloniki · Plywood structure · Histology

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Introduction

Soft-shelled turtles (Pan-Trionychidae Joyce et al. 2004) form a group of highly aquatic cryptodires, which first appeared in the Early Cretaceous of Asia, and spread to the other continents over the course of Late Cretaceous and Cenozoic (Danilov 2005; Scheyer et al. 2012; Danilov and Vitek 2013, and references therein). They possess a flat leathery shell, a long neck and only three clawed digits in each limb (Meylan 1987). The surface of the shell of pan-trionychids has a distinctive sculpturing, which makes it easy-to-diagnose shell fragments in the fossil record (Meylan 1987). Although pan-trionychid fossils are very common in Europe, they have been considered absent in the Greek fossil record to date (de Lapparent de Broin 2001; Danilov 2005). Our current knowledge of the morphology and distribution (geographical and temporal) of fossil turtles (i.e. Testudinata) in Greece is delimited by poor preservation and scarce published material.

Pan-trionychids have received special attention recently due to their distinctive bone histology. Based on the pioneering work of T. Scheyer (Scheyer 2007; Scheyer et al. 2007; 2012; Delfino et al. 2010; among others), it has become clear that soft-shelled turtles are characterized by the so-called plywood structure in the external cortex of the bone of their shell. This morphology, which can be observed in detail in thin sections, is considered to be an unambiguous synapomorphy of the Pan-Trionychidae (Scheyer et al. 2007). Scheyer (2007) and Scheyer et al. (2007) discussed possible biomechanical advantages for the presence of the "plywood" structure but noted that more sampling is needed to clarify the taxonomic value of this feature.

Here we report the first pan-trionychid fossils from Greece as it has been noted during the revision of the Greek fossil chelonians (Vlachos 2015), from the new locality of Gefira Axios (GAS). Gefira village is located in the lower Axios River valley near Thessaloniki (Central Macedonia, Greece) (Fig. 1a). The fossil pan-trionychid remains studied in this paper were discovered by M. Savvelis during work in a sand-pit in 2006 and were subsequently donated to the Aristotle University of Thessaloniki (AUTH). Several mammalian fossils were associated with the fossil pan-trionychids, most of which are attributed to the gomphothere *Anancus arvernensis*, including two semi-mandibles with the third lower molar, a vertebra (axis), a complete femur and a complete tibia. Among the remaining mammalian fossils (hipparions, cervids, large bovids), the most notable findings are two partial crania of a new Boselaphini with both horn-cores. In this paper, we describe the fossil pan-trionychids and present information on their bone histology from observations in thin sections.

Geological setting

The study area is located in the lower Axios valley, near Thessaloniki, northern Greece. In this area, a significant amount of Neogene–Quaternary deposits occur, with many important fossil mammal localities of Late Miocene age (Koufos 2006). Similar continental deposits have been found further to the south in the eastern part of the Thermaikos Gulf, the western Chalkidiki deposits (Syrides 1990). These two areas are the present-day remnants of a broader basin, the Axios–Thermaikos basin, which existed during Neogene.

According to Koufos and Pavlides (1988), the deposits between the villages Gefira and Vathylakkos (lower Axios valley) belong to the Gefira Member, which consists mainly of alternating gravels and sands. The Gefira Member overlies the Megalo Emvolon Member and both are included in the Angelochori Formation. Megalo Emvolon Member is exposed in the cape of Megalo Emvolon (also known as Karaburun or Karabouroun), near Thessaloniki in the western Chalkidiki peninsula, and consists of reddish marls and grey sandy marls alternated with gravels. Koufos and Pavlides (1988) mention that the Megalo Emvolon Member is of an Early Ruscinian age (MN 14), whereas the Gefira Member is younger and terminates probably in the Pliocene (MN 15–16). The Auvergne's mastodon is widespread in continental Greece and in the eastern Aegean islands during Pliocene/Early Villafranchian (see Tsoukala and Mol in press and references therein). Also, the association of *A. arvernensis* with hipparions, cervids and Boselaphini in the accompanying fauna of the pan-trionychid indicates a Late Pliocene age for the turtle. Additional material attributed to *Coelodonta antiquitatis praecursor* (MNQ 24), from sand deposits in the broader Gefira area, shows that parts of this formation could extend to the Middle Pleistocene (Tsoukala 1991).

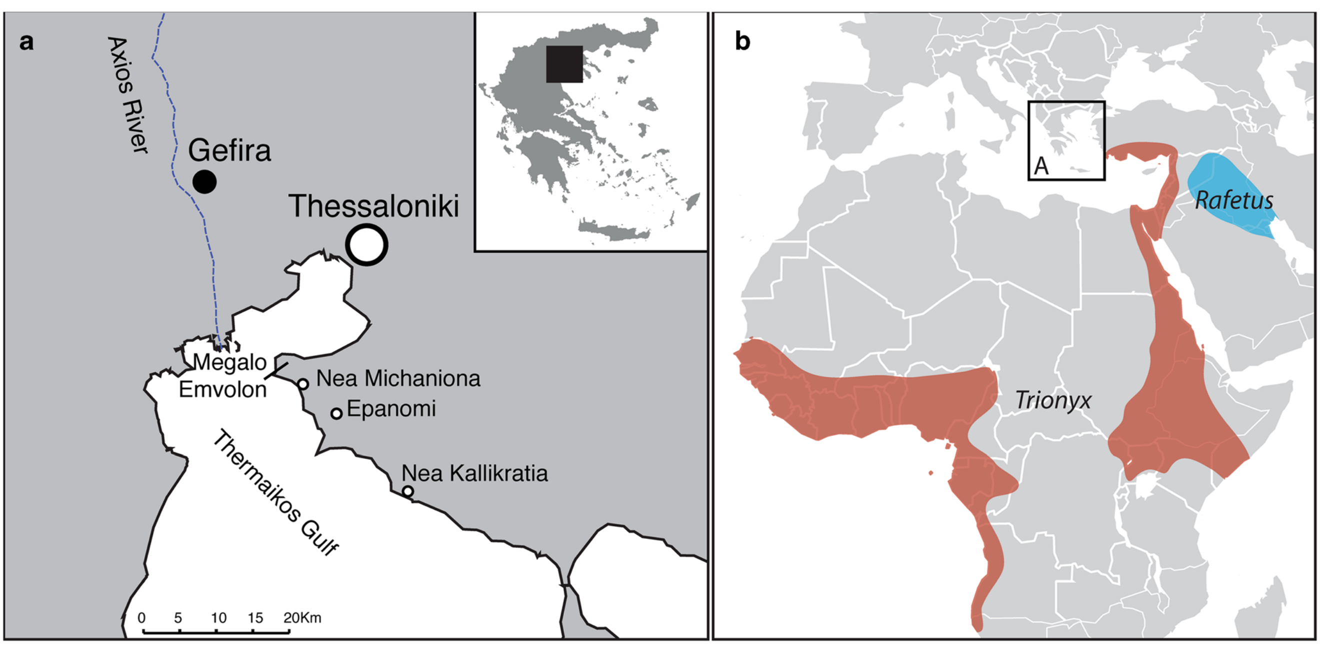

Fig. 1 **a** Map of Greece, showing the Gefira locality (GAS), in the lower Axios valley. **b** Map of Europe and Africa showing the ranges of the extant trionychids *Trionyx triunguis* (*red*) and *Rafetus euphraticus* (*blue*), following Iverson (1992)

Material and methods

The pan-trionychid material is deposited in the collections of the School of Geology, Aristotle University of Thessaloniki, Greece (LGPUT), under the inventory numbers LGPUT GAS 8a–e. For comparative material, we used extant and fossil pan-trionychid specimens from the collections of the Department of Paleontology, University of Vienna (IPUW) and Naturhistorisches Museum, Vienna, Austria (NHMW). Measurements were taken using a BMI calliper (0.01 mm precision). Length and thickness were measured at the medial margin of bones, width at the maximum preserved. For the description of the various features of the carapace, we follow the work and terminology of Gardner and Russell (1994). Taxonomy follows Joyce et al. (2004).

The thin sections were prepared following the methodology of Scheyer (2007), in the Laboratory of Petrography of the School of Geology, AUTH. The costals were sampled in X (transversely, perpendicular to the anteroposterior axis) and L (parallel to the anteroposterior axis) sections, following standard petrographic procedure. All samples were processed into standard petrographic thin sections. The study of the sections was realized using two LEICA DMLP® compound polarizing microscope (magnifications ×40, ×100, ×400; normal transmitted and polarized light) at LGPUT. Additional study and photographic documentation were made using a Nikon E400® compound polarizing microscope (magnifications ×20, ×40, ×100, ×400; normal transmitted and polarized light) at Museo Paleontológico Egidio Feruglio, Trelew, Chubut, Argentina (MEF). Some of the sections were analysed using a scanning electron microscope (by Ass. Prof. L. Papadopoulou, AUTH) and in X-ray diffraction (by MSc. I.M. Zougrou, AUTH), and these results will be presented elsewhere.

Systematic palaeontology

Testudines Batsch, 1788

Cryptodira Cope 1868

Pan-Trionychidae Joyce et al. 2004

(Figs. 2–3)

Locality: Gefira, lower Axios valley (Central Macedonia, Greece); GPS: 40° 44′ 58.5″ N, 022° 40′ 01.8″ E, Hellenic Grid, EGSA ‘87.

Material: LGPUT GAS 8a (Fig. 2(A)), right part of nuchal; LGPUT GAS 8b (Fig. 2(B)), right part of right first costal; LGPUT GAS 8c (Fig. 2(C)), left second costal; LGPUT GAS 8d (Fig. 3(A)), left part of left fourth costal; LGPUT GAS 8e (Fig 3(B)), right part of left fifth costal.

Age: Late Pliocene (MN 16).

Results

Description All plates have a size and morphology that is consistent as deriving from a single individual. They were found in the same locality, do not preserve overlapping parts of the carapace, and show similar state of fossilization and color. Moreover, some of the plates were recovered articulated (nuchal and first costal). These allow us to conclude that they belong to the same individual that probably exceeded 400 mm in carapacial disk length (Table 1 for measurements). All specimens are heavy mineralized and preserved in relatively good condition. From the nuchal plate (LGPUT GAS 8a), the right part is preserved, showing that the anterior carapace margin is shallowly concave, while the width is at least three times greater than the length of the plate (Fig. 2(A)). The remaining preserved elements are from the costals. The right part of the right first costal (LGPUT GAS 8b; Fig. 2(B)) has a well-developed sutural surface for contact with the nuchal. The sculpturing on the dorsal surface is not visible in some parts probably as a result of erosion. The left second costal (LGPUT GAS 8c) is almost complete (Fig. 2(C)) and only lacks a small part of the anterior medial margin. The dorsal surface exhibits distinctive sculpturing consisting of small pits that are either isolated or connected into larger associations (Fig. 2(C1)). This pattern is quite similar to that observed in most other Pan-Trionychidae, both fossil and extant. This costal plate is anteroposteriorly longer laterally than medially. The anterior border is shallowly concave, whereas the posterior border for articulation with the third costal is almost straight. Based on this element, the transverse profile of the carapace is broadly convex (Fig. 2(C3)). The thickness of this costal varies from 9.1 mm medially to 9.4 mm laterally (Table 1). From the left fourth costal (LGPUT GAS 8d), the left part is preserved (Fig. 3(A)). Both the anterior and posterior borders are straight, whereas the lateral edge is medially concave in transverse view (Fig. 3(A3)). The dorsal surface is eroded, but some sculpturing still remains. Of the left fifth costal (LGPUT GAS 8e), the right part is preserved (Fig. 3(B)). The anterior border is straight, whereas the posterior margin is anteriorly convex. Again, the sculpturing on the dorsal surface is partially eroded. The medial margin is preserved, showing the former presence of a long fifth neural with straight lateral borders and a sixth with short anterior sides. Ventrally, the ends of the costal ribs can be observed. The ends of the costal rib are convex but probably they do not project laterally (Fig. 3(B2)). However, this morphology needs to be checked with more specimens and especially in a complete shell and not in disarticulated fragments. In the remaining costal plates, the medial margin is not preserved, disallowing the estimation of the shape of the corresponding neurals. The lateral margins of the costals are rounded and show no evidence of a sutural surface, suggesting the absence of peripherals. The dimensions of the preserved parts of this carapace (Fig. 3(C)) are given in Table 1.

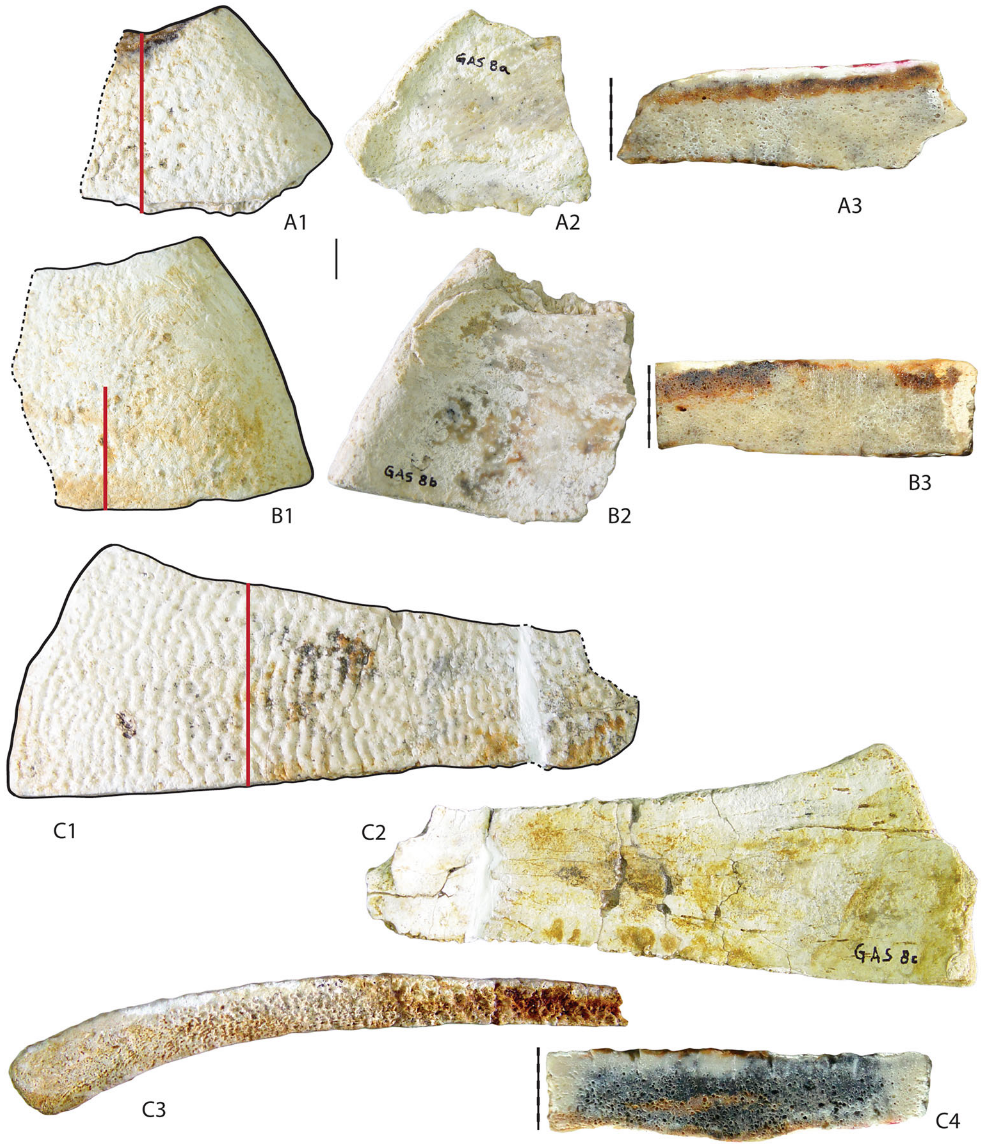

Fig. 2 Carapace plates of Pan-Trionychidae from Gefira locality, Late Pliocene, N. Greece. *A* LGPUT GAS 8a, nuchal in *A1* dorsal, *A2* visceral and *A3* section views. *B* LGPUT GAS 8b, 1st right costal plate in *B1* dorsal, *B2* visceral and *B3* section views. *C* LGPUT GAS 8c, 2nd left costal plate in *C1* dorsal, *C2* visceral, *C3* transversal and *C4* section views. *Scale bar*=10 mm

Bone histology All plates exhibit a diploe structure, in which external and internal cortex frame an internal core of cancellous bone. The external cortex (ECO) is composed of two distinct zones (outer and inner). The outer zone is composed of avascular lamellar bone tissue, in which each lamella follows the external surface outline (Fig. 4a). Bone cell lacunae are flattened or circular in section. Sharpey's fibres are present throughout the outer zone (Fig. 4b). These extrinsic fibres are long and they insert perpendicularly to the outer surface. In LGPUT GAS 8a, long Sharpey's fibres are also present in the (anterior) margin. Primary vascular spaces are absent. Few secondary osteons are scattered in some regions of the outer cortex. The inner zone is composed of inter-crossed structural fibre bundles (structural fibres sensu Scheyer and Sander 2004). While in some areas (e.g. in the boundary with the outer zone) these fibres exhibit a disorganized arrangement, in others they show the typical plywood organization described previously for pan-trionychid taxa (Scheyer 2007; Scheyer et al. 2007, 2012; Delfino et al. 2010). This plywood pattern consists of three orthogonal systems of fibre bundles, one vertical and two horizontal (Fig. 4c–f). Horizontal systems are organized in successive layers. In each layer, fibre bundles are parallel to one another, but the direction changes from one layer to the next, forming angles of 90°. These layers are crossed perpendicularly by a vertical system of fibre bundles. Transversally, sectioned horizontal systems possess a quadrangular shape. Bone cell lacunae show a prolate ellipsoid shape, and they are oriented following the arrangement of the fibres in which they are imbedded. The inner zone of the outer cortex is vascularized by simple vascular canals and primary and secondary osteons. The transition between the external cortex and cancellous bone is not clearly defined because of the amount of scattered secondary osteons and erosion cavities.

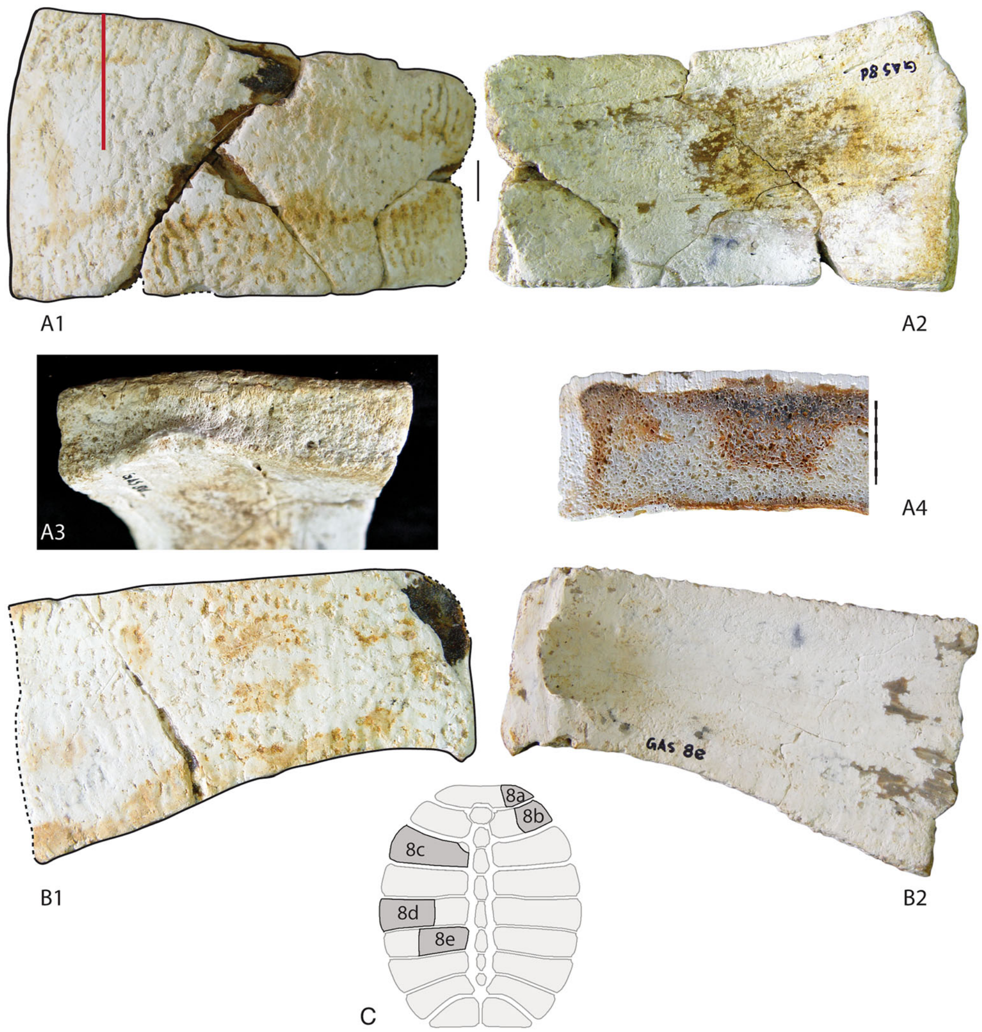

Fig. 3 Carapace plates of Pan-Trionychidae from Gefira locality, Late Pliocene, N. Greece. *A* LGPUT GAS 8d, left 4th costal plate in *A1* dorsal, *A2* visceral, *A3* lateral and *A4* section views. *B* LGPUT GAS 8e, 5th left costal plate in *B1* dorsal and *B2* visceral views. *C* drawing, showing the position of the studied specimens on a generalized trionychid carapace. *Scale bar*=10 mm

The cancellous bone consists of short and thick trabeculae and relatively small inter-trabecular spaces (Fig. 5a). Some of these small spaces coalesce to form larger spaces of irregular shape. In the LGPUT GAS 8b, a large sub-circular space is present within the cancellous tissue. Some variation with regard to the degree of development of the cancellous bone is observed. In LGPUT GAS 8c, the internal spaces of the "cancellous" bone are relatively smaller and less abundant compared with the other bones and the entire bone exhibits a rather compact structure. Bony trabeculae are exclusively composed of secondary lamellar bone tissue formed during different generation of continuous resorption and deposition (Fig. 5b).

The internal cortex (ICO) is relatively thin and consists of avascular parallel fibred bone (Fig. 5c). The ICO contains at least ten lines of arrested growth (LAGs), which are more clearly observed using a high contrast in the normal light incidence (Fig. 5d, arrowheads).

The histological data obtained from the Gefira trionychid are not only valuable for the systematic assignment of the material but also allow the discussion on other issues,

Table 1 Measurements of the preserved parts of the Gefira pan-trionychid carapace

	LGPUT				
	GAS 8a Nuchal	GAS 8b 1st right costal	GAS 8c 2nd left costal	GAS 8d 4th left costal	GAS 8e 5th left costal
Preserved length (mm)	42.9	61	33.7	55.3	48.1
Preserved width (mm)	63.9	66.7	149.9	121.4	114.4
Thickness (mm)	10.6–13.2	10.4–12.1	9.1–9.4	7.1–11	6.8–7.3

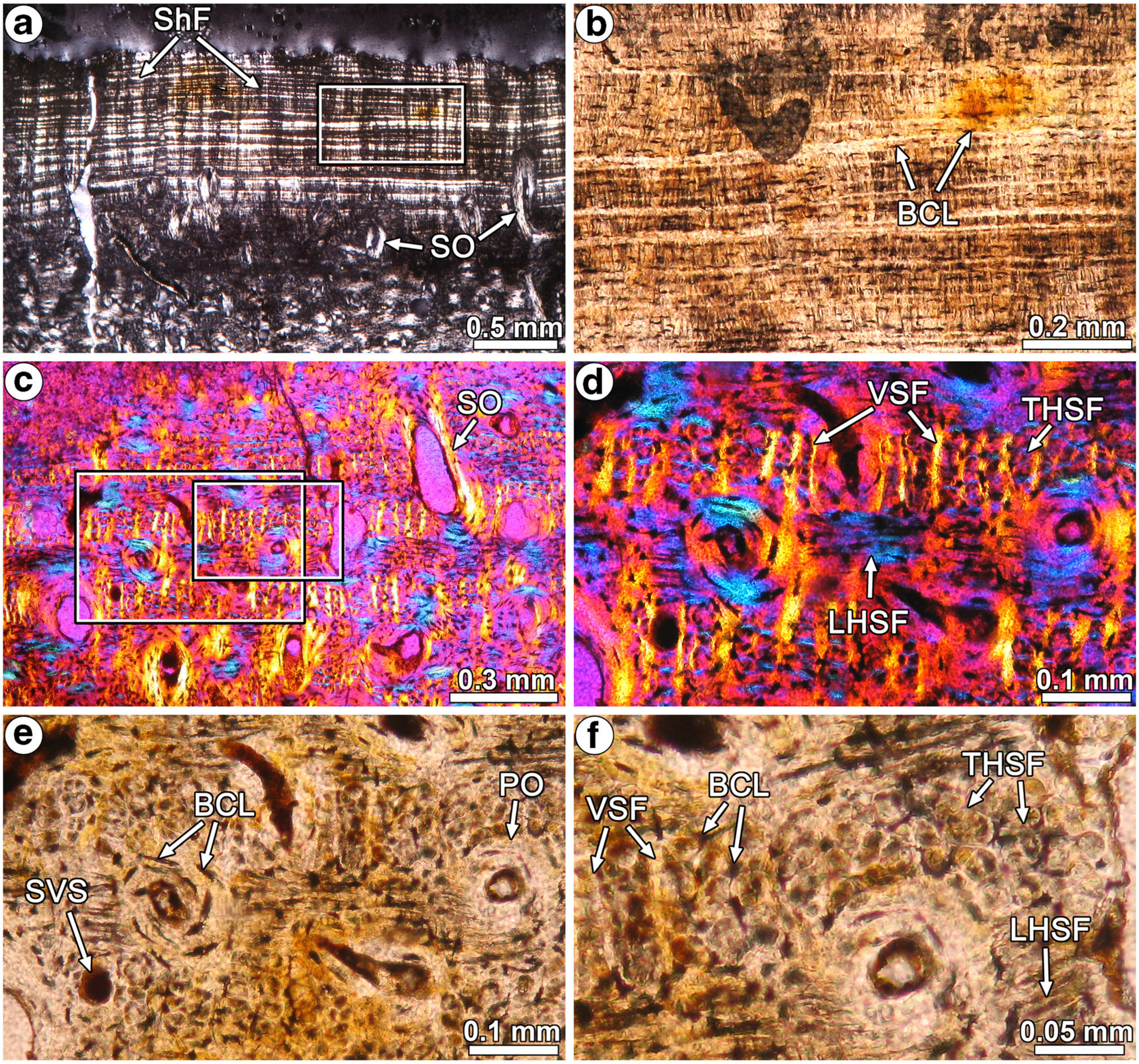

Fig. 4 Bone shell histology of Pan-Trionychidae from Gefira locality, Late Pliocene, N. Greece. **a** Outer region of the external cortex composed of lamellar bone tissue in LGPUT GAS 8c. Note the abundance of Sharpey's fibres oriented perpendicularly to the surface. Cross-polarized light. **b** Detailed view of the same specimen (*box inset* in (**a**)). Normal transmitted light. **c** General view of the inner portion of the external cortex LGPUT GAS 8b. Structural fibres are organized forming a plywood arrangement. Cross-polarized light with lambda compensator. **d**, **e** detailed view of the same specimen (*large box inset* in (**c**)) viewed under cross-polarized light with lambda compensator (**d**) and normal transmitted light (**e**). **f** Enlarged view of the structural fibres (*small box inset* in (**c**)). Normal light. *BCL* bone cell lacunae, *LHSF* horizontally oriented structural fibres longitudinally sectioned, *PO* primary osteons, *ShF* Sharpey's fibres, *SO* secondary osteons, *SVS* simple vascular space, *THSF* horizontally oriented structural fibres transversally sectioned, *VSF* vertically oriented structural fibres

including inter-elemental and inter-specific variation of the shell histology. Regarding the degree of histological inter-elemental variation within the shell, previous studies (Scheyer et al. 2007; Delfino et al. 2010) showed that, with exception of the prenuchal bones of *Cyclanorbis senegalensis*, which possess SFB in both external and internal cortices, the main histological features in the plates are invariable. The most distinctive histological variation in our sample, besides the variation described above, was recorded in the anterior margin of the nuchal plate (LGPUT GAS 8a), which possesses abundant and long Sharpey's fibres. These extrinsic fibres are continuous with those observed in the outer portion, and they are most likely related to the anchoring between the shell and the overlying integument.

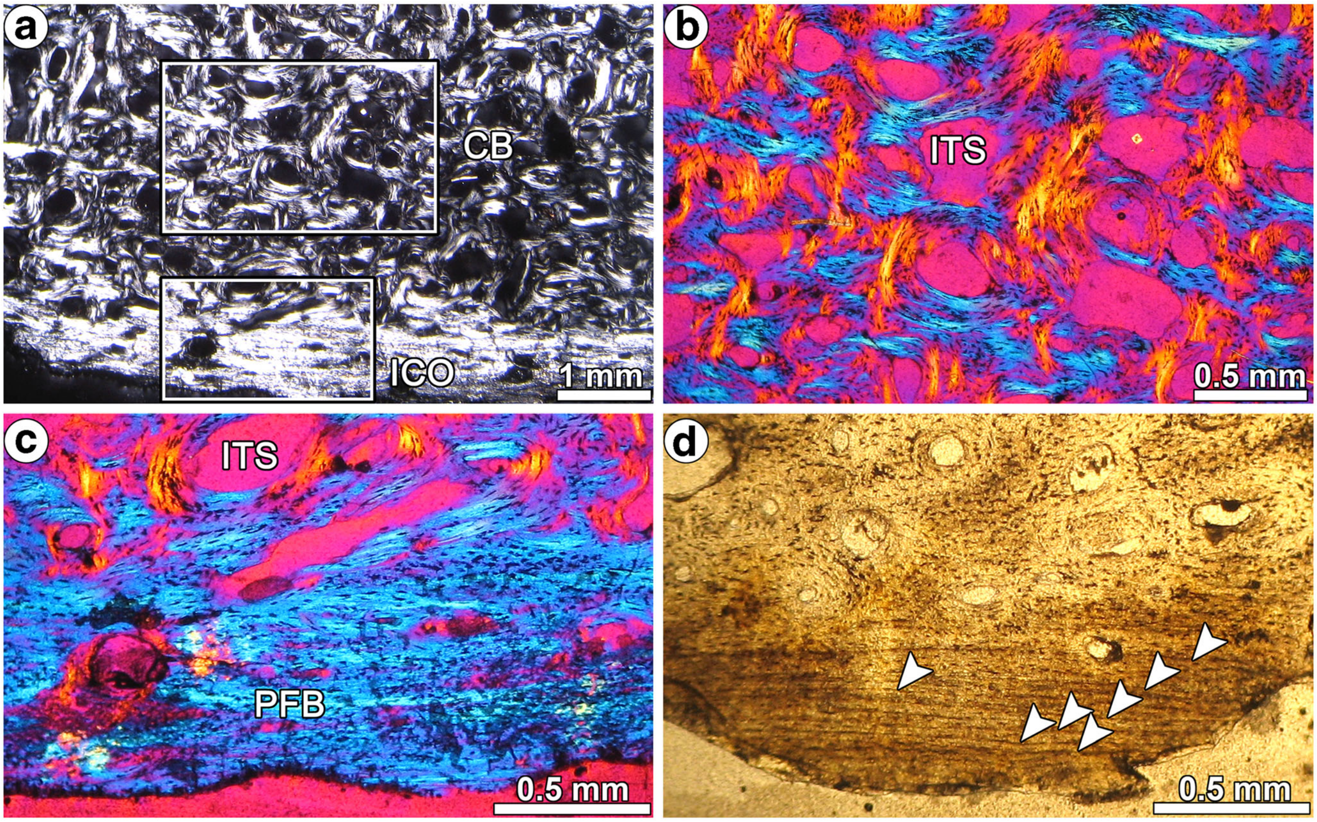

Fig. 5 Bone shell histology of Pan-Trionychidae from Gefira locality, Late Pliocene, N. Greece. **a** General view of the transition between the cancellous bone and the internal cortex in LGPUT GAS 8b. Cross-polarized light. **b** Detailed view of the cancellous bone (*large box inset* in (**a**)). Cross-polarized light with lambda compensator. **c** Detailed view of the internal cortex (*small box inset* in (**a**)), which is entirely composed of parallel fibred bone. Cross-polarized light with lambda compensator. **d** lines of arrested growth in the internal cortex (*arrowheads*). Normal light. *CB* cancellous bone, *ICO* internal cortex, *ITS* inter-trabecular spaces, *PFB* parallel fibred bone

Discussion

Taxonomic identification of the Gefira trionychid The morphology of the preserved carapace fragments of the Gefira individual allows attribution to a single individual of Pan-Trionychidae, based on the absence of scute sulci, absence of peripherals and the sculpturing on the outer shell surface. Given the scarcity of the material, further identification is not possible beyond the family level. Gardner and Russel (1994) showed that numerous carapacial features are variable and therefore cannot be of high diagnostic value. Especially, the standard sculpturing described above for the fragments from Gefira is only diagnostic up to the level of Pan-Trionychidae (see Vitek and Joyce (2015) for examples of taxa that can be diagnosed by shell sculpturing). The other line of evidence for the attribution of the Gefira individual to the Pan-Trionychidae comes from the bone shell histology. In this sense, the plywood arrangement of the structural fibres in the inner portion of the external cortex is considered an exclusive histological character of this group (Scheyer et al. 2007; Delfino et al. 2010).

Our current knowledge of the taxonomy of European pan-testudinids is not clear. Lapparent de Broin (de Lapparent de Broin 2001) reports that most fossil trionychids from the Cenozoic of Europe are too poorly preserved to be identified as members of the *Trionyx* s.l. group (meaning unclear, probably refers to extinct members of *Trionyx* that are different from *Trionyx triunguis*). According to same author, the presence of the Egyptian *Rafetus* cannot be excluded in the European material, whereas *T. triunguis* is restricted in Europe to the Pliocene of South France–Italy. In the classification of Meylan (1987), the clade *Trionychinae* is characterized (among other characters not preserved in the Gefira material) by a nuchal that is at least three times wider than long. Consequently, the Gefira trionychid could be a member of *Trionychinae* (sensu Meylan 1987), based on the dimensions of the preserved nuchal, but more specimens are needed to allow a more detailed identification.

Histological descriptions of trionychid shell bone elements have been provided by previous studies (Scheyer et al. 2007; Delfino et al. 2010; Hirasawa et al. 2013; Lyson et al. 2013), which allow a comparison with the Gefira sample. Since no histological descriptions of trionychid nuchal plates exist to our knowledge, we restrict the comparison only to the costal bones. The main histological variations appear to be related with the relative thickness of the internal cortex and the plywood layer of structural fibres. Whereas in the Gefira bones the internal cortex occupies around the 5 % of the thickness of the plates, this value is notably increased (around 25 %) in other taxa in other taxa (e.g. cf. *Aspideretoides* sp.; Late Cretaceous Judith River Group, Kennedy Coulee, North of Goldstone, MT, USA in Scheyer 2007 and Scheyer et al. 2007). Regarding the relative proportion of the plywood layer, this is comparatively lower (10 %) in the Gefira trionychid compared with other taxa (25 % in cf. *Aspideretoides* sp.; same as above). These variations could be related to different, non-mutually exclusive causes, including biomechanical and ontogenetic factors.

Implications for trionychid distribution in the southern Balkans Up to the present, fossil pan-trionychids were unknown from Greece and this was highlighted as one of the main differences between Greek and Turkish fossil assemblages (de Lapparent de Broin 2002). This clade is also absent from the genuine extant chelonian fauna of Greece, with the only reported members found in the waters near Kos (Taskavak et al. 1999) and other Aegean Islands such as Rhodes, Kalymnos, Leros and Kos (Dimaki 2002), originating most probably from nearby Turkish populations. These individuals are usually accidentally caught in fishing nets by local fishers. In Turkey, extant trionychids are represented by two species, *T. triunguis* (Forsskål 1775) and *Rafetus euphraticus* Dauden 1802 (from Taskavak and Akcinar 2009; Fig. 1b).

These mainly carnivorous turtles are known for their ability to cover large distances. Floods and overflows can sweep extant trionychids into the ocean, and by tolerating salty water they can disperse into another river system (Taskavak and Akcinar 2009 and references therein). Soft-shelled turtles usually prefer rivers and lakes with sandy bottoms, which are particularly important for their hatchlings (Plummer et al. 2008). Gefira deposits are characterized by large quantities of sands, while the presence of *A. arvernensis* reveals the presence of a forested environment with significant bodies of water and large quantities of plants during the Pliocene in the area. This paleoenvironment is consistent with the ecosystem to which trionychids are adapted. Moreover, the fact that these findings are reported from the mainland of Greece allows us to propose that the presence of a pan-trionychid in the Pliocene is not random find as in the present-day fauna, but an original member of the Greek fossil chelonian fauna. However, more specimens are needed to provide a confident assessment of such claim.

In the rest of Europe, relatively few fossil pan-trionychids are known from Pliocene localities. An extensive overview of the distribution of the soft-shelled turtles of Europe was given by Karl (1999). Regardless of the taxonomic attribution of these occurrences in Karl (1999), pan-trionychids are present in the Pliocene of Germany, Romania, South France (see also Broin F de 1977) and Italy (for detailed information see also Delfino 2002 and Chesi 2008). The available information on the fragmented record of European Neogene soft-shelled turtles needs a detailed revision. Nevertheless, it is evident that the occurrence of a pan-trionychid in the South Balkans expands the distribution of this clade during the Pliocene and is the southernmost finding in Europe towards the end of Neogene. During the Late Miocene, pan-trionychids are reported also as south as Murcia (Spain; Pérez García et al. 2011). This could be in accordance with the retraction of the range of *Trionyx* outside Europe, being found at present in Central Africa and all along the Nile River, Middle East and southern Turkey (Iverson 1992; Fig. 1b). Also, the Gefira findings are important for the temporal distribution of trionychids in the Paleoarctic, as it could be among the last trionychids in Europe, along with *Trionyx* (*Amyda*) cf. *pliopedemontanus* (Kotsakis 1980; *Trionyx* sp. in Chesi 2008) from the Late Pliocene–Late Pleistocene of Colombaiolo and Montecarlo, Tuscany (Italy).

Conclusions

In this paper, we present the first fossil record of a soft-shelled turtle from Greece, from the Late Pliocene sand deposits of the Gefira Member, in the lower Axios valley, northern Greece. These five fragments of a carapace are diagnosed as belonging to Pan-Trionychidae, based on the distinctive sculpturing, the absence of scute sulci on the dorsal surface of the shell, and the plywood arrangement of the structural fibres in the external cortex of the plates. Extensive sampling allows the documentation of variation in the histology within the carapace, especially between the nuchal and the costal plates. All fragments combined are interpreted as belonging to the same individual estimated to exceed 400 mm in carapacial disk length. This turtle probably lived in a forested environment as indicated by the associated fauna, with significant quantities of water resources. These findings expand the paleobiogeographic range of Pan-Trionychidae to the southern Balkans and increase the diversity of the fossil chelonian fauna of Greece. In contrast to the extant fauna where the presence of soft-shelled turtles is accidental from Turkish populations, the Gefira findings show that trionychids were established in Greece in the past. According to the mammalian association, the sand deposits in Gefira are dated to the Late Pliocene. Therefore, the material presented here, along with coeval material from Italy, could represent one the last trionychids in Europe. These findings will hopefully stimulate researchers to discover more of these easily recognizable turtles, to investigate the reasons for the extinction of this clade in southern Balkans and to trace their past temporal distribution in Greece.

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