Fossil *Aphanius* (Teleostei, Cyprinodontiformes) from southwestern Anatolia (Turkey): a contribution to the evolutionary history of a hotspot of freshwater biodiversity

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ABSTRACT
Until recently, only a single fossil species of the cyprinodontiform genus *Aphanius* was known from Anatolia (Turkey), mainly based on fossil otoliths. As Anatolia is a diversity hotspot of this genus, it was of peculiar interest to investigate recently found fossil otoliths of ?upper Pliocene-lower Pleistocene age from the Yassigüme section located in the Burdur Basin in southwest Anatolia. We studied the morphological relations to otoliths of extant *Aphanius anatoliae sureyanus* inhabiting present-day Lake Burdur by conducting Fourier shape and statistical analyses (principal components analysis [PCA], canonical discriminant analysis [CDA]). For further comparisons, we included a population of *A. a. anatoliae* (at Lake Salda) nearby Lake Burdur and one population of *A. danfordii* from the Kızılırmak River in northern central Anatolia. The contour of the fossil otoliths closely resembles that seen in the otoliths produced by the extant subspecies *A. a. sureyanus* from Lake Burdur. On the other hand, the fossil otoliths are distinctly different from those of the extant species *A. danfordii*. Furthermore, the fossil otoliths reveal more proximity to *A. a. sureyanus* than to *A. a. anatoliae* of that region, and both extant subspecies show a certain distance to one another in the CDA; thus we suggest that this might be explained by a diversification of the subspecies that had begun before the investigated fossil populations existed. Based on the strong similarity of the fossil otoliths with *A. a. sureyanus*, we conclude that they do not represent a new fossil species, and thus are preliminary denominated as †*A.* cf. *anatoliae sureyanus*.

KEY WORDS
Teleostei,
Cyprinodontiformes,
otolith,
Neogene,
Lake Burdur,
Aphanius,
Anatolia,
Fourier analysis,
freshwater biodiversity.

RÉSUMÉ

Des Aphanius *fossiles (Teleostei, Cyprinodontiformes) du Bassin de Burdur au sud-ouest de l'Anatolie (Turquie). Une contribution à l'histoire évolutive d'un centre de diversification de biodiversité des faunes d'eau douce.*

Jusqu'à présent le genre *Aphanius* n'était connu en Anatolie (Turquie) que par une seule espèce fossile surtout représentée par quelques otolithes. L'Anatolie étant un important centre de diversification pour ce genre, il est intéressant d'étudier de nouveaux otolithes fossiles récemment récoltés dans le Pliocène (inférieur ou supérieur ?) à Yassigüme dans le bassin de Burdur (sud-ouest de l'Anatolie). Nous avons comparé les morphologies de ces otolithes fossiles avec celles des otolithes de l'espèce actuelle *Aphanius anatoliae sureyanus* qui vit dans le lac de Burdur. La morphométrie a été menée par analyse de Fourier des contours et les résultats ont été traités statistiquement par une analyse en composante principale (ACP) complétée par une analyse canonique discriminante (ACD). Pour enrichir le champ des comparaisons, nous avons également pris en compte une population d'*A. a. anatoliae* provenant du Lac Salda proche du lac de Burdur et une population d'*A. danfordii* provenant du fleuve Kızılırmak situé dans la partie septentrionale de l'Anatolie centrale. Les contours extérieurs des otolithes fossiles sont morphologiquement très proches de ceux des *A. a. sureyanus* qui vivent actuellement dans le lac de Burdur. Par contre les otolithes fossiles sont clairement distincts de ceux de l'espèce actuelle *A. danfordii*. En outre, la similitude morphologique est plus forte avec *A. a. sureyanus* qu'avec *A. a. anatoliae*, deux sous-espèces actuelles qui vivent à peu de distance l'une de l'autre mais qui, comme le montre l'ACD, n'occupent pas exactement la même partie de l'espace morphologique. Ces résultats nous conduisent à supposer que la différenciation sous-spécifique était déjà bien avancée lors de l'épisode de fossilisation du Pliocène. La forte similitude morphologique qui existe entre les otolithes fossiles et *A. a. sureyanus* ne permet pas de considérer les formes fossiles comme une nouvelle espèce. Nous préférons donc, au moins provisoirement, les désigner comme †*A.* cf. *anatoliae sureyanus.*

MOTS CLÉS
Teleostei,
Cyprinodontiformes,
otolithe,
Néogène,
Lac Burdur,
Aphanius,
Anatolie,
analyse de Fourier,
biodiversité des faunes
d'eau douce.

INTRODUCTION

Otoliths are composed primarily of aragonite and of some organic material. They lie in membranous sacs of the inner ear of teleostean fishes where they are involved in the senses of balance and hearing (cf. Popper *et al.* 2005). Three different otolith types on each side of the head can be distinguished: the utricular, the saccular, and the lagenar otolith (cf. Nolf 1985), which are named after the three types of otolithic endorgans (utriculus, sacculus, and lagena) in which they are located. Based on the symmetry of the inner ear in the head, a left and a right member of each otolith type can be identified. In most Teleostei, the saccular otolith is the largest or most robust otolith, and thus usually has the potential of becoming fossilized. As the saccular otolith has a species-specific morphology, studies on its contour and characteristics have contributed considerably to our knowledge and understanding of fossil and extant teleost diversity (e.g., Koken 1884; Nolf 1995; Reichenbacher *et al.* 2007). In the following, the saccular otolith is referred to as "otolith".

ZOOGEOGRAPHY OF THE ANATOLIAN *APHANIUS* TAXA

The Anatolian part of Turkey is one of the diversity hotspots of the cyprinodontiform genus *Aphanius* Nardo, 1827, which occurs in this area with four endemic species, i.e. *Aphanius anatoliae*

(Leidenfrost, 1912), *A. danfordii* (Boulenger, 1890), *A. villwocki* Hrbek & Wildekamp, 2003, and *A. asquamatus* (Sözer, 1942) (Wildekamp *et al.* 1999; Hrbek & Wildekamp 2003). *Aphanius anatoliae* is subdivided into four different subspecies: *A. anatoliae anatoliae* (Leidenfrost, 1912), whose populations live in the southwest and the western central part of Anatolia, *A. a. splendens* (Kosswig & Sözer, 1945), which is now restricted to Lake Salda (Fig. 1A), and *A. a. sureyanus* (Neu, 1937) and *A. a. transgrediens* (Ermin, 1946), which are endemic to Lake Burdur (Fig. 1A) and to Lake Acı, respectively (Wildekamp 1993; Wildekamp *et al.* 1999). Analysis of mitochondrial DNA indicates that *Aphanius a. sureyanus*, *A. a. splendens*, *A. a. transgrediens*, and several populations of *A. a. anatoliae* from the Lakes District (southwestern Anatolia) belong to a single clade (Hrbek *et al.* 2002; Hrbek & Meyer 2003). Based on crossbreeding experiments, Villwock (1964, 1982) had already demonstrated that three different population groups of *A. anatoliae* exist (Wildekamp *et al.* 1999), i.e. a southwestern group, which includes the first three of the above listed subspecies and several populations of *A. a. anatoliae* from the Lakes District, a second group that is composed of the western central populations of *A. a. anatoliae*, and a third group that links the first and second groups with one another.

Hrbek *et al.* (2002) and Hrbek & Meyer (2003) hypothesized that the zoogeographic distribution of *Aphanius* is a result of the complex geological history of Turkey. Adding support to this hypothesis is the fact that the zoogeographic distribution pattern of the cyprinid genus *Pseudophoxinus* from Turkey is very similar to that observed for *Aphanius* (Hrbek *et al.* 2004). Based primarily on studies of *A. anatoliae*, however, Villwock (1964, 2004) postulated that the separation events occurred relatively recently, i.e. during the Plio-Pleistocene (about 1.8 Ma ago), or are of postglacial age, and resulted from climatic changes that led to a considerable reduction in size of the lakes. Conversely, Hrbek & Meyer (2003) suggested that the separation events took place at least several million years ago; molecular clock estimates indicate that the diversification of *A. anatoliae* occurred some 11.79 ± 0.52 Ma ago, and the diversification of the *A. anatoliae* populations in the Lakes District 7.48 ± 0.49 Ma ago.

The fossil record of *Aphanius* in Turkey

Fossil remains of *Aphanius* from Anatolia can assist in answering the above raised uncertainties concerning the separation events. However, until recently only a single fossil species of this genus was known from Turkey, i.e. *Aphanius kayai* Reichenbacher & Rückert, 2002 from the upper Miocene-lower Pliocene of Manisa near Izmir (Rückert *et al.* 2002), which is known almost exclusively from otoliths. As a result, no suitable fossil record of the genus *Aphanius* existed from the Lakes District in southwest Anatolia. In addition, only a few studies have focused on *Aphanius* otoliths, e.g., Malz (1978), Reichenbacher & Sienknecht (2001), and Schulz-Mirbach *et al.* (2006). Therefore, it was of special interest to study fossil *Aphanius* otoliths from the ?upper Pliocene-lower Pleistocene of the Burdur Basin in the Lakes District.

This study addresses the following questions: a) Do distinct differences exist in otolith contour between the fossil specimens and the extant *A. anatoliae* from the Lakes District and *A. danfordii*, respectively? b) Which parts of the otolith contour are most significant for distinguishing the *Aphanius* groups? and c) How similar or dissimilar are the otoliths from the two fossil samples and extant *A. anatoliae* subspecies to one another and what does this possibly imply with regard to the diversification models of the *A. anatoliae* populations in the Lakes District?

MATERIAL AND METHODS

Sampling and preparation

Extant otoliths

Otoliths from each of the populations of *A. anatoliae anatoliae*, *A. a. sureyanus* and *A. danfordii* were obtained from wild catches (bycatches) of the Süleyman Demirel University (Isparta, Turkey) and the University of Hamburg (Germany) (Fig. 1A; Table 1). Skulls of fishes were opened ventrally and left and right otoliths were removed. Otoliths were cleaned from organic residues by soaking in a 1% potassium hydroxide solution for four hours and subsequent rinsing with distilled water for 12 hours.

TABLE 1. — Overview of species (subspecies), sample number, number of otoliths, length range of otoliths (L_{ot}) used in Fourier analysis (FA), locality, and age. The first numeral in brackets represents the number of left otoliths; the second numeral specifies the number of right otoliths and the third numeral after the semicolon the number of intact whole otoliths. Bold numerals in bold brackets represent the specimens used in Fourier analysis. Abbreviations: ***A.***, *Aphanius*.

Taxon	Sample no. (Fig. 2)	No. of otoliths	L_{ot} [µm] for FA	Locality (Fig. 1)	Age
A. anatoliae anatoliae (Leidenfrost, 1912)	–	(29/29) **(11/0)**	623-888	Nearby Lake Salda	extant
A. anatoliae sureyanus (Neu, 1937)	–	(45/45) **(15/0)**	600-838	Lake Burdur	extant
A. danfordii (Boulenger, 1890)	–	(36/36) **(34/0)**	751-970	Karpuzatan	extant
†*A.* cf. *anatoliae sureyanus*	T01 259	(20/12;23) –	–	Burdur Basin	early Pleistocene
†*A.* cf. *anatoliae sureyanus*	T01 258	(1/3;4) –	–	Burdur Basin	early Pleistocene
†*A.* cf. *anatoliae sureyanus*	T01 257	(2/3;5) –	–	Burdur Basin	early Pleistocene
†*A.* cf. *anatoliae sureyanus*	T01 256	(15/13;25) **(7/11)**	636-879	Burdur Basin	?Plio-Pleistocene
†*A.* cf. *anatoliae sureyanus*	T97 242	(15/14;22) **(9/7)**	545-798	Burdur Basin	?Plio-Pleistocene
†*A.* cf. *anatoliae sureyanus*	T01 255	(6/5;9) –	–	Burdur Basin	?Plio-Pleistocene

Fossil otoliths

Otoliths come from sediments that crop out near Yassigüme (Fig. 1B), to the south of the present-day Lake Burdur (cf. Sen *et al.* pers. com.). The samples (Fig. 2) were screen-washed with peroxide, sieved, and microfossils were sorted under a stereo-microscope. A total of 108 otoliths of fossil *Aphanius* were obtained. Thirty-four otoliths from samples T97 242 and T01 256 were selected for Fourier shape and the statistical analyses (cf. Table 1). The otoliths from sample T01 259 were not included in the quantitative analysis because they mainly represent juvenile or sub-adult/adolescent specimens. The fossil otoliths did not show any deformation and exclusively intact otoliths were included in the analysis.

Preparation

Fossil and extant otoliths were stored dry in small plastic boxes (FEMA-cells). Otoliths are deposited in the Bavarian State Collection for Palaeontology and Geology in Munich (BSPG-2003 IV 167-260), Germany.

SEM IMAGES

For qualitative description of the otolith contours, SEM images were taken with a LEO 1430 VP at the Zoological State Collection in Munich (ZSM). The nomenclature of otolith features follows Chaine & Duvergier (1934) and Nolf (1985) (see Figure 3).

DIGITISING OF CONTOURS

Left otoliths of the extant taxa, and left and right otoliths of fossil *Aphanius* were positioned (with their outer face down) on plasticine, and digital images were taken with a magnification of 152×. Images were imported and measured with a Leica Image Software (IMAGIC 1000) via a CCD camera connected to a PC. Contours were processed in Adobe Photoshop CS2 with a final contrast of 100% (white object on black background). Digitising of the contours was applied in tpsDig vers. 2.0 (Rohlf 2004) with the tip of the rostrum used as the starting point and saving raw x-y values. Prior to the digitalization of the contours, images of right otoliths were mirrored. With the fossil specimens, it was not possible to use only left

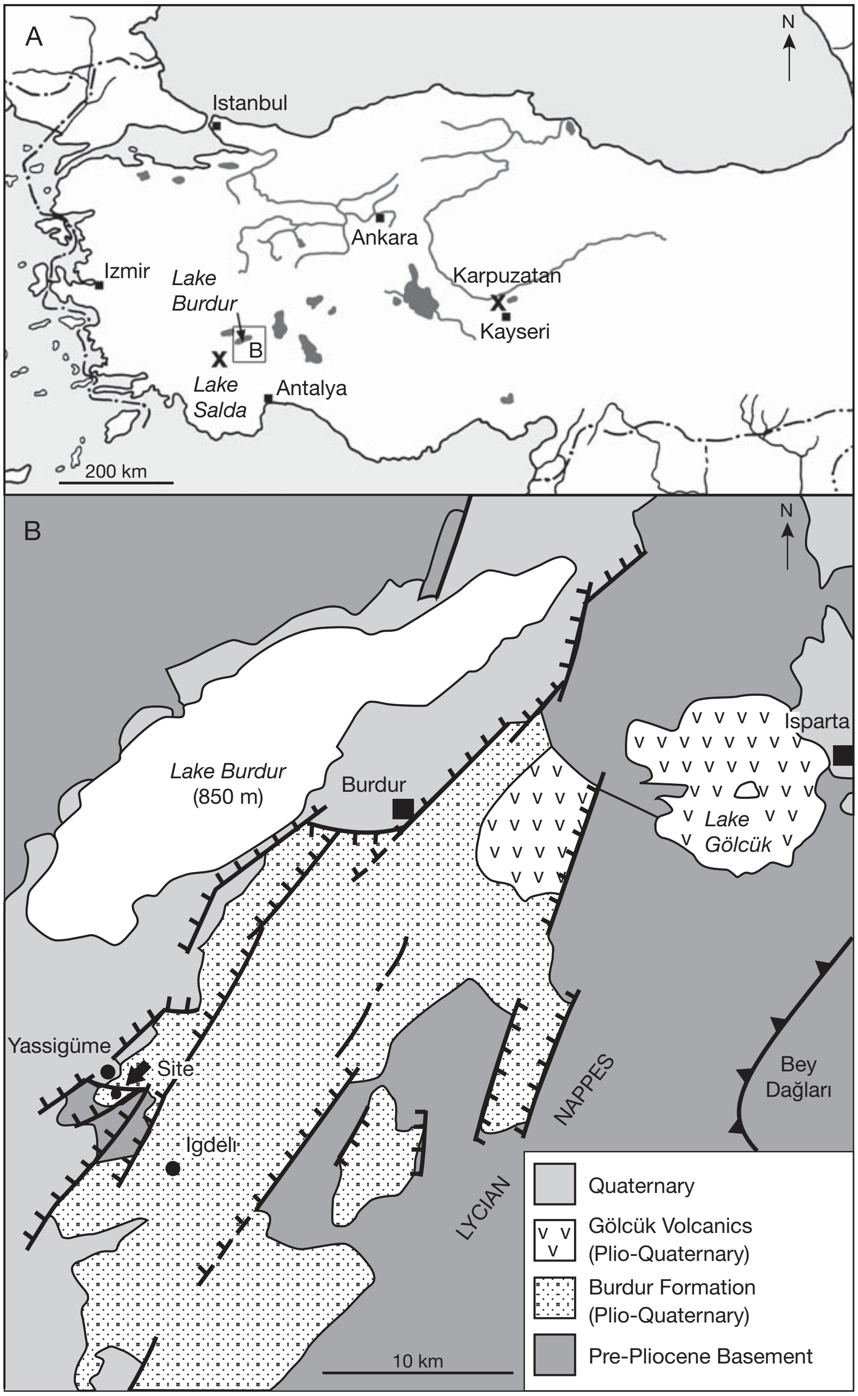

FIG. 1. — **A**, Map of Turkey showing the sample sites of the extant species; **B**, geological map of the northern part of the Burdur Basin with the sampling site at Yassigüme.

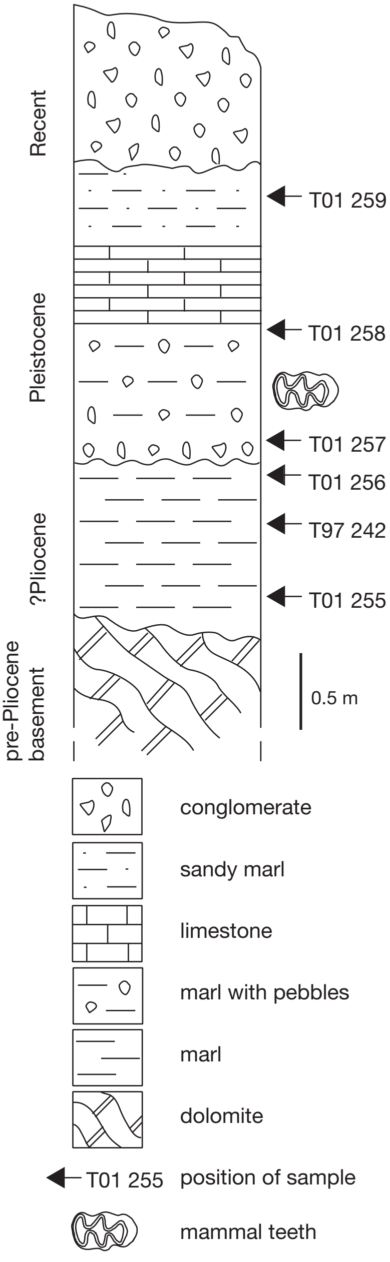

FIG. 2. — Schematic overview of stratigraphy, lithology, and position of the samples from the cross section at Yassigüme near Lake Burdur.

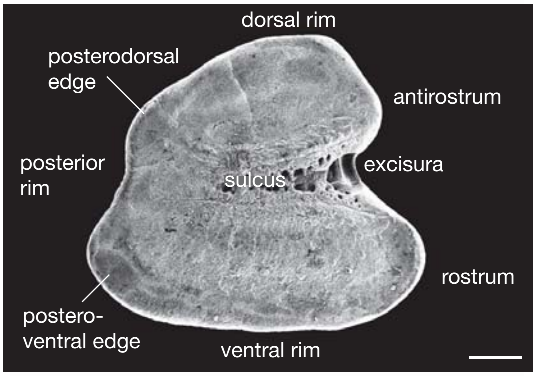

FIG. 3. — Left saccular otolith of *Aphanius anatoliae sureyanus* (Neu, 1937) (BSPG-2003 IV 167), showing the most important morphological features of the contour and inner face. Scale bar: 100 µm.

otoliths because otherwise the sample sizes would not have been sufficiently large enough for statistical analyses (< 10 specimens). Otoliths with a length of approximately 600 to 900 µm (*A. anatoliae* and fossil *Aphanius*) and 700 to 1000 µm (*A. danfordii*) were selected for further analyses to minimize size-dependent effects (see also Table 1). These otolith length ranges correspond to total lengths of adult fish of the extant species between 27 and 50 mm. Juvenile or sub-adult specimens (TL < 27 mm) were not included in the shape analysis to avoid ontogenetic effects and otoliths of old and large individuals (TL > 50 mm) were omitted as well. Old and large specimens possess otoliths that tend to display (strongly) crenulated otolith rims whereas rims are smooth in otoliths of fish with TL values between 27 and 50 mm.

FOURIER ANALYSIS

Images were processed in the Hshape Software of Crampton & Haines (1996) based on a Fast Fourier Transform (FFT) algorithm. This software consists of the three programs HANGLE, HMATCH, and HCURVE. In HANGLE, the Fourier functions are fitted to a function of the tangent angle dependent of the arc length that is connected to the x-y values. It results in two computationally independent Fourier descriptors per harmonic (Haines & Crampton 2000). Moreover, Fourier descriptors of higher number harmonics are not downweighted as in elliptic Fourier analysis. Therefore, Fourier descriptors of high number harmonics considerably contribute to the overall contour (Haines & Crampton 2000) which is especially important with regard to the statistical analyses (see below).

Normalization of size was performed automatically in HANGLE (harmonics: 20; smoothing iterations: 13), and normalization of orientation was attained by HMATCH for the entire sample set because the sum of eigenvalues of variance-covariance based PCA was lowest for fitting all contours by HMATCH (see also Haines & Crampton 2000 for quantitative determination of best fitting method). A number of at least 20 harmonics necessary for the analysis was indicated by the amplitude vs harmonic number plot and by visual comparison of back-calculated contours (HCURVE) with 5 to 30 harmonics and the original contour.

Average contours: Fourier descriptors (FDs) of every group (*A. a. anatoliae*, *A. a. sureyanus*, *A. danfordii*, fossil samples T97 242 and T01 256) were averaged and 1024 x-y values were back-calculated in HCURVE.

STATISTICAL ANALYSES

A variance-covariance-based principal components analysis (PCA) was applied to the FDs in SPSS vers. 14.0 (SPSS Inc. 2005). In Hshape also FDs of higher number harmonics explain a partially important amount of the contour and therefore are of more or less "equal" scale. Hence, in this study, it did not seem to be reasonable to standardize original variables (FDs), i.e. to perform a correlation-based PCA (cf. Crampton 1995). The number of relevant PCs that explain more variability than would be explained by chance alone was determined based on the method outlined in Jackson (1993: 2207; Fig. 2). 95%-confidence ellipses for groups and for group means of the PC-plots were calculated based on the formulas provided in Sokal & Rohlf (1995).

For visualization of morphospace of PC-plots, Haines & Crampton (2000) proposed calculation of synthetic model shapes that may represent real contours as well as extreme artificial shapes. Model shapes for all plots were calculated from -2 to +2 S.D.

A canonical discriminant analysis (CDA) was conducted with the first three PCs for five groups (*A. a. anatoliae*, *A. a. sureyanus*, *A. danfordii*, and the fossil samples T97 242 and T01 256) and for three groups (the subspecies of *A. anatoliae* merged, *A. danfordii*, and both fossil samples merged). The first three principal components were used instead of the Fourier descriptors (number of FDs = 38) because the CDA requires considerably more specimens in the smallest groups than variables characterising these specimens (Ponton 2006). With the principal components as new variables this basic assumption was fulfilled: the number of individuals in the smallest group was 11 compared to three variables (PCs) used in the CDA. The assumption of homogeneity of the group covariance matrix was examined by the Box's M test.

ABBREVIATIONS

BSPG	Bayerische Staatssammlung für Paläontologie und Geologie (Bavarian State Collection for Palaeontology and Geology, Munich);
CDA	Canonical discriminant analysis;
FA	Fourier analysis;
FD	Fourier descriptor;
FFT	Fast Fourier Transform;
L_{ot}	Length range of otoliths;
PC	Principal component;
PCA	Principal components analysis;
S.D.	Standard deviation;
TL	Total length;
ZSM	Zoologische Staatssammlung München (Bavarian State Collection for Zoology, Munich).

RESULTS

PRINCIPAL COMPONENTS ANALYSIS (PCA)

The three first PCs explain more variability than would be explained by chance alone (Fig. 4). The three first PCs account for approximately 44.9% of the overall variance of the dataset with PC 1 covering 27%, PC 2 10.1% and PC 3 7.8%.

95%-confidence ellipses of the group means

The PC 1 vs PC 2 (Fig. 5A) and PC 1 vs PC 3 (Fig. 5C) plots show that the two extant species *A. danfordii* and *A. anatoliae* (*A. a. anatoliae* and *A. a. sureyanus*) are clearly separated, whereas the minor overlap that occurs between *A. danfordii* and *A. a. sureyanus* is visible in the PC 2 vs PC 3 plot (Fig. 5E). The fossil samples do not display overlap with *A. danfordii*, with exception of the PC 1 vs

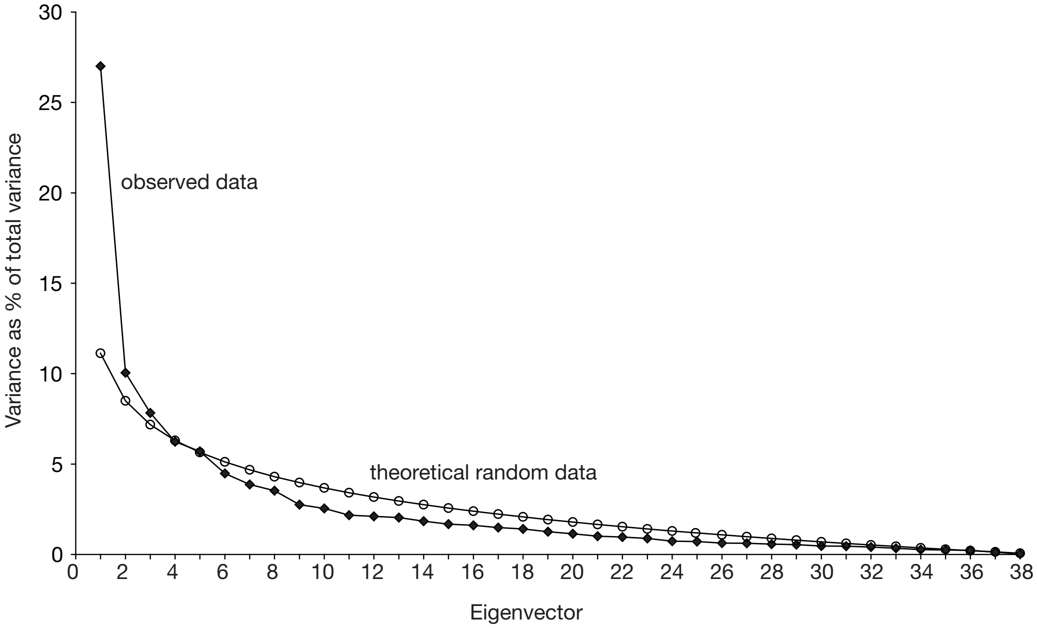

FIG. 4. — Eigenvector vs variance as % of total variance plot showing the position of the eigenvalues of the eigenvectors with respect to the theoretical eigenvalues expected by chance alone. Theoretical eigenvalues were determined based on the formula provided in Jackson (1993: 2207 "Broken Stick").

PC 3 plot (Fig. 5C) where sample T01 256 depicts a somewhat intermediate position between both extant species with the affinities tending towards *A. danfordii*. On the other hand, the fossil samples are characterized by large overlap between one another, and show overlap, or at least more proximity, to *A. anatoliae*, which is especially true of sample T97 242.

95%-confidence ellipses of the groups

The overlap between *A. danfordii* and the remaining groups in PC 1 vs PC 2 plot is relatively small, with exception of the fossil sample T01 256 (Fig. 5A). Considerable overlap occurs between *A. danfordii* and the other four groups in the PC 1 vs PC 3 and PC 2 vs PC 3 plots.

Outliers or data points near the boundary of the confidence ellipses of the groups contain references with regard to the later interpretation of the morphospace generated by the PC-plots. The PC 1 vs PC 2 plot (Fig. 5A) displays "extreme outlines", one of which belongs to *A. danfordii*, and a second to the fossil sample T01 256. Moreover, the latter falls into the 95%-confidence ellipse of the group mean of *A. danfordii*. Both otoliths reveal a distinct shape of the posteroventral edge, a relatively deep excisura and a prolonged rostrum. The five "extreme contours" of the PC 1 vs PC 3 plot (Fig. 5C) are peculiar in that they are located in-between their groups due to the shape of the antirostrum, deepness of the excisura and development of the posterodorsal edge. The PC 2 vs PC 3 plot (Fig. 5E) again indicates that the shape of the antirostrum and the posteroventral and posterodorsal edges are of importance for the extraordinary positions of the four "extreme outlines".

SYNTHETIC MODEL SHAPES

The PC 1 vs PC 2 and PC 1 vs PC 3 plots (Fig. 5B, D) indicate that the more negative the PC 1 values are, the deeper the excisura and the longer the rostrum become. In addition, the more negative the values of PC 1 are, the more prolonged the tip of the rostrum becomes. More positive values along

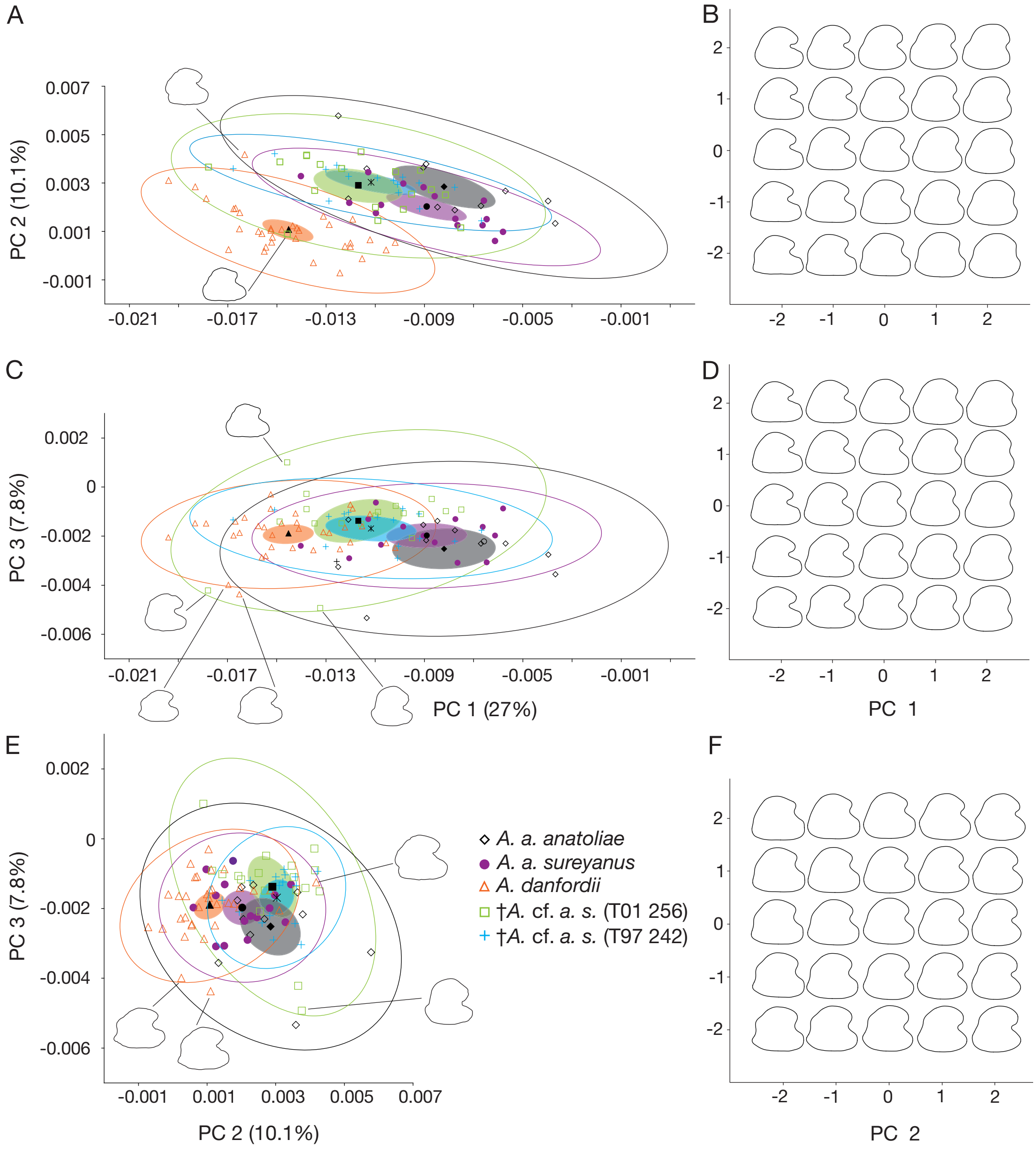

FIG. 5. — **A**, PC 1 vs PC 2 scatter plot; **B**, synthetic model shapes for the PC 1 vs PC 2 plot in units of S.D.; **C**, PC 1 vs PC 3 scatter plot; **D**, synthetic model shapes for the PC 1 vs PC 3 plot in units of S.D.; **E**, PC 2 vs PC 3 scatter plot; **F**, synthetic model shapes for the PC 2 vs PC 3 plot in units of S.D; **A**, **C**, **E**, open ellipses represent 95%-confidence ellipses of the groups, shaded ellipses show the 95%-confidence ellipses of the group means. "Extreme contours" are indicated by original outlines of the respective otoliths. Abbreviations: ***A.***, *Aphanius*; ***a.***, *anatoliae*; ***s.***, *sureyanus*.

the PC 2 axis (Fig. 5B, F) account for a more ventrally bent antirostrum and a less angular and more pointed tip of the rostrum. Furthermore, the PC 2 explains the degree of curvature/angularity of the posteroventral edge, whereas the PC 3 (Fig. 5D, F) characterizes the development of the posterodorsal

TABLE 2. — Jackknifed classification matrix of the canonical discriminant analysis of all five investigated groups of *Aphanius*. The percentages in rows represent the classification into the groups given in columns; the corresponding number of specimens is given in brackets. The percentages of correctly classified individuals are in bold. Overall classification success is 63.8% (Wilks'λ = 0.169). Abbreviations: ***A.***, *Aphanius*; ***a.***, *anatoliae*; ***s.***, *sureyanus*.

Groups	***A. a. anatoliae***	***A. a. sureyanus***	***A. danfordii***	**†*A.* cf. *a. s.* (T01 256)**	**†*A.* cf. *a. s.* (T97 242)**
A. a. anatoliae (Leidenfrost, 1912)	**72.7 (8)**	18.2 (2)	0.0 (0)	9.1 (1)	0.0 (0)
A. a. sureyanus (Neu, 1937)	26.7 (4)	**46.7 (7)**	0.0 (0)	13.3 (2)	13.3 (2)
A. danfordii (Boulenger, 1870)	0.0 (0)	0.0 (0)	**94.1 (32)**	5.9 (2)	0.0 (0)
†*A.* cf. *a. sureyanus* (T01 256)	5.6 (1)	16.7 (3)	11.1 (2)	**38.9 (7)**	27.8 (5)
†*A.* cf. *a. sureyanus* (T97 242)	12.5 (2)	18.8 (3)	0.0 (0)	31.3 (5)	**37.5 (6)**

TABLE 3. — Jackknifed classification matrix of the canonical discriminant analysis of the three groups: *Aphanius anatoliae* (*A. a. anatoliae* and *A. a. sureyanus*), *A. danfordii* and †*A.* cf. *a. sureyanus* (T97 242 and T01 256). The percentages in rows represent the classification into the groups given in columns; the corresponding number of specimens is given in brackets. The percentages of correctly classified individuals are in bold. Overall classification success is 78.7% (Wilks'λ = 0.197). Abbreviations: ***A.***, *Aphanius*; ***a.***, *anatoliae*.

Groups	***A. anatoliae***	***A. danfordii***	**†*A.* cf. *a. sureyanus***
A. anatoliae (Leidenfrost, 1912)	**69.2 (18)**	0.0 (0)	30.8 (8)
A. danfordii (Boulenger, 1870)	0.0 (0)	**97.1 (33)**	2.9 (1)
†*A.* cf. *a. sureyanus*	26.5 (9)	5.9 (2)	**67.6 (23)**

edge and posterior rim. The more negative the values become along the PC 3 axis, the more dorsally bent the posteroventral edge is. This is particularly well visible in the PC 2 vs PC 3 plot (Fig. 5F).

CANONICAL DISCRIMINANT ANALYSIS (CDA)

The Box's M test yields no significant result ($p > 0.1$) for both CDA plots, and thus the assumption of homogeneity of the group covariance matrix cannot be rejected. The plots of the CDA reveal both a distinct separation of *A. danfordii* and the *A. anatoliae* subspecies and the fossil samples (Fig. 6). According to the jackknifed classification matrices, there is no misclassification between the extant species *A. danfordii* and *A. anatoliae* (Tables 2; 3). Minor misclassification occurs between *A. danfordii* and the merged fossil samples (Table 3), and *A. danfordii* and the fossil sample T01 256 (Table 2). In comparison to *A. danfordii*, which always has more than 90% correct classification, the individual subspecies as well as the subspecies and the fossil samples show correct classification rates of 37.5% (T97 242) to 72.7% (*A. a. anatoliae*) for all groups (Table 2) and about 70% (67.6% fossil *Aphanius* and 69.2% *A. anatoliae*) for the merged groups (Table 3).

AVERAGE CONTOURS (FIG. 7)

The development of the dorsal and posterior rims in the fossils more closely correspond to that seen in *A. anatoliae* than to that of *A. danfordii* (for comparison see also Fig. 8C, F vs H, I). The average contour of *A. danfordii* is marked by a distinct posteroventral edge. The fossil otoliths and *A. danfordii* are characterized by a relatively deeply incised narrow excisura, while the excisura of *A. a. anatoliae* and *A. a. sureyanus* is wider and flatter. The shape of the tip of the antirostrum in the fossils resembles that seen in *A. danfordii*, but the overall curvature, especially in sample T97 242, parallels that of *A. a. anatoliae* and *A. a. sureyanus* (Fig. 8C, D, G vs H). The rostrum of the fossils is not as round as in *A. a. anatoliae* and also not as broad and rectangular as in *A. danfordii*, but very similar to *A. a. sureyanus*. The contours of the fossil samples and *A. a. sureyanus* are similar in the region of the dorsal and the posterior rim, with exception of the posteroventral edge where the outlines of the extant subspecies show almost the same course.

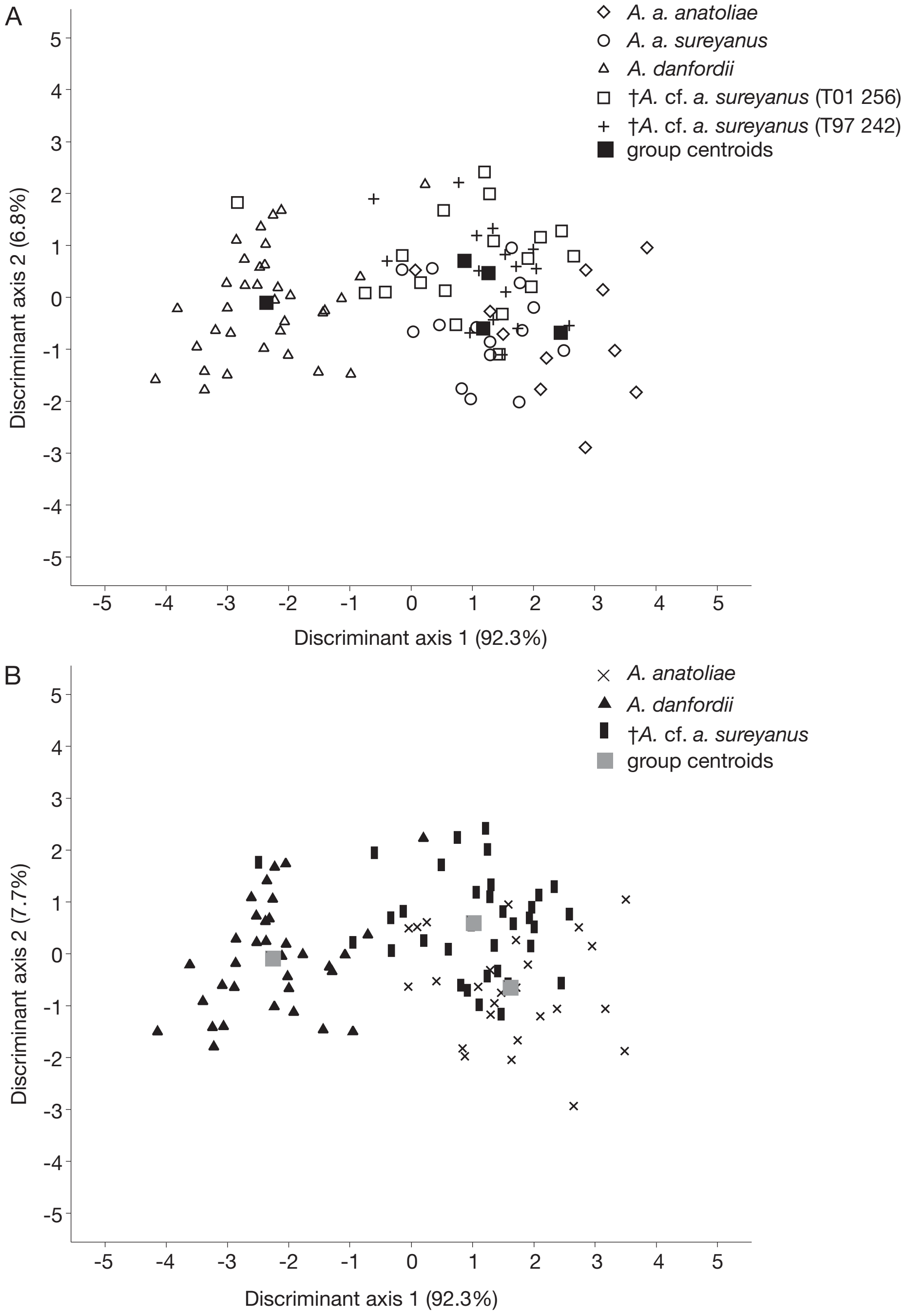

FIG. 6. — Discriminant function scores for the first three principal components based on 38 Fourier descriptors generated by the program Hshape: **A**, for all five investigated groups; **B**, for three groups, the subspecies of *Aphanius anatoliae* (Leidenfrost, 1912) and the fossil samples merged, respectively. Abbreviations: ***A.***, *Aphanius*; ***a.***, *anatoliae*.

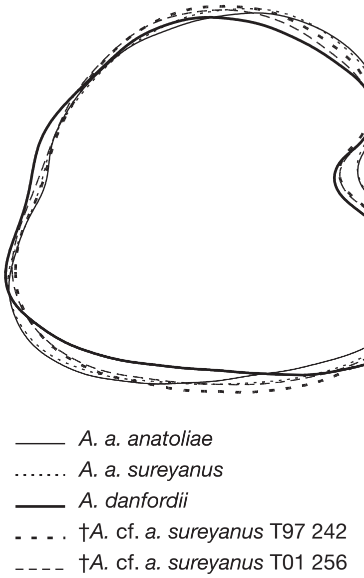

FIG. 7. — Average contours based on the averaged 38 Fourier descriptors for every group, back-calculated with the program HCURVE (1024 x-y values for each contour) of *A. a. anatoliae* (Leidenfrost, 1912), *A. a. sureyanus* (Neu, 1937), *A. danfordii* (Boulenger, 1870), and the two fossil samples of †*A.* cf. *a. sureyanus* (T97 242 and T01 256). Abbreviations: ***A.***, *Aphanius*; ***a.***, *anatoliae*.

DISCUSSION

In this study, *A. danfordii* primarily served as "outgroup" for a more accurate similarity estimate between the fossil *Aphanius* and the extant *A. anatoliae* otoliths. In general, the results of the CDA (Fig. 6; Tables 2; 3) show that the fossil *Aphanius* und *A. anatoliae* are distinct from *A. danfordii*. The differences between the two extant species concur with the results of mtDNA analyses presented by Hrbek *et al.* (2002), and Hrbek & Meyer (2003), the crossbreeding studies conducted by Villwock (1964), and the qualitative analysis of otolith morphology by Schulz-Mirbach *et al.* (2006).

The average contour of *A. danfordii* otoliths appears to be slightly rotated to the average contours of the other groups due to the fitting by the program HMATCH (Fig. 7). One might argue that this may be the reason for the differences between *A. danfordii* and the other groups. However, fitting the contours by HMATCH not only was the best method (see chapter "Material and methods") for this purpose, but also takes indirectly into account that the sulcus of *A. danfordii* often runs obliquely compared to the median straight sulcus of the *A. anatoliae* subspecies and fossils (Fig. 8H vs A, B, E, G).

Group-specific traits of the otolith contour

The morphological features of the otolith contour explained by the PC 2 are significant in distinguishing *A. danfordii* from *A. anatoliae* and the fossil *Aphanius*. These are: the shape of the antirostrum and curvature/angularity of the posteroventral edge. Moreover, the qualitative analysis of *A. danfordii* indicates that most of the otoliths are characterized by a tapering posteroventral edge and straighter and slightly dorsally pointed antirostrum (Figs 7; 8H). On the other hand, the otoliths of the fossil *Aphanius* and extant *A. anatoliae* populations are usually marked by a ventrally bent antirostrum and broad and angularly shaped posteroventral edge (Figs 7; 8C, D, F).

Aphanius danfordii, the fossil *Aphanius*, and *A. anatoliae* are separated mainly along the PC 1 axis, which explains the deepness of the excisura and length of the rostrum. However, especially the depth and shape of the excisura vary considerably within the *A. anatoliae* populations as can be seen by comparing series of individual otoliths from each of the populations. As a result, this feature must be regarded less significant in the discrimination of the individual groups, and may also explain the somewhat unexpected proximity of the fossil samples (especially T01 256) to *A. danfordii*. There are several fossil otoliths in sample T01 256 that display a distinctly deepened excisura (see extreme original contours in Fig. 5C).

Morphological features of the contour explained by the PC 3 (e.g., the development of the posterodorsal edge) seem to play only a subordinate role in separating the investigated groups from each other, which is expressed in the great overlap of all groups in the PC 2 vs PC 3 plot (Fig. 5E).

Diversification of *A. anatoliae* in the Lakes District

If all three PCs are taken into consideration for the CDA, both extant species are well separated

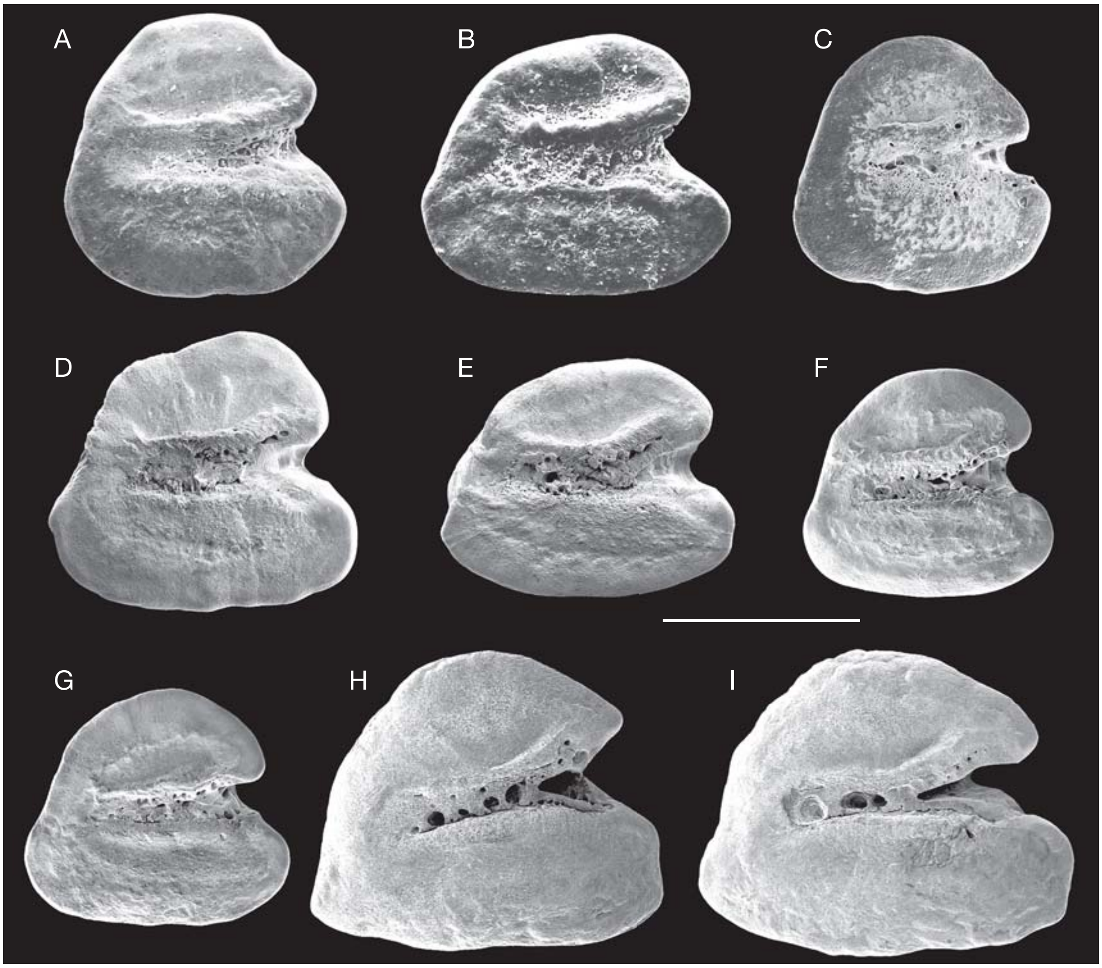

FIG. 8. — SEM pictures of: **A**, †*Aphanius* cf. *anatoliae sureyanus* (T97 242), right otolith (mirrored); **B**, †*A.* cf. *a. sureyanus* (T97 242); **C**, †*A.* cf. *a. sureyanus* (T01 256), right otolith (mirrored); **D**, *A. a. anatoliae* (Leidenfrost, 1912), Yeşilova at Lake Salda, TL (total fish length) 45 mm, female; **E**, *A. a. anatoliae*, in-between Salda and Doğanbaba at Lake Salda, TL 38 mm, male; **F**, *A. a. sureyanus*, Lake Burdur, TL 31 mm, male; **G**, *A. a. sureyanus* (Neu, 1937), Lake Burdur, TL 37 mm, female; **H**, *A. danfordii* (Boulenger, 1870), Karpuzatan, TL 39 mm, male; **I**, *A. danfordii*, Karpuzatan, TL 39 mm, male; **A**, **B**, BSPG-2003 IV 245-246; **C**, BSPG-2003 IV 227; **D**, **E**, BSPG-2003 IV 182-183; **F**, **G**, BSPG-2003 IV 168-169; **H**, BSPG-2003 IV 193. If not mentioned otherwise, the pictures show left otoliths. Scale bar: 0.5 mm.

(Fig. 6), and the fossil samples show great proximity to one another and also seem to have affinities with the extant *A. a. sureyanus* (Fig. 6A). The fossils are slightly more similar to *A. a. sureyanus* than to *A. a. anatoliae* (see for example Figs 6A; 7; Tables 2; 3), and these two extant subspecies of *A. anatoliae* reveal a certain distance in the CDA (Fig. 6A; Table 2). Based on these results, we suggest that *A. a. anatoliae* and *A. a. sureyanus* had begun to develop independently from each other already before the fossil populations established. However, we have to bear in mind that ecological influences may also play a certain role with regard to the formation of differences in otolith morphology. The distance between *A. a. anatoliae* and *A. a. sureyanus* could be also due to ecological differences in the respective habitats or differences in the life history. The sampled *A. a. anatoliae* population inhabits shallow vegetation-rich freshwater at the shores of Lake Salda, while *A. a. sureyanus* displays a limnetic (open-water dwelling) phenotype (Hrbek & Meyer 2003) in the brackish Lake Burdur that does not contain large amounts of vegetation (Wildekamp *et al.* 1999). Volpedo & Echeverría (2003) demonstrated that in certain marine fishes, the substrate type, along with bottom oriented vs pelagic life, is reflected in the rostrum length to maximum otolith length ratio. Moreover, in some marine fishes, complexity of otolith outline and within-population variability was shown to decrease from deep to shallow water habitats (Gauldie & Crampton 2002). Although these studies focused on marine fishes and the investigated depth ranges encompass hundreds or thousands of metres, they demonstrated that certain ecological parameters are affecting otolith contour.

Moreover, Gauldie & Crampton (2002) hypothesized that particularly high levels of symmetry between left and right otoliths may point to a largely genetically determined otolith morphology. Left and right *A. a. anatoliae* otoliths are morphologically quite different, indicated by a CDA with the raw FDs (data not shown). As environmental parameters always affect the entire animal, one would expect to find the left and right otoliths shaped in exactly the same way. As a result, it is unlikely that the asymmetry between the left and right *A. a. anatoliae* otoliths is caused by environmental influences. Rather, the asymmetry appears to be due to local differences in the concentration of intrinsic factors involved in the developmental processes (see Yoshioka *et al.* 2004) that are responsible for the formation of the otoliths.

In future studies, populations of the second (e.g., Lake Eğirdir) and third population-groups (western central Anatolia) of *A. anatoliae* will be incorporated in the CDA to elaborate on the relevance of the difference between *A. a. anatoliae* and *A. a. sureyanus* presented in this study.

TAXONOMICAL CONCLUSIONS

Based on the fact that the fossil *Aphanius* and *A. anatoliae* are not distinctly separated, but rather show more or less similarity to each other in the CDA plots (Fig. 6B; Table 3), and because the fossils and the extant populations display a certain degree of variability with regard to otolith contour indicated by qualitative analyses (for variability of extant populations of *A. anatoliae*, see Schulz-Mirbach *et al.* 2006), it does not seem reasonable to establish a new species for the fossil *Aphanius* otoliths. We suggest that the fossil otoliths can be assigned to *A. anatoliae* or the subspecies *A. a. sureyanus*. However, the nomenclatural situation within *A. anatoliae* is problematic insofar as the subspecies *A. a. sureyanus*, *A. a. splendens*, *A. a. transgrediens*, and populations of *A. a. anatoliae* of the Lakes District form a single clade according to Hrbek *et al.* (2002), and show total fertility between one another in the crossbreeding experiments of Villwock (1958, 1964), whereas other *A. a. anatoliae* populations in the western central part of Anatolia form a separate clade and are marked by different degrees of sterility to those populations of the Lakes District. Therefore, Villwock (2004) proposed to regard the various population-groups of *A. anatoliae* as "species *in statu nascendi*" and hence, to denominate them as *A. anatoliae* ssp. On the other hand, Hrbek & Meyer (2003) hypothesized that the identified *A. anatoliae* clades might represent new species. Based on the biospecies concept, the population-groups should only be regarded as distinct subspecies because they do not reveal total sterility among each other, which, however, is an essential criterion in the determination of true species. With regard to the classification of the fossils, there are two nomenclatural possibilities: they could be denominated as *A.* cf. *anatoliae* ssp. "Lakes District", which would be impracticable, or as †*A.* cf. *anatoliae sureyanus*, which is preferred because of the close correspondences between the fossil otoliths and those of the extant subspecies *A. a. sureyanus*.

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