

Combined Otolith Morphology and Morphometry for Assessing Taxonomy and Diversity in Fossil and Extant Killifish (*Aphanius*, †*Prolebias*)

Bettina Reichenbacher,^{1*} Ulrike Sienknecht,² Helmut Küchenhoff,³ and Nora Fenske³

¹Department of Earth and Environmental Sciences, Section Palaeontology, Ludwig-Maximilians-University Munich, 80333 Munich, Germany

²Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907, USA

³Statistical Consulting Unit, Department of Statistics, Ludwig-Maximilians-University Munich, Akademiestraße 1, 80799 Munich, Germany

ABSTRACT Systematic assignment of fossil otoliths is virtually always based on studies of otolith morphology and subsequent comparisons with otoliths from collections and/or literature. Although this usually represents a practical method, comparisons and subsequent evaluation may be biased by subjective criteria used in the individual descriptions. Quantitative morphometric studies focusing on variations in the otolith morphology of extant fishes have been conducted in fisheries research, mostly based on Fourier shape analysis and related methods. However, with regard to fossil otoliths, these approaches are generally not suitable, mainly due to preservation-related problems. Here we present a new approach for quantifying otolith variation between species and populations of killifish (cyprinodontiforms) in the genera *Aphanius* Nardo and †*Prolebias* Sauvage that can be used with both extant and fossil otoliths. Our new approach includes the definition of 10 variables from linear and angle measurements of an otolith and statistical analyses. Best results were obtained by presorting the otoliths into three groups based on sulcus shape (straight, bent, S-shaped). In this case, canonical discriminant analysis (CDA) with jackknifed cross-validation yielded an overall species classification success of 86–96%. The three groups based on sulcus shape separate according to zoogeographic patterns (i.e., Mediterranean *Aphanius*, Arabian *Aphanius*, European †*Prolebias*) and probably reflect phylogenetic lineages. Application of CDA to compare otolith variation between populations resulted in an overall classification success (jackknifed) of 33–83%. High levels of variation were observed for *Aphanius dispar* and †*P. malzi*, but not for *A. fasciatus* and †*P. weileri*. We suggest that otolith variation between populations results predominantly from geographic separation. Combination of qualitative characters (sulcus morphology) with quantitative approaches (otolith morphometry) presents a new approach for obtaining a better understanding of the taxonomy, diversity, and zoogeography of both fossil and extant killifishes. Moreover, the method may also be suitable for assessing taxonomy and diversity in other species-rich groups like the atheriniforms and many perciforms because these groups display otolith Bauplans that are similar to those seen in killifishes. *J. Morphol.* 268:898–915, 2007. © 2007 Wiley-Liss, Inc.

KEY WORDS: cyprinodontiforms; *Aphanius*; †*Prolebias*; otolith morphology; morphometry; diversity

In the inner ear of teleost fishes are three pairs of largely aragonitic mineralizations, which are

termed otoliths. The present study focuses on the saccular otolith, which is the largest and/or most massive of the three types of otoliths in most groups of teleost fishes (e.g., Nolf, 1985; Assis, 2005). Otoliths have an important biological function because they enable the inner ear to mediate the senses of hearing and balance (see Popper et al., 2005 for a recent overview). The characteristic morphology of a saccular otolith of any given teleost fish is composed of a specific complement of traits (Fig. 1a), which includes overall shape, size, and contour of the sulcus, and forms, sculptures and proportions of distinct segments of the otolith.

Otolith morphology represents a valuable parameter in species discrimination of many extant teleost fishes (e.g., Koken, 1884; Chaîne and Duvergier, 1934, 1942; Nolf, 1985; Härkönen, 1986; Williams and McEldowney, 1990; Schwarzhans, 1993, 1999; Smale et al., 1995; Volpedo and Echeverría, 2000; Campana, 2004). However, many of these studies, and almost all studies focusing on fossil otoliths, are based on comparative descriptions of otolith morphology, and thus depend on subjective criteria (cf. Lombarte and Castellon, 1991). On the other hand, studies conducted by research groups working with commercially important fishes have used Elliptic Fourier analysis and landmarks in the quantification of otolith variation between species, populations, and even stocks (Torres et al., 2000; Monteiro et al., 2005; Parisi-Baradad et al., 2005; Stransky and

Contract grant sponsor: Deutsche Forschungsgemeinschaft; Contract grant number: RE 1113/6-1; Contract grant sponsor: GeoBio-Center of the Ludwig-Maximilians-University, Munich.

*Correspondence to: Bettina Reichenbacher, Department of Earth and Environmental Sciences, Section Palaeontology, Ludwig-Maximilians-University Munich, Richard-Wagner-Str. 10, 80333 Munich, Germany. E-mail: b.reichenbacher@lrz.uni-muenchen.de

Published online 2 August 2007 in
Wiley InterScience (www.interscience.wiley.com)
DOI: 10.1002/jmor.10561

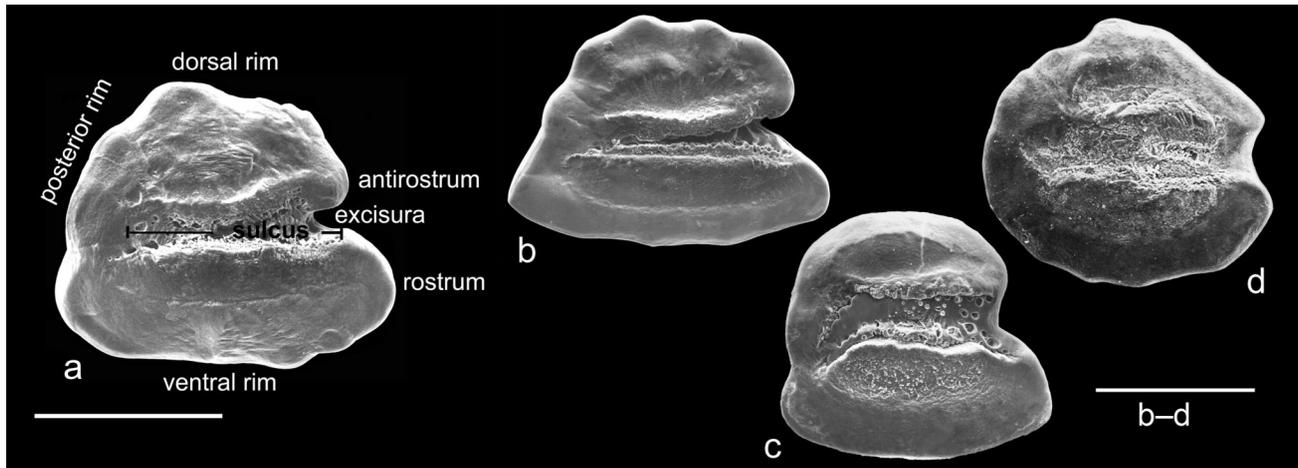


Fig. 1. General morphology of cyprinodontiform otoliths and sulcus morphologies. All figures are SEM micrographs of left otoliths (medial). Scale bar = 0.5 mm. TL, total length in cm. BSPG 2004 II 1–3 (a–c), 2003 XVIII 183. (a) Terminology of otolith characters; *Aphanius fasciatus*, Ravenna, Italy (female, TL 3.85). (b) Otolith with straight sulcus; *Aphanius fasciatus*, Fanjo Delta, Corsica (female, TL 4.4). (c) Otolith with terminally bent sulcus; *Aphanius dispar* from Al Khari, Saudi Arabia (male, TL 3.8). (d) Otolith with slightly S-shaped sulcus; †*Prolebias malzi* from the Hanau Basin.

MacLellan, 2005; Ponton, 2006). But similar approaches have not been developed to date for fossil otoliths because the limited availability of fossils of similar size and preservation-related problems (i.e., fossil otoliths are often slightly corroded, which conflicts with the approach of shape discrimination) usually place serious constraints on any quantitative analysis of fossil otoliths.

The study presented here was inspired by previous studies of otolith-based fossil fish faunas from Oligo-Miocene euryhaline and freshwater environments (Reichenbacher and Weidmann, 1992; Reichenbacher, 2000). The high variability of certain fossil otolith-based species, mainly killifishes (cyprinodontiforms) and silversides (atheriniforms), produced difficulties with regard to species discrimination, and raised the question as to whether variations in otolith morphology occur exclusively between species or may also occur between populations of a single species. Detailed information about intraspecific otolith variability is important with regard to considerations on palaeodiversity, palaeozoogeography, and palaeogeography.

The intention of this study was to develop an appropriate approach that permits quantification of otolith variations both between species and individual populations of a single species. The approach includes extant and fossil cyprinodontiform otoliths, and focuses on *Aphanius* as a representative for extant cyprinodontiforms because it represents the nearest living relative of the Oligo-Miocene †*Prolebias* and †*Palaeolebias* (see Villwock, 1999). For a better understanding of otolith morphology and variation, we present illustrations and short descriptions of the otoliths of extant *Aphanius* species from the Mediterranean region, Arabian Peninsula and Persian Gulf region. The

otoliths of the *Aphanius* species from these areas have not previously been documented, with the exception of *A. iberus* and *A. apodus* from the westernmost Mediterranean (cf. Reichenbacher and Sienknecht, 2001).

MATERIALS AND METHODS

Sampling

The otoliths used in this study come from ten euryhaline cyprinodontiform species, including seven extant *Aphanius*, one fossil *Aphanius*, and two fossil *Prolebias* species (Table 1). Specimens from different populations were studied for *Aphanius fasciatus* (Valenciennes, in Humboldt and Valenciennes, 1821), *A. dispar* Rüppell, 1829, †*Prolebias weileri* von Salis, 1967 and †*P. malzi* Reichenbacher and Gaudant, 2003. Dissected otoliths are deposited in the collections of the Senckenberg Museum, Frankfurt (SMF PO) and the Bavarian State Collection for Palaeontology and Geology in Munich (BSPG 2004 II), Germany.

Figure 2 indicates the geographical position of the sample localities where the extant fishes and fossils have been collected; Table 1 surveys the numbers and localities of extant specimens and fossils, and summarizes selected references. The extant species were collected by Wolfgang Villwock (Hamburg University), the second author and other colleagues during several research expeditions. *Aphanius fasciatus* (Valenciennes, in Humboldt and Valenciennes, 1821), *A. mento* (Heckel, in Russeger, 1843) and *A. ginaonis* Holly, 1929 were obtained directly from wild catches, while *A. iberus* Valenciennes, in Cuvier and Valenciennes, 1846, *A. baeticus* Doadrio, Carmona and Fernández-Delgado, 2002, *A. dispar* Rüppell, 1829 and *A. sirhani* Villwock, Scholl and Krupp, 1983 come from cultivated stocks. Only otoliths from sexually mature individuals (TL > 2.5 cm) were used to minimize tainting of the data by ontogenetic variations in otolith morphology. Fishes were killed by a lethal dose anesthetic (tricaine methanesulfonate, MS222) and stored in the lab frozen at -20°C (aquarium stock material) or in the field in 99.9% ethanol (wild catches) until dissection. Otoliths were dissection and cleaned from organic residues by incubation in 1% KOH solution for 6 h and subsequent washing in distilled water for 12 h. For the collection of fossil otoliths sediment samples

TABLE 1. Species names, localities, and references, number of dissected specimens (for the extant species), numbers of otoliths, and stratigraphic age (of the fossils)

Recent Species ($n = 186$ specimens)	Locality, Reference	Specimens
<i>Aphanius iberus</i> Valenciennes, in Cuvier and Valenciennes, 1846	Valencia, Spain (1, 2)	16 (12 f, 4 m)
<i>Aphanius baeticus</i> Doadrio, Carmona and Fernández-Delgado, 2002	Lebrija, Spain (1, 2)	16 (7 f, 9 m)
<i>Aphanius fasciatus</i> (Valenciennes, in Humboldt and Valenciennes, 1821)	Ganzirri, NE-Sicily Marina di Modica, SE-Sicily Fano Delta, Corsica Golfo di Palmas, SW-Sardinia	12 (8 f, 4 m) 11 (4 f, 7 m) 18 (16 f, 2 m) 7 (5 f, 2 m)
<i>Aphanius mento</i> (Heckel, in Russegger, 1843)	Kirk Göz, Turkey	28 (12 f, 16 m)
<i>Aphanius dispar</i> Rüppell, 1829	Al Khari, Saudi-Arabia Faluja, Iraq	16 (8 f, 8 m) 28 (8 f, 20 m)
<i>Aphanius ginaonis</i> Holly, 1929	Genu, Iran	19 (10 f, 8 m, 1 j)
<i>Aphanius sirhani</i> Villwock, Scholl and Krupp, 1983	Azraq, Jordan (3)	21 (6 f, 15 m)
Fossil Species ($n = 87$ otoliths)	Locality, Age, Reference	Otoliths
<i>Aphanius kayai</i> Reichenbacher and Rückert-Ülkümen, 2002	W-Turkey, Manisa, 5 Ma, (4)	20
† <i>Prolebias malzi</i> Reichenbacher and Gaudant, 2003	Mainz Basin, Weisenau, 23 Ma, (5) Hanau Basin, Nieder-Erlenbach, 22 Ma, (5)	18 18
† <i>Prolebias weileri</i> von Salis, 1967	Molasse Basin S-Germany, Illerkirchberg, 17.3 Ma (6) Molasse Basin Switzerland, Mauensee, 17 Ma (7) Molasse Basin Switzerland, Schmiedrue, 17 Ma (7)	6 11 14

F, female, m, male. References: (1) Sienknecht, 1999, (2) Reichenbacher and Sienknecht, 2001, (3) Villwock et al., 1983, (4) Rückert et al., 2002, (5) Reichenbacher, 2000, (6) Reichenbacher et al., 2004, (7) Jost et al., 2006.

were processed in H_2O_2 , screen washed, and dried. Otoliths were picked from the residue larger than 0.4 mm in diameter.

Otolith Morphology and Dimensions

SEM images of several otoliths from each species and population were prepared for comparative investigation of the morphology with a Cambridge SEM at the Laboratorium für Elektronenmikroskopie of the Technical University Karlsruhe, and a Leo Gemini SEM at the Zoological State Collection, Munich. The digital images that were used for the measurements were taken with a stereoscope to which a Leica DC 200 digital camera was attached; for photography, the otoliths were oriented with the outer/lateral side down and ventral rim parallel to a horizontal line (Fig. 3). Digital images were taken of the left otolith of each extant specimen to assure that one sample represents one fish; from the fossil material, mostly the left otoliths were selected and photographed. We used the Leica IMAGIC software for the measurements.

Eight linear lines and three angles were measured for each otolith (see Fig. 3). Six out of the eight linear lines are located according to objective criteria (with the otolith orientated as described above), the two remaining lines and the three angles are located by reference to them. These six lines are:

– a horizontal line, located at the intersection with the most prominent point of the rostrum (l–l' in Fig. 3);

- a horizontal line, located at the intersection with the most prominent point of the antirostrum (d–d' in Fig. 3);
- a horizontal line, located at the intersection with the most incised point of the excisura (m–m' in Fig. 3);
- a vertical line, located at the intersection with the highest point of the dorsal rim (h–h' in Fig. 3);
- a vertical line, located at the intersection with the most incised point of the excisura and running to the dorsal rim (m–a in Fig. 3);
- a vertical line, located at the intersection with the most incised point of the excisura and running to the ventral rim (m–r in Fig. 3);

The two further lines, which are related to the previous lines, are: a horizontal line, running from the intersection of line d–d' with m–a to the most prominent point of the antirostrum (al–d in Fig. 3); and a horizontal line, running from the intersection of line l–l' with m–r to the most prominent point of the rostrum (rl–l in Fig. 3). The locations of the three angles (PV, P, and E) are explained by their illustration in Figure 3.

The measurements of these lines and angles provide numeric data, which can be treated statistically. For that purpose, 10 otolith variables were defined as proportional and absolute numerical values based on the measurements (Table 2). We used proportional values in the indication of distances to assure size correction of the measured data, and absolute values in the indication of angles. The measurement error was empirically determined by repeated measurements and amounted to ~2.5% of a distance or an angle.

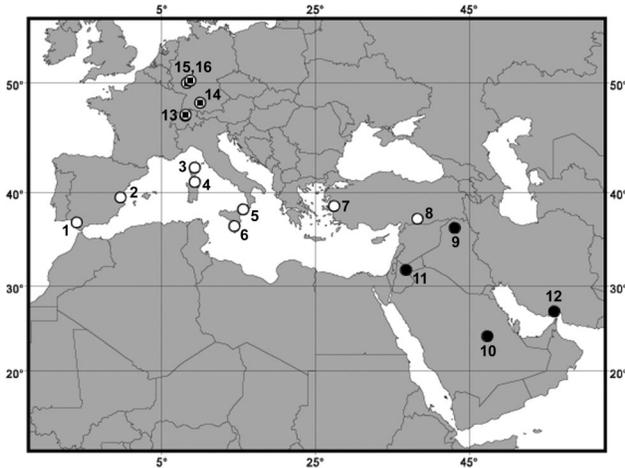


Fig. 2. Locations of the extant and fossil cyprinodontiforms studied. Open circles = Sampling sites of *Aphanis* from the Mediterranean and Turkey; closed circles = Sampling sites of *Aphanis* from the Arabian Peninsula and the Persian Gulf region; circles with squares = Sampling sites of †*Prolebias* species. 1, Lebrija (*Aphanis baeticus*); 2, Valencia (*Aphanis iberus*); 3, Fanjo Delta (*Aphanis fasciatus*); 4, Sardinia (*Aphanis fasciatus*); 5, Ganzirri Lake (*Aphanis fasciatus*); 6, Marina di Modica (*Aphanis fasciatus*); 7, Manisa (†*Aphanis kayai*); 8, Kirk Göz (*Aphanis mento*); 9, Faluja (*Aphanis dispar*); 10, Al Khari (*Aphanis dispar*); 11, Azraq (*Aphanis sirhani*); 12, Genu (*Aphanis ginaonis*); 13, Mauensee and Schmiedrued (†*Prolebias weileri*); 14, Illerkirchberg (†*Prolebias weileri*); 15, Mainz-Weisenau (†*Prolebias malzi*); 16, Nieder-Erlenbach (†*Prolebias malzi*).

Statistics

The 10 variables obtained from each of the extant and fossil otoliths served as input for the statistical analysis, which was conducted with SPSS 14.00 for Windows (SPSS, 2005). A negative correlation (Pearson $-0.7/-0.8$) occurs with the following variables: excisura vs. relative rostrum length, excisura vs. relative antirostrum length, and relative rostrum length vs. relative medial length. Positive correlations (Pearson $+0.7/+0.8$) occur with regard to excisura vs. relative medial length, relative dorsal length vs. relative medial length, relative antirostrum height vs. relative antirostrum length, and relative rostrum height vs. relative rostrum length. Nonparametric tests (Mann-Whitney-U, Kolmogorov-Smirnov, $P < 0.05$) and one-way ANOVA with post-hoc tests (Tamhane-T2, Dunnett T3, $P < 0.05$) were used to compare the means of a particular otolith variable between species and populations. Canonical discriminant analysis (CDA) was applied to test the significance of the 10 otolith variables for species and population discrimination; classification success was tested by jackknifed cross validation. In addition, stepwise variable selection was used to determine those otolith variables that are most relevant in species and population discrimination.

RESULTS

In the following sections, otolith variables are indicated in italics, but the term “relative” is generally omitted to make the text easier readable; for example, instead of the expression “relative dorsal length” we just use “dorsal length.”

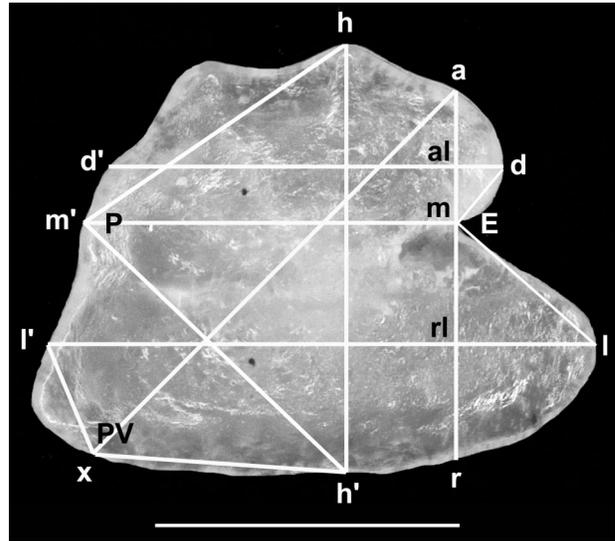


Fig. 3. Left otolith of *Aphanis fasciatus* with measured distances and angles. See text for further explanation. Scale bar = 0.5 mm. TL, total length in cm. BSPG 2004 II 4 (Fanjo Delta, female, TL 3.7).

Shape of the Sulcus

Cyprinodontiform otoliths usually bear a well developed sulcus (Fig. 1a) that is subdivided into a small, slightly deepened, ovate or U-shaped ostium and a long cauda. Based on the form of the sulcus, the studied otoliths can be assigned to three groups (Fig. 1b–d): Group I consists of otoliths that have a straight sulcus (cf. Fig. 4); the group includes the Mediterranean species *Aphanis baeticus*, *A. iberus*, *A. fasciatus*, *A. mento*, and the Turkish species †*A. kayai*. Group II consists of oto-

TABLE 2. Otolith variables and relations to otolith measurements (see Fig. 3)

Variable	Measurement (see Fig. 3)	Description
D, relative dorsal length	d–d'	$(d-d'/l-l')*100$
M, relative medial length	m–m'	$(m-m'/l-l')*100$
A, relative antirostrum height	m–a	$(m-a/h-h')*100$
R, relative rostrum height	m–r	$(m-r/h-h')*100$
AL, relative antirostrum length	al–d	$(al-d/l-l')*100$
RL, relative rostrum length	rl–l	$(rl-l/l-l')*100$
LH, L/H-index of previous studies	l–l' and h–h'	$l-l'/h-h'$
P, Posterior angle	Angle h–m'–h	Posterior angle
E, Excisura angle	Angle d–m–l	Excisura angle
PV, Posteroventral angle	Angle l'–x–h'	Posteroventral angle

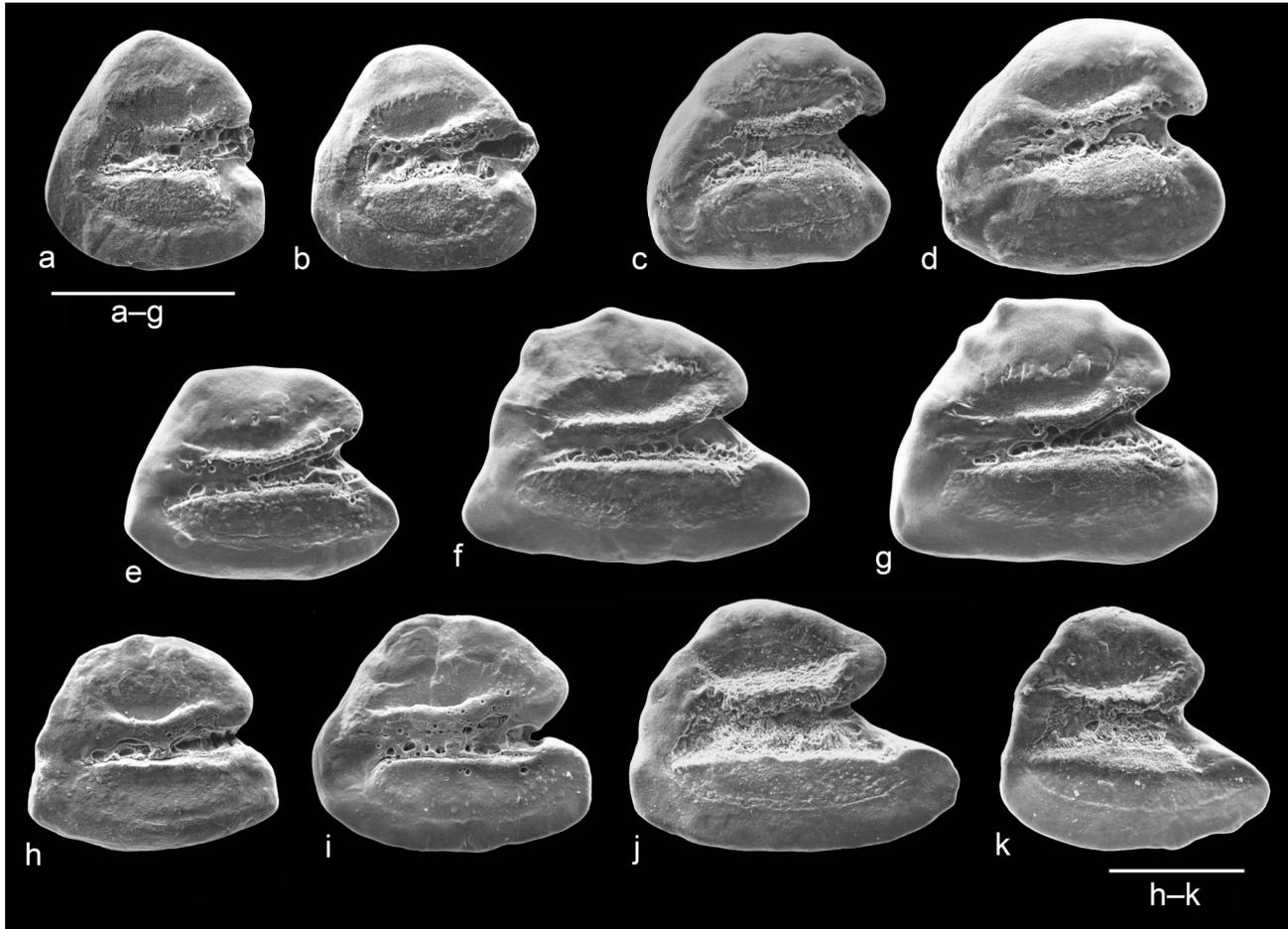


Fig. 4. Otoliths of the studied *Aphanius* species from the Mediterranean and Turkey. All figures are SEM micrographs of left otoliths (medial). Scale bar = 0.5 mm. TL, total length in cm. SMFPO 64271, -73, -78 (d, c, a), BSPG 2004 II 5–10 (b, e–i), BSPG 1980 X 1150, -59 (k, j). (a, b) *Aphanius iberus*, Valencia, Spain (males, TL 2.5, 2.7). (c, d) *Aphanius baeticus*, Lebrija, Spain (male [c] and female, TL 2.8, 3.1). (e–g) *Aphanius fasciatus*, Fanjo Delta, Corsica (females [e–f] and male, TL 3.1, 3.8, 3.7). (h, i) *Aphanius mento*, Kirk Göz, Turkey (females, TL 4.2, 5.3). (j, k) †*Aphanius kayai*, Manisa, Turkey (~5-million years old).

liths with a sulcus that is distinctly bent terminally (cf. Fig. 7); the Arabian species *Aphanius dispar*, *A. ginaonis*, and *A. sirhani* belong to this group. Otoliths in Group III are characterized by a slightly S-shaped sulcus, and include †*Prolebias malzi* and †*P. weileri* (cf. Fig. 9). As a result, sulcus shape provides a parameter for preliminary assignment of the studied cyprinodontiform otoliths to distinct groups. This fact was considered in the subsequent statistical analysis.

Variation Between Species

Group I otoliths (straight sulcus). Otoliths of *Aphanius iberus* are characterized by a triangular to rounded shape and short, truncated rostrum (Fig. 4a,b). *Excisura angle*, *dorsal length*, *length/height*, *rostrum height*, and *rostrum length* (Fig. 5a,d,e,h,j) are distinctly different in comparison with the other *Aphanius* species in Group I, which is supported by one-way ANOVA.

Otoliths of *Aphanius baeticus* are lop-sided-triangular and possess a strong and pointed antirostrum (Fig. 4c,d). The unique shape of antirostrum is reflected by the values of *antirostrum height* and *antirostrum length* (Fig. 5g,i). In addition, one-way ANOVA determines these variables as relevant. Otoliths of *A. baeticus* show the greatest *antirostrum height* and *antirostrum length*, also in comparison with the studied fishes of Groups II and III; solely *A. ginaonis* (Group II) shows a slightly greater *antirostrum height* (Fig. 5g).

Otoliths of *Aphanius fasciatus* are almost triangular and typified by an elongate, long rostrum (Fig. 4e–g). In our data set, this species is represented by four populations (Fig. 10, Table 1), which, in part, is responsible for the high variability of the *excisura angle*, *posterior angle*, *posteroventral angle*, and *length/height* (Fig. 5a–c,e; see below). Nevertheless, the *length/height* (Fig. 5e) is most relevant for the classification of *A. fasciatus*, which is supported by one-way ANOVA.

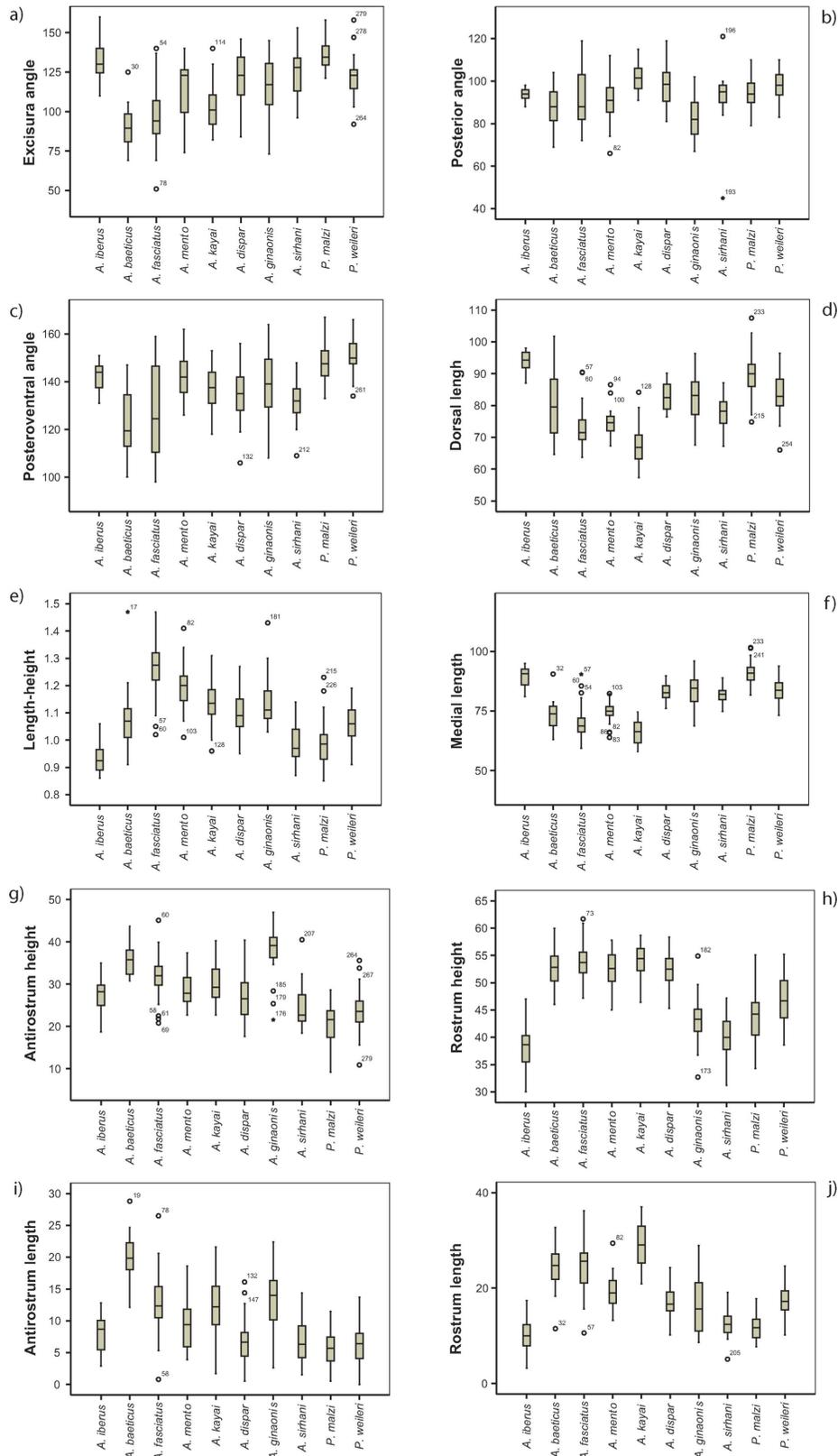


Fig. 5. Box plots showing the median (line within the box), the 25th and 75th percentiles, the data range for each otolith variable, and the variance of the respective otolith variable between the studied species. Open circles respectively stars refer to outliers within respectively without the 100th percentile.

TABLE 3. Jackknifed classification matrix of the CDA for the Group I *Aphanius* species (straight sulcus)

n	Species	Predicted classification				
		<i>A. iberus</i>	<i>A. baeticus</i>	<i>A. fasciatus</i> ^a	<i>A. mento</i>	† <i>A. kayai</i>
16	<i>A. iberus</i>	93.8 (15)	0	0	6.3 (1)	0
16	<i>A. baeticus</i>	0	93.8 (15)	6.3 (1)	0	0
18	<i>A. fasciatus</i> ^a	0	0	72.2 (13)	22.2 (4)	5.6 (1)
28	<i>A. mento</i>	0	3.6 (1)	17.9 (5)	78.6 (22)	0
20	† <i>A. kayai</i>	0	0	5.0 (1)	0	95.0 (19)

The percentages in rows represent the classification into the species given in columns (correctly classified species are bold-faced); corresponding numbers of individuals are given in brackets. *n*, number of otoliths. Overall classification success is 85.7% (Wilks' $\lambda = 0.01$).

^aPopulation from Fanjo Delta.

Otoliths of *Aphanius mento* have an almost quadratic to slightly triangular shape with a truncated rostrum of medium size (Fig. 4h,i). *Length/height* and *rostrum length* (Fig. 5e,j) are most characteristic, which is confirmed by one-way ANOVA, with the exception of *length/height* in *A. mento* vs. †*A. kayai* ($P = 0.3$).

Otoliths of †*Aphanius kayai* are elongated-triangular in shape and bear a long rostrum (Fig. 4j,k), which is even longer than that seen in *A. fasciatus* (see also Fig. 5j). In addition, the *posterior angle* is relevant to discriminate †*A. kayai* from other *Aphanius* species of Group I (Fig. 5b). One-way ANOVA adds support for both variables, with the exception of *rostrum length* in †*A. kayai* vs. *A. baeticus* ($P = 0.07$).

Species discrimination power of the otolith variables was estimated by CDA (see Table 3). Four functions were calculated: Function 1 captures 58.3% of the variation, Functions 2 and 3 capture 24.5 and 12.9%, and Function 4 confines 4.3%. The plot of the discriminant function scores documents two separate clusters, one of which (Fig. 6, right cluster) contains the otoliths of *Aphanius iberus*, the second (Fig. 6, left cluster) those of the other four species. Within the left cluster, †*A. kayai* and *A. baeticus* form the end-points and do not overlap with one another. *Aphanius fasciatus* and *A. mento* are situated in the middle and overlap with one another and, to a lesser extent, also with †*A. kayai* and *A. baeticus*. The overall classification success based on jackknifed cross-validation is 85.7% (Table 3). Misclassification was mainly due to confusions of *A. fasciatus* with *A. mento*.

Group II otoliths (bent sulcus). Otoliths of *Aphanius dispar* are trapezoid in overall shape and have a short to medium-sized rostrum (Fig. 7q-t). *Rostrum height* (Fig. 5h) separates this species from *A. ginaonis* and *A. sirhani*, which is supported by one-way ANOVA. Although two populations of *A. dispar* (Fig. 12) are included, considerable variability in the otolith variables does not occur (Fig. 5).

Aphanius ginaonis is characterized by otoliths that are triangular to rounded-triangular in shape and bear a comparatively thin, medium-sized rostrum, whereas the antirostrum is prominent (Fig. 7i-p). *Antirostrum height* and *antirostrum length* (Fig. 5g,i) differ from those seen in the remaining *Aphanius* from Group II, which is supported by one-way ANOVA. In addition, sexual dimorphism is observed in *A. ginaonis*, but could not be tested quantitatively because sample sizes were too small. Otoliths of males (Fig. 7m-p) are more rounded than those seen in the females (Fig. 7i-l). This explains the high variability of the measurements of the *posteroventral angle* in *A. ginaonis* (Fig. 5c), since this otolith variable depends on the overall otolith shape.

Otoliths of *Aphanius sirhani* are high-triangular in shape, with a short and slightly pointed rostrum (Fig. 7a-h). *Length/height* and *rostrum height* (Fig. 4e,h) seem to be suitable for species identification; however, one-way ANOVA exclusively supports the *length/height* relevance. *Aphanius sirhani* also displays sexual dimorphism. Otoliths of males are more slender in their dorsal portion (Fig. 7e-h) than otoliths of females (Fig. 7a-d).

CDA calculated two functions with Function 1 capturing 66.5% of the variation. The plot of the

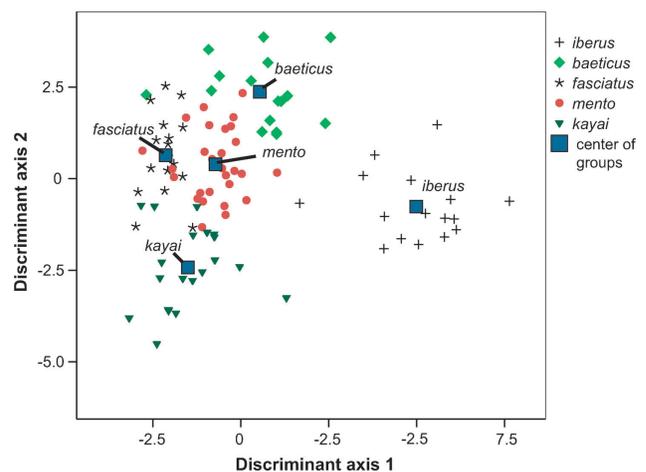


Fig. 6. Discriminant function scores for the otolith variables of the studied *Aphanius* species from the Mediterranean and Turkey (Group I otoliths).

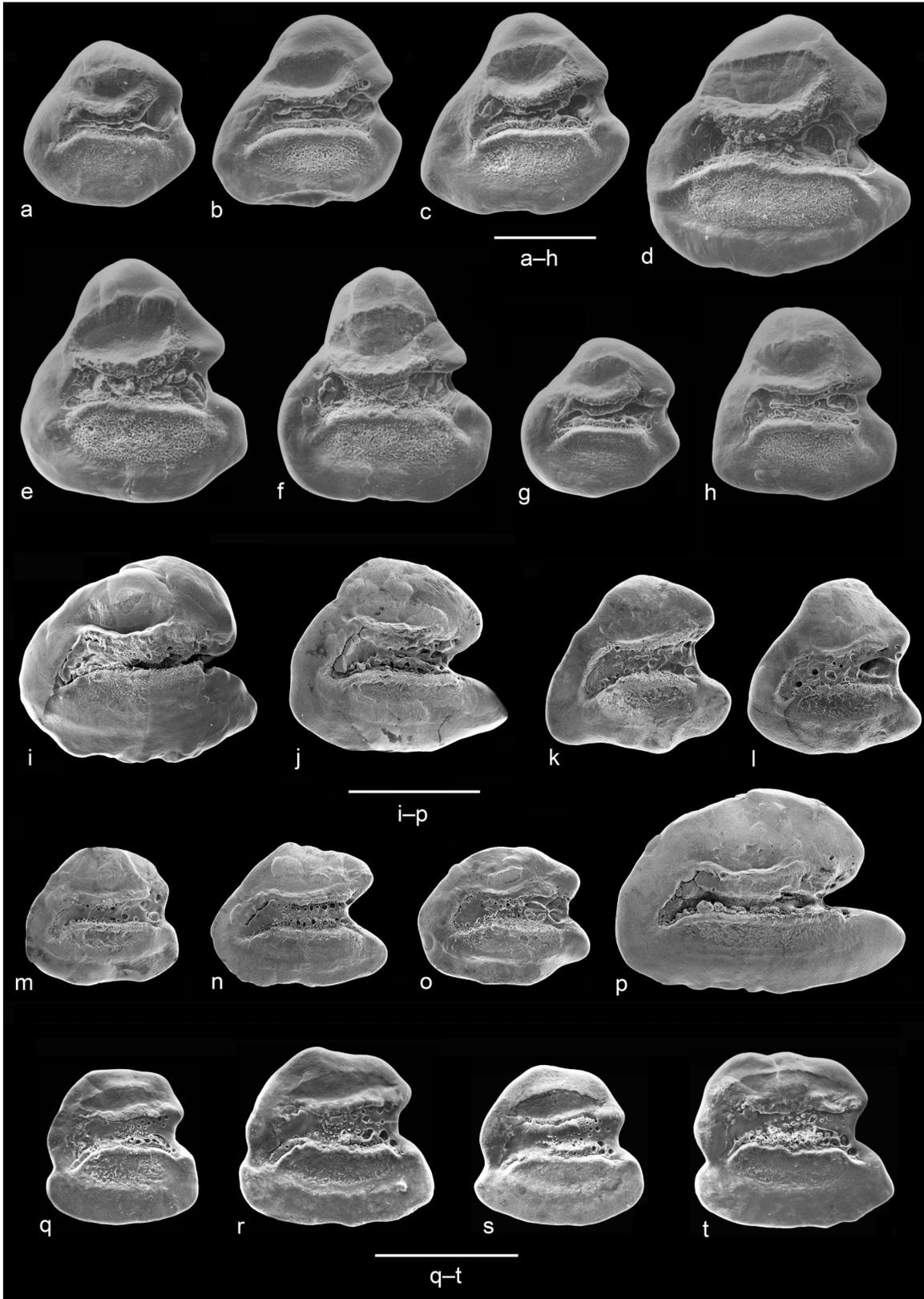


Fig. 7. Otoliths of the studied *Aphanius* species from Arabia and the Persian Gulf region. All figures are SEM micrographs of left otoliths (medial), except (i), which is a right otolith that was mirrored for better comparison. Scale bar = 0.5 mm. TL, total length in cm. BSPG 2004 II 11–30. (a–h) *Aphanius sirhani*, Azraq, Jordan (females [a–d] and males, TL 3.4, 3.8, 4.1, 5.1, 3.2, 3.5, 4.3, 4.6). (i–p) *Aphanius ginaonis*, Genu, Iran (females [i–l] and males, TL 4.1, 3.8, 3.7, 3.8, 2.5, 2.5, 2.7, 4.5). (q–t) *Aphanius dispar*, Al Khari, Saudi Arabia (females [q–r] and males, TL 2.5, 3.7, 2.8, 4.0).

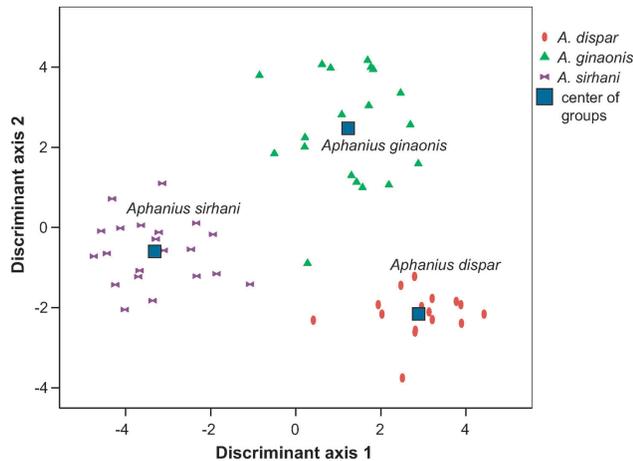


Fig. 8. Discriminant function scores for the otolith variables of the studied *Aphanius* species from Arabia and the Persian Gulf region (Group II otoliths).

discriminant function scores (Fig. 8) reveals three clusters with very little overlap, which concurs with the excellent classification success (jackknifed) for all species (96.4%, see Table 4).

Group III otoliths (s-shaped sulcus). Otoliths of †*Prolebias weileri* are characterized by a rounded-elongate shape and dorsally prolonged tip and medium-sized rostrum (Fig. 9a–d). †*P. malzi* is rounded with a small dorsal tip and a truncated, short rostrum (Fig. 9e–h). *Excisura angle*, *length/height*, *medial length*, and *rostrum length* (Fig. 5a,e,f,j) indicate the morphological differences between †*P. weileri* and †*P. malzi*. This was supported by nonparametric tests, which, moreover, determined the variable *dorsal length* as relevant.

CDA with jackknifed cross-validation achieved best overall species classification success (93.8%, Wilks' $\lambda = 0.19$) if †*Prolebias malzi* from Mainz Basin and †*P. weileri* from Schmiedruef were analysed, and “worse” (but still valuable) results if †*P. malzi* from Mainz Basin and †*P. weileri* from Illerkirchberg were used (75.0%, Wilks' $\lambda = 0.25$). CDA for all †*P. malzi* and †*P. weileri* specimens (including all populations) displays a classification success of 83.3% for †*P. malzi* and 93.5% for †*P. weileri* (Wilks' $\lambda = 0.3$).

Otoliths of all groups. CDA was successful even without preassignment of the species to groups, but discrimination power was adversely affected (Table 5). Nine functions were calculated with Function 1 capturing 66.5% of the variation and Functions 2 and 3 each confining ~18% of the variation. Species classification success (Table 5) remained equal or worsened slightly for some species, but strongly declined for *Aphanius dispar*, *A. ginaonis*, †*Prolebias malzi*, and †*P. weileri*.

Variation Between Populations

***Aphanius fasciatus*.** Otoliths from four populations (see Table 1) were compared. Otoliths from

the population in Marina di Modica (SE Sicily, Fig. 10i–l) are rounded-triangular in outline, due primarily to their rounded posterior margin and rostrum. In contrast, otoliths from the population in Sardinia (Fig. 10e–h) are narrow-triangular and have a pointed rostrum. Otoliths from the populations in the Fanjo Delta (Corsica, Fig. 10a–d) and Ganzirri (NE Sicily, Fig. 10m–p) are high-triangular and display an intermediate shape in comparison with the angular otoliths from Sardinia and rounded forms from Marina di Modica. The similarities and differences can be supported and complemented by descriptive statistics (Fig. 11), and one-way ANOVA indicates different means as follows:

- *excisura angle* for Marina di Modica vs. Sardinia otoliths,
- *posterior angle* for Ganzirri vs. Fanjo Delta otoliths,
- *posterior angle* and *posteroventral angle* for Ganzirri vs. Sardinia otoliths,
- *length/height* for Marina di Modica vs. Fanjo Delta otoliths.

CDA calculated three functions: Function 1 captures 42.4% of the variation, Function 2 confines 38.1%, and Function 3 still 19.5%. The plot of the discriminant function scores (not figured) generates a single cluster with overlap of all populations, with the exception of the Ganzirri population that is positioned slightly distant from the others. Accordingly, the population classification success is moderate (Table 6).

***Aphanius dispar*.** Otoliths from the Al Khari locality (Saudi Arabia, Fig. 12f–i) possess a slightly longer rostrum in comparison with otoliths of the same species from Faluja (Iraq, Fig. 12a–e). This is supported by descriptive statistics (*rostrum length*, Fig. 11j), which also indicate slight differences with regard to *excisura angle*, *posteroventral angle*, and *length/height* (Fig. 11a,c,e). Nonparametric statistics determines the same four variables as significant. CDA generates a classification success (jackknifed) of 92.9% for the otoliths from Faluja, and 87.5% for those from the Al Khari population (Wilks' $\lambda = 0.1$).

TABLE 4. Jackknifed classification matrix of the CDA for the Group II *Aphanius* species (bent sulcus)

n	Species	Predicted classification		
		<i>A. dispar</i> ^a	<i>A. ginaonis</i>	<i>A. sirhani</i>
16	<i>A. dispar</i> ^a	100 (16)	0	0
19	<i>A. ginaonis</i>	5.3 (1)	89.5 (17)	5.3 (1)
21	<i>A. sirhani</i>	0	0	100 (21)

Percentages in rows represent the classification into the species given in columns (correctly classified species are bold-faced); corresponding numbers of individuals are given in brackets. *n*, number of otoliths. Overall classification success is 96.4% (Wilks' $\lambda = 0.02$).

^aPopulation from Al Khari.

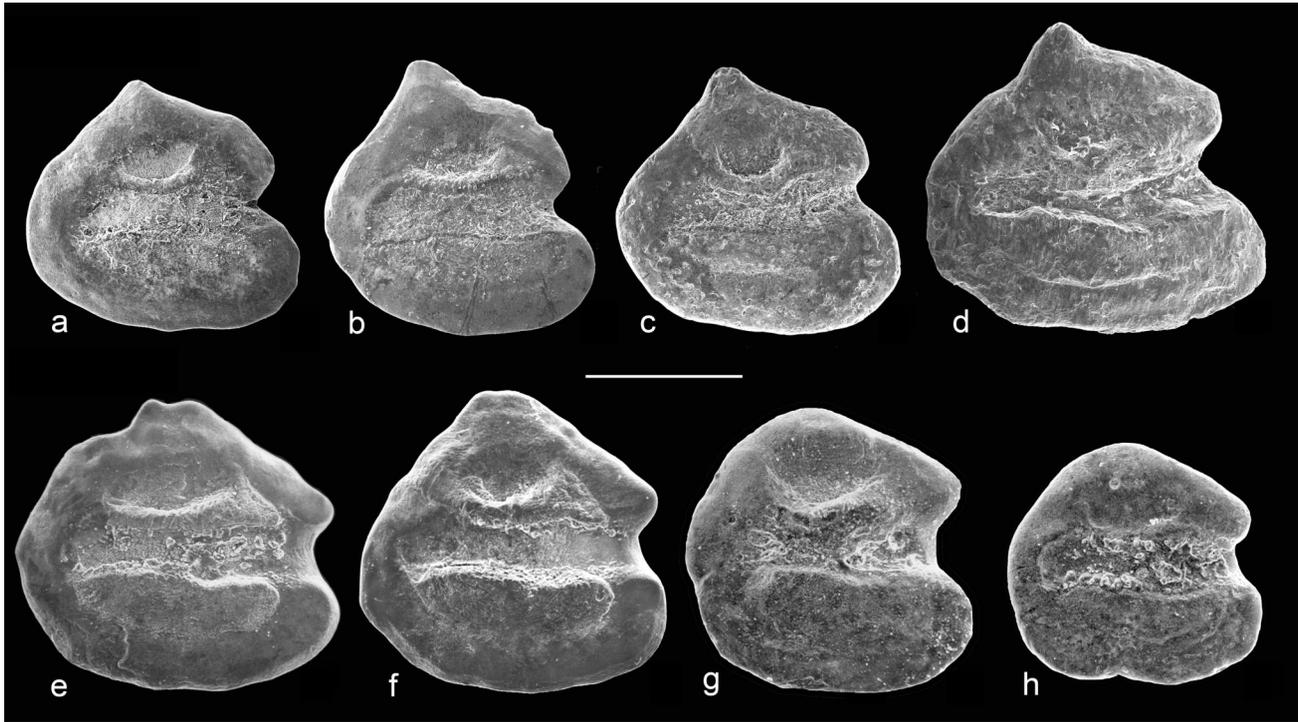


Fig. 9. Otoliths of the studied †*Prolebias* species from Germany and Switzerland. All figures are SEM micrographs of left otoliths (medial), except (a, b) which are right otoliths that were mirrored for better comparison. Scale bar = 0.5 mm. BSPG 2003 XXVIII 175-182. (a-d) †*Prolebias weileri* (~17.3-million years old). (a, b) Illerkirchberg, Germany; (c) Mauensee, Switzerland; d. Schmiedrued, Switzerland. (e, f) †*Prolebias malzi*, Hanau Basin, Germany (~22-million years old). (g, h) †*Prolebias malzi*, Mainz Basin, Germany (~23-million years old).

TABLE 5. Jackknifed classification matrix of the CDA for all species

n	Species	Predicted classification									
		Group I (straight sulcus)					Group II (bent sulcus)			Group III (S-shaped sulcus)	
		<i>A. iberus</i>	<i>A. baeticus</i>	<i>A. fasciatus</i> ^a	<i>A. mento</i>	† <i>A. kayai</i>	<i>A. dispar</i> ^b	<i>A. ginaonis</i>	<i>A. sirhani</i>	† <i>P. malzi</i> ^c	† <i>P. weileri</i> ^d
16	<i>A. iberus</i>	87.5 (14) [-6.3]	0	0	6.3 (1)	0	0	0	0	0	6.3 (1)
16	<i>A. baeticus</i>	0	87.5 (14) [-6.3]	6.3 (1)	0	0	0	6.3 (1)	0	0	0
18	<i>A. fasciatus</i> ^a	0	0	72.2 (13) [0]	27.8 (5)	0	0	0	0	0	0
28	<i>A. mento</i>	0	3.6 (1)	10.7 (3)	71.4 (20) [-7.2]	0	7.1 (2)	7.1 (2)	0	0	0
20	† <i>A. kayai</i>	0	0	5.0 (1)	5.0 (1)	90 (18) [-5]	0	0	0	0	0
16	<i>A. dispar</i> ^b	0	0	0	6.3 (1)	0	87.5 (14) [-12.5]	0	0	6.3 (1)	0
19	<i>A. ginaonis</i>	0	21.1 (4)	5.3 (1)	0	0	0	68.4 (13) [-11.1]	0	5.3 (1)	0
21	<i>A. sirhani</i>	4.8 (1)	0	0	0	0	0	0	95.2 (20) [-4.8]	0	0
18	† <i>P. malzi</i> ^c	5.6 (1)	0	0	0	0	5.6 (1)	0	0	83.3 (15) [-11.1]	5.6 (1)
14	† <i>P. weileri</i> ^d	0	0	0	7.1 (1)	7.1 (1)	0	0	0	14.3 (2)	71.4 (10) [-21.5]

Percentages in rows represent the classification into the species given in columns (correctly classified species are bold-faced); corresponding numbers of individuals are given in brackets. In angular brackets is the difference (in %) with regard to the classification success where pre-assignment to groups was included. n, number of otoliths. Overall classification success is 81.2% (Wilks' λ = 0.003).

^aPopulation from Fanjo Delta.

^bPopulation from Al Khari.

^cPopulation from Mainz Basin.

^dPopulation from Schmiedrued.

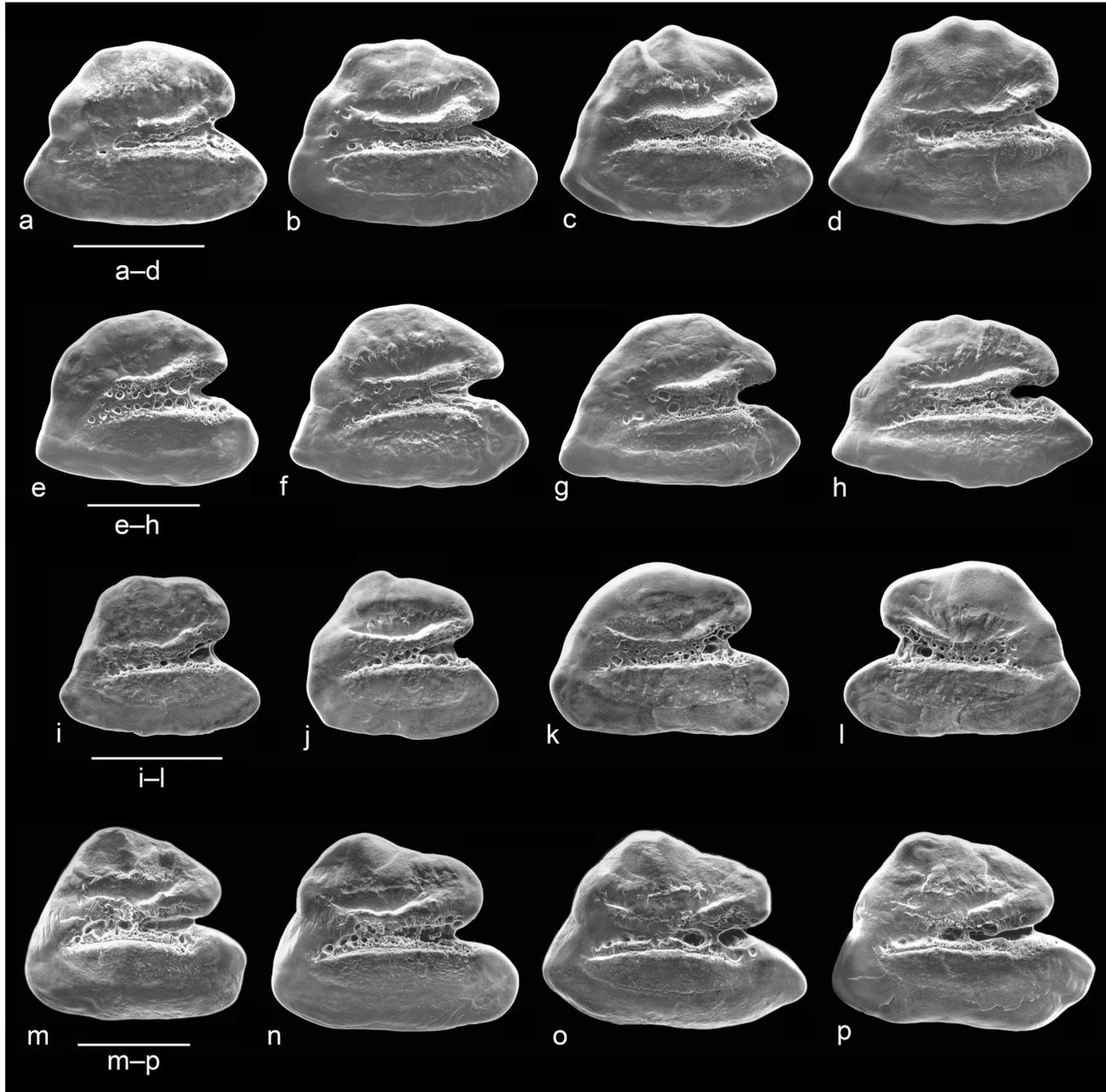


Fig. 10. Otoliths of the studied *Aphanius fasciatus* populations. All figures are SEM micrographs of left otoliths (medial), except (l) which is a right otolith. Scale bar = 0.5 mm. TL, total length in cm. BSPG 2004 II 31–46. (a–d) Fanjo Delta, Corsica (females [b–d] and male, TL 3.9, 3.7, 4.0, 4.3). (e–h) Sardinia (females, TL 4.4, 4.2, 4.7, 4.9). (i–l) Marina di Modica, Sicily (female [i] and males, TL 3.0, 3.0, 4.15). (m–p) Ganzirri, Sicily (females [n–p] and male, TL 5.4, 5.9, 5.9, 5.6).

†*Prolebias malzi*. Within †*Prolebias malzi* the main difference in otolith morphology between populations is the overall shape: Otoliths from the Hanau Basin (Germany, Fig. 9e,f) are rounded, whereas those from the Mainz Basin (Germany, Fig. 9g,h) appear more triangular. Furthermore, the antirostrum is more pronounced in otoliths from the Mainz Basin. This is supported and complemented by descriptive statistics, which indicates minor differences with regard to the pos-

terior angle, posteroventral angle, and length/height (all related to the overall shape) (Fig. 13b,c,e), as well as with regard to antirostrum height, antirostrum length, and rostrum height (Fig. 13g,i,j). However, nonparametric statistics only supports the significance of antirostrum height, antirostrum length, and rostrum height. CDA with jackknifed cross-validation reveals a classification success of 83.3% for both populations (Wilks' $\lambda = 0.2$).

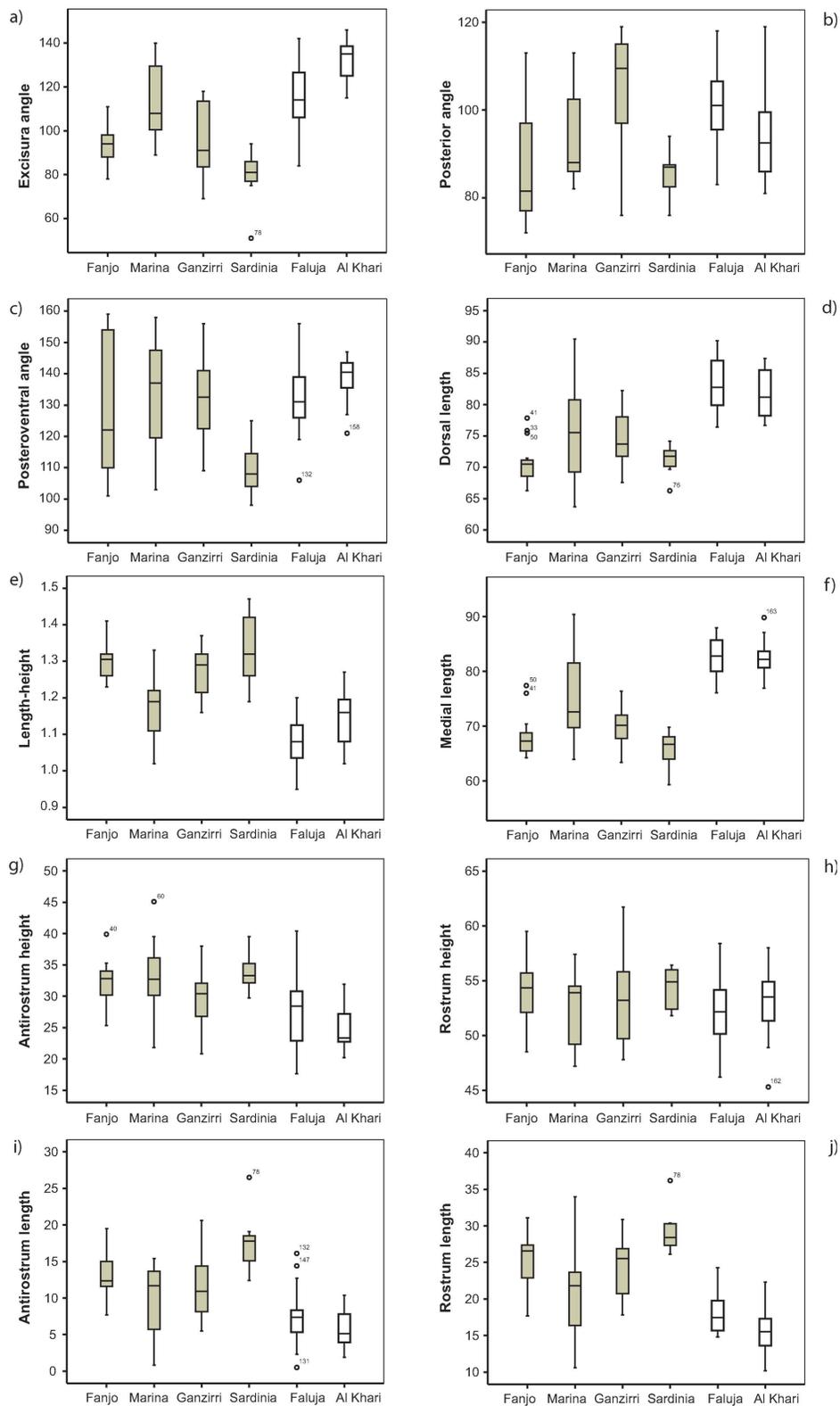


Fig. 11. Box plots showing the median (line within the box), the 25th and 75th percentiles, the data range for each otolith variable, and the variance of the respective otolith variable between the studied populations of *Aphanis fasciatus* (grey boxes) and *Aphanis dispar* (white boxes). Open circles refer to outliers within the 100th percentile.

TABLE 6. Jackknifed classification matrix of the CDA for four *Aphanius fasciatus* populations

<i>n</i>	Population	Predicted classification			
		Fanjo Delta	Marina di Modica	Ganzirri	Sardinia
18	Fanjo Delta	50.0 (9)	16.7 (3)	16.7 (3)	16.7 (3)
11	Marina di Modica	36.4 (4)	45.5 (5)	18.2 (2)	0
12	Ganzirri	8.3 (1)	0	66.7 (8)	25.0 (3)
7	Sardinia	14.3 (1)	14.3 (1)	0	71.4 (5)

Percentages in rows represent the classification into the populations given in columns (correctly classified populations are bold-faced); corresponding numbers of individuals are given in brackets. *n*, number of otoliths. Overall classification success is 56.3% (Wilks' $\lambda = 0.1$).

†*Prolebias weileri*. Macroscopical or SEM analysis of individual †*Prolebias weileri* populations have failed to yield distinct inter-population differences with regard to otolith morphology (Fig. 9a–d). Descriptive statistics indicate a slight variation of the *excisura angle* and *rostrum height* (Fig. 13a,h). One-way ANOVA provides additional support for differences in *rostrum height*. CDA generated two functions with Function 1 capturing 85.4% of the variation. Classification success with jackknifed cross-validation is 33.3% for †*P. weileri* from Illerkirchberg, 45.5% for the otoliths from Mauensee, and 64.3% for the Schmiedrued otoliths. Overall classification success (jackknifed) is 51.6% (Wilks' $\lambda = 0.2$).

DISCUSSION

Sulcus Morphology

Many previous studies on fossil and extant otoliths have demonstrated that sulcus morphology

usually is consistent between the species of a single genus (Nolf, 1985), and thus this feature is likely controlled genetically (Gauldie, 1988). However, with respect to *Aphanius* species studied here there are distinct and characteristic sulcus morphologies allowing species classifications into groups. The principal difference concerns the curvature of the sulcus (see Fig. 1), which is straight in the *Aphanius* species from the Mediterranean area and Turkey (Figs. 4, 10), but terminally bent in species from the Arabian Peninsula, Iraq, and Iran (Figs. 7, 12). Interspecific variation in sulcus morphology has previously been recorded for only a few other genera. For example, in *Merluccius* (Merlucciidae), interspecific sulcus variation separates the American from the Euro–African species, and hence sulcus variation parallels zoogeography and phylogeny (Torres et al., 2000). However, sulcus variation has been shown to concur with specialisation in hearing abilities, and thus interspe-

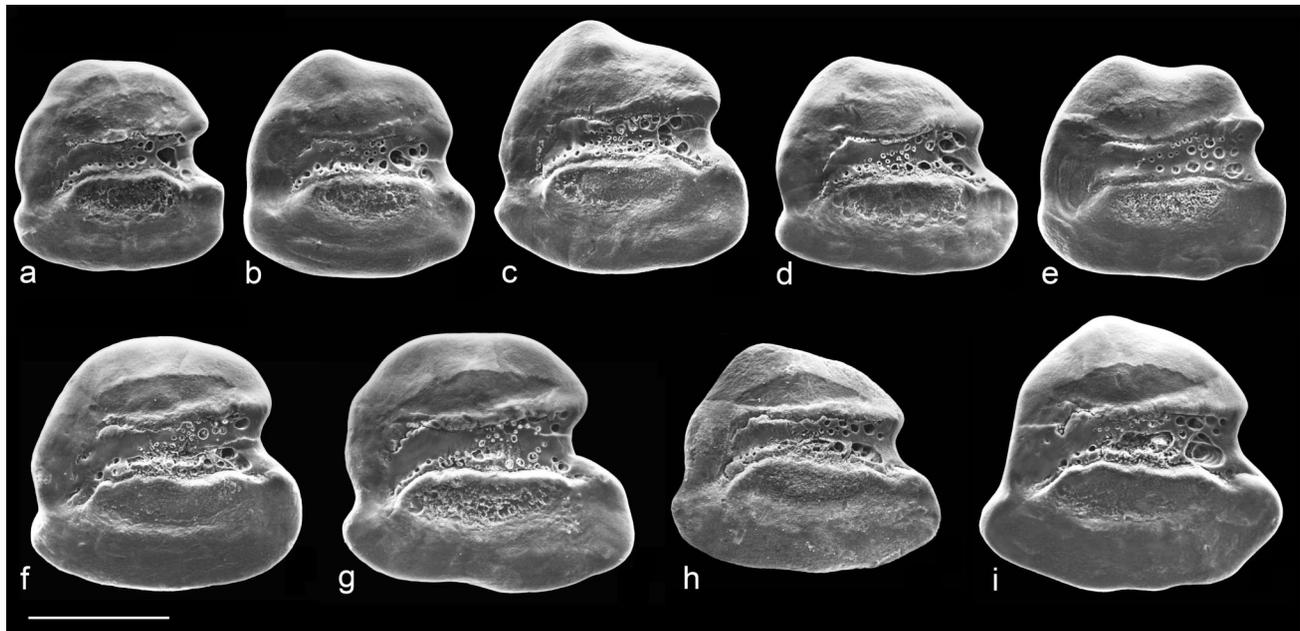


Fig. 12. Otoliths of the studied *Aphanius dispar* populations. All figures are SEM micrographs of left otoliths (medial), except (a), which is a right otolith that was mirrored for better comparison. Scale bar = 0.5 mm. TL, total length in cm. BSPG 2004 II 47–55. (a–e) Faluja, Iraq (females [d–e] and males, TL 2.6, 2.8, 3.4, 2.8, 3.2). (f–i) Al Khari, Saudi Arabia (females [h–i] and males, TL 3.7, 4.05, 3.0, 4.0).

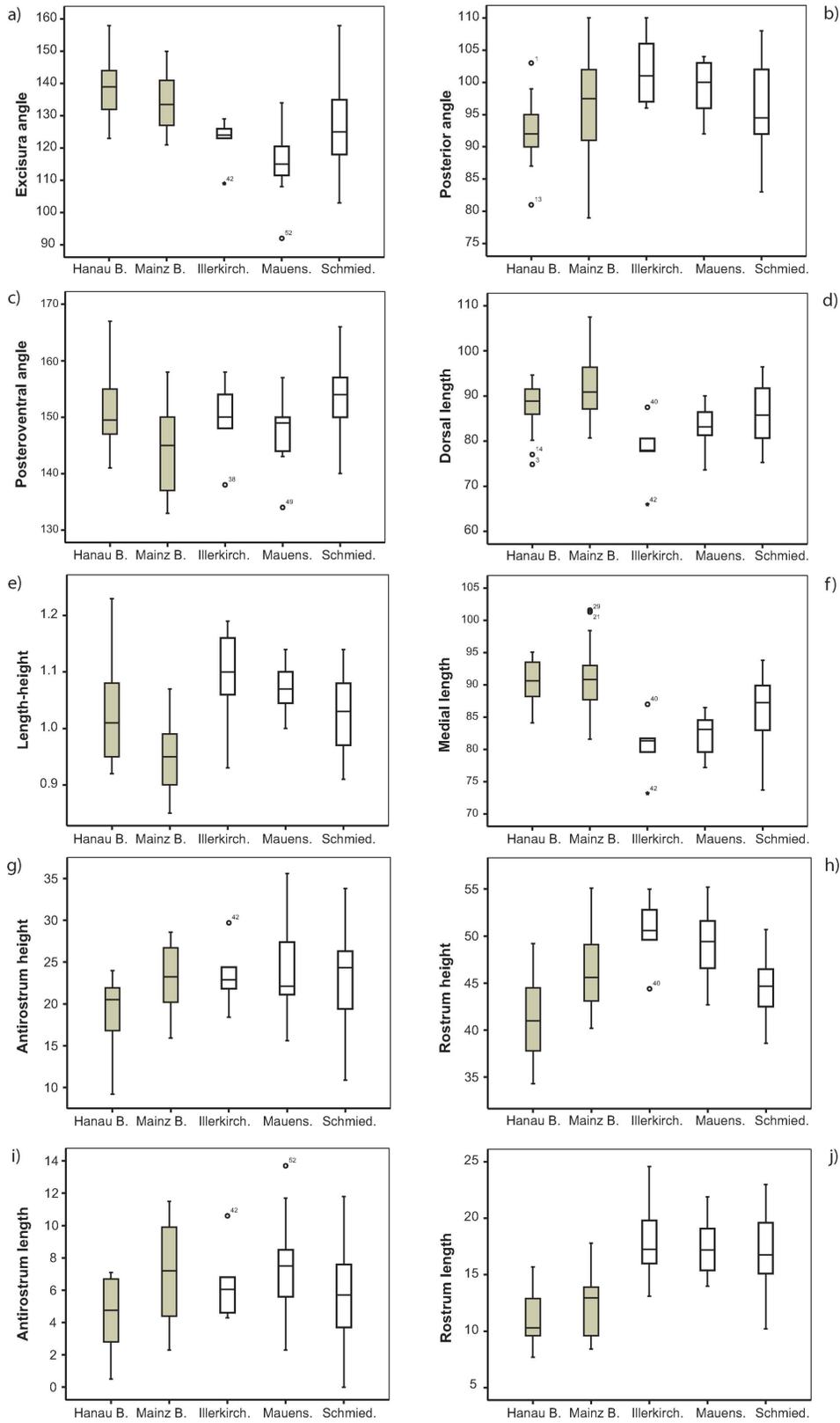


Fig. 13. Box plots showing the median (line within the box), the 25th and 75th percentiles, the data range for each otolith variable, and the variance of the respective otolith variable between the studied populations of *Prolebias malzi* (grey boxes) and *Prolebias weileri* (white boxes). Open circles refer to outliers within the 100th percentile.

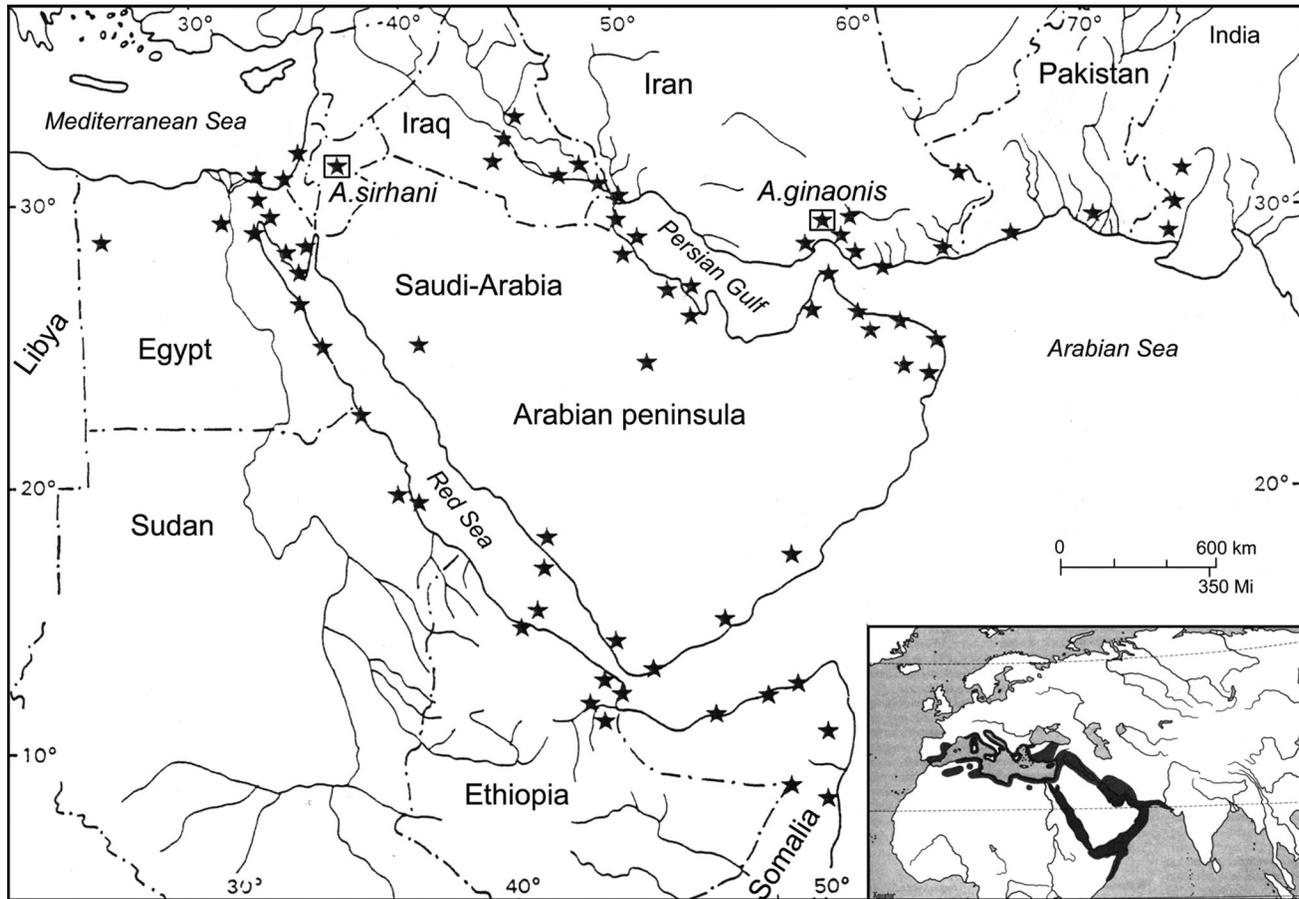


Fig. 14. The geographic distribution of *Aphanius* Group II (after Wildekamp, 1993). Stars indicate the distribution of *A. dispar*, if not mentioned otherwise. The inset indicates the general geographic distribution of *Aphanius* (after Sterba, 1990).

cific sulcus variation may also result from ecomorphological adaptations (Ramcharitar et al., 2004; Popper et al., 2005). For example, an ecomorphological influence on sulcus morphology is reflected in the ratio of the sulcus area to the total otolith area; the ratio increases in species from deeper water environments (Lombarte, 1992; Tuset et al., 2003).

This raises the question if sulcus differences between *Aphanius* species occur as a result of ecological adaptation. Most *Aphanius* species are considered as ecological generalists that tolerate highly variable habitat conditions such as changing water temperatures and salinity (Villwock, 1977, 1985; Wildekamp, 1993). This is also true in the Mediterranean *Aphanius fasciatus* (with a straight sulcus) and Arabian *A. dispar* (with a bent sulcus), which, however, appear sympatrically in the Bardawil Lagoon of the northern Sinai (Villwock, 1985). Thus, we conclude that interspecific sulcus variation in *Aphanius* species does not result from ecomorphological adaptation. Instead, zoogeography leads to a coherent picture because the geographical distribution of the Group I *Apha-*

nus species (straight sulcus) along the Mediterranean Sea and throughout Turkey does not overlap with that seen in the Group II *Aphanius* species (bent sulcus) (see Fig. 14), which inhabit the adjacent regions to the south-east, with the single exception of the Bardawil Lagoon mentioned above.

The discrimination of Groups I and II *Aphanius* species established here is also supported by molecular data (cf. Hrbek and Meyer, 2003). However, Hrbek and Meyer (2003) sorted one species from Group I, i.e., *Aphanius mento*, into the clade consisting of *A. dispar*, *A. ginaonis*, and *A. sirhani*, which represent our Group II. As a result, *A. mento* perhaps represents an intermediate form between the two *Aphanius* groups or clades. On the other hand, Hoedeman (1959) excluded *A. dispar* from *Aphanius* and transferred the species to the new genus *Aphaniops* based primarily on the absence of a genital pocket at the anterior base of the anal fin. This taxonomic separation corresponds to our observation of sulcus morphology. However, meristic data of *A. dispar*, *A. ginaonis*, and *A. sirhani* do not reveal diagnostic features that support the separate status of a

TABLE 7. Relevant otolith variables (+) in species and population discrimination selected by the canonical discriminant analysis with stepwise variable selection

	Variation between species			Variation between populations			
	<i>Aphanius</i> Group I	<i>Aphanius</i> Group II	† <i>Prolebias</i> Group III	<i>A. fasciatus</i>	<i>A. dispar</i>	† <i>P. malzi</i>	† <i>P. weileri</i>
Excisura angle				+	+		
Posterior angle			+	+			
Posteroventral angle							
Dorsal length							
Length/height	+	+				+	
Medial length					+		
Antirostrum height		+				+	
Rostrum height	+	+				+	+
Antirostrum length					+		
Rostrum length	+		+				

genus *Aphaniops* (Villwock et al, 1983; Wildekamp, 1993). Rather, thus further morphological or molecular data are required to finally resolve the status of *Aphaniops*.

Significance of the New Otolith Variables

The variable *length/height* was already considered as useful parameter for species separation in fossil otoliths by previous workers (e.g., Weiler, 1963). The other nine otolith variables presented here are new. Statistical analyses (Figs. 5, 11) indicate their different power for quantifying variation between species and populations. This is also supported when applying CDA with a stepwise variable selection (Table 7): The variables *rostrum height* and *length/height* have high separation power for *Aphanius* species, regardless of whether the species belongs to Group I or II. On the other hand, the same variables are not relevant to describe variations between species of †*Prolebias*; rather, they are important to define the variation between populations of †*P. malzi* (both variables) and †*P. weileri* (only *rostrum height*). Conversely, differences between populations of *Aphanius fasciatus* and of *A. dispar* are mainly indicated by the *excisura angle*, and this variable again has no discrimination power for species separation, neither in *Aphanius* nor †*Prolebias*.

The importance of the 10 otolith variables for quantifying variation between species and populations was additionally tested by using the k-nearest-neighbor method and the classification tree (see Hastie et al., 2003). Both methods yielded reliable results but, however, were not as successful as CDA in discriminating species and populations.

Apart from sulcus morphology, a correlation between particular otolith features (e.g., rostrum, antirostrum proportions) and biological functions such as swimming ability, feeding, or other activities has not yet been established (Popper et al., 2005). Considering the whole variety of teleost fishes there might be some correlation between the otolith

rostrum length and swimming ability (Nolf, 1985; Volpedo and Echeverría, 2003), but this feature has not been shown to be significant in the discrimination of closely related species. A study on Sciaenid fishes demonstrates that interspecific shape differences established by landmarks and contour analysis correspond with presumed phylogenetic relationships, but not with the environment (Monteiro et al., 2005). With regard to killifishes, the fact that *Aphanius* and †*Prolebias* live/lived in similar habitats (cf. Reichenbacher, 2000) argues against functional differences as an explanation for the variations in otolith morphology. We hypothesize that otolith characters and thus otolith variables among killifishes are predominantly dependent on genetic factors. As a result, we conclude that i) in closely related cyprinodontiform genera or groups (in this case *Aphanius*) particular otolith variables reflect interspecific otolith variation, whereas other variables indicate variation between populations, and ii) the diagnostic significance of individual otolith variables may vary considerably when examining a more distant phylogenetic group (like †*Prolebias*). This is important to bear in mind when assessing character variations in fossil cyprinodontiform otoliths, and perhaps in other teleost otoliths.

Intraspecific Otolith Variation

Otolith variations within a single species have previously been shown to occur as a result of i) ontogeny (Lombarte and Castellón, 1991; Monteiro et al., 2005), ii) sexual dimorphism (Morales-Nin et al., 1998), and iii) geographical and phylogenetical isolation (Torres et al., 2000; Stransky, 2005). However, all these studies focused on marine fishes. For killifishes, data on ontogenetic otolith variation have not become available to date, sexual dimorphism of otoliths is known to occur in *Aphanius iberus* and *A. apodus* (Reichenbacher and Sienknecht, 2001), and otolith variation in zoogeographically isolated populations, mainly consistent with phylogeny, has been reported for *Aphanius*

anatoliae anatoliae by Schulz-Mirbach et al. (2006).

With regard to *Aphanius dispar*, both studied populations come from similar freshwater environments located in a semiarid region with unstable water temperature and water chemistry (see Wildekamp, 1993). Thus, distinct ecological conditions or different environments for each population cannot have affected otolith morphology. The most likely reason for this otolith variation is zoogeographic isolation (see Fig. 2) and allopatric divergence. Regarding the two †*Prolebias malzi* populations, no geographic separation occurs (see Fig. 2), and a corresponding shallow brackish water environment in semiarid conditions has been suggested for both taxa (Reichenbacher, 2000). However, the populations are separated by a 1-million-year age difference (see Table 1). We suggest that otolith characters were gradually deviating in time and that different phylogenetic age was the primary factor leading to otolith variation in *P. malzi*.

Otolith variations in the studied populations of *Aphanius fasciatus* and †*Prolebias weileri* are less significant, especially in comparison to *A. dispar* and †*P. malzi*. This is consistent with the (palaeo) zoogeography (see Fig. 2) since geographic isolation does not occur. Moreover, the populations are of more or less the same age (see Table 1 for references). Studies on the genetic divergence of *A. fasciatus* have recorded high genetic divergence in some, but not all *A. fasciatus* populations from the Mediterranean Sea (Maltagliati, 1999; Maltagliati et al., 2003; Tigano et al., 2006). The here analyzed *A. fasciatus* population from Sardinia possesses a certain individuality because the otoliths are more angular (Fig. 10e–h), which is confirmed by a comparatively low misclassification rate of these fishes (see Table 6). Thus, *A. fasciatus* from Sardinia may belong to such a genetically divergent population.

Combined Otolith Morphology and Morphometry—Conclusion

Fourier shape analysis and landmarks represent highly successful tools for studying otolith variation in extant fishes (e.g., Parisi-Baradad et al., 2005; Ponton, 2006; Stransky and MacLellan, 2005). However, these methods have not been applied to fossil otoliths to date, due probably to preservation-related problems and limited numbers of fossils of similar sizes. As a result, taxonomic discrimination of fossil otoliths is sometimes regarded as suspicious since it depends on subjective criteria and missing quantification (Lombarte and Castellón, 1991). The present study shows that descriptive criteria such as sulcus morphology can be combined with the quantification of otolith variables. The use of “classical” otolith descriptions combined with morphometry and multivariate

analysis represents a new approach that provides greater and more detailed information on fossil teleost diversity and related topics on palaeoecology, palaeozoogeography, and palaeogeography. Although this study is restricted to otoliths of cyprinodontiforms, we assume that the presented combined approach can be equally successful applied to other teleost otoliths, especially atheriniform and many perciform otoliths, in which the general otolith Bauplan is similar to that seen in cyprinodontiforms.

ACKNOWLEDGMENTS

Wolfgang Villwock (Hamburg University), Ruud Wildekamp (Gemert, Netherlands), and Concetta Tigano (Catania, Italy) provided the samples of the extant *Aphanius* species. Four additional specimens of *Aphanius baeticus* from aquarium stocks were provided by Heiko Kaerst (Gaggenau). August Ilg (Düsseldorf) helped with the production of the Figure 1 and Ruud Wildekamp allowed us to use his map for the Figure 14. Volker Zibat and Renate Preiss (both Technical University Karlsruhe) assisted with SEM and reprints of photographs. Roland Melzer (Zoological State Collection Munich) helped with digital SEM. Christian Rink and Caroline Stahl (both Ludwig-Maximilians-University, Munich) contributed to the statistical analyses. To all, we offer our sincere thanks. Many thanks go also to Michael Krings (Palaeontological State Collection Munich), Alexander Altenbach and Tanja Schulz-Mirbach (both Ludwig-Maximilians-University, Munich) for constructive discussions. Finally we thank Dirk Nolf and an anonymous reviewer, as well as the editor of the Journal of Morphology, Frederick W. Harrison, for their constructive remarks.

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