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Genetic and Morphological Characterization of a Newly Found *Aphanius* Population (Cyprinodontidae) from Kaklik, Turkey, and of Populations from Lakes Aci, Burdur and Salda

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Abstract

Turkey is the hotspot for *Aphanius*, a cyprinodont genus, occurring in brackish and freshwater around the Mediterranean and in the Near East. Especially a region in the southwest of Turkey, also called the Lakes District, is known for its high diversity in this taxon. To characterize a newly found *Aphanius* population in this region, we studied genetic and morphological variation in six populations. A total number of 209 specimens was included in this study, comprising the following species: *A. anatoliae* and *A. splendens*, Lake Salda, *A. sureyanus*, Lake Burdur, *A. transgrediens*, Lake Aci, *A. maeandricus*, Işıklı spring, and *A.* sp. "Kaklik cave", Kaklik. Four different methods were used: six microsatellite markers, a mitochondrial marker encoding the d-loop including four out-group species, Amplified Fragment Length Polymorphism (AFLP) and geometric morphometrics for analysing photographs of each specimen. All methods show that the new population in Kaklik is distinct from the Lakes District populations, but genetically related to *A. maeandricus*. The high number of fixed private fragments within the AFLP method in *A. transgrediens* suggests that this population is isolated, but is close to *A. sureyanus*, representing the geographic situation. The populations in Lakes Salda and Burdur exhibit a complex relationship: mitochondrial haplotypes of *A. anatoliae* were found in *A. splendens* and *sureyanus*, even though *A. sureyanus* occurs isolated as only species in Lake Burdur. Due to the number of fixed private fragments in *A. anatoliae*, we suggest that this population was the ancestral population. *Aphanius splendens* occurs syntopically with *A. anatoliae*, but genetic results suggest two separated gene pools.

Keywords: Anatolia, amplified fragment length polymorphism, biogeography, geometric morphometric, microsatellite, mitochondrial marker

Zusammenfassung

Die Türkei ist Hotspot für Arten der Gattung *Aphanius* (Cyprinodontidae), die im Süß- und Brackwasser rund um das Mittelmeer, als auch im Nahen Osten vorkommt. Im Besonderen der Seendistrikt, eine Seenlandschaft im Südwesten der Türkei, ist bekannt für die hohe Diversität. Um eine neuentdeckte *Aphanius*-Population zu charakterisieren, haben wir sowohl genetische als auch morphologische Variation in sechs Populationen untersucht. Insgesamt wurden 209 Individuen folgender Arten miteinbezogen: *A. anatoliae* und *A. splendens*, Saldasee, *A. sureyanus*, Burdursee, *A. transgrediens*, Lake Aci, *A. maeandricus*, Quelle in Işıklı, und *A.* sp. "Kaklik cave", Kaklik. Im

Ganzen wurden vier Methoden verwendet: sechs Mikrosatelliten, der D-loop als mitochondrialer Marker, Amplified Fragment Length Polymorphism (AFLP) und eine geometrisch-morphometrische Methode, um Lateralbilder der Fische zu analysieren. Alle Methoden zeigten, dass sich die Kaklik population von den Populationen im Seendistrikt abhebt und mit *A. maeandricus* näher verwandt ist. Die hohe Anzahl von fixierten privaten Fragmenten in *A. transgrediens* zeigt, dass diese Population von den anderen Seen getrennt ist, aber *A. sureyanus* am nächsten steht, was der geografischen Ausgangslage entspricht. Die Verwandtschaft der Populationen im Salda- und Burdursee ist komplex: mitochondriale Haplotypen von *A. anatoliae* können in beiden anderen Populationen gefunden werden, obwohl die Burdurpopulation isoliert ist. Aus der Anzahl der fixierten privaten Fragmente von *A. anatoliae* schließen wir, dass diese Population der Vorfahre gewesen sein könnte. *Aphanius splendens* lebt syntop im gleichen See wie *A. anatoliae*, die genetischen Ergebnisse deuten jedoch auf zwei getrennte Genpools hin.

Introduction

The killifish genus *Aphanius* is a member of the family Cyprinodontidae, which is part of the toothcarps (Cyprinodontiformes), and encompasses numerous species (Nelson, 2006). They recently occur in brackish and freshwater habitats around the Mediterranean Sea revealing the former extent of the Tethys Sea across Europe and the Near East. Due to the closing of the Tethys 25 to 5 Million years ago (Mya) and the accompanying geological changes, the initial ancestor species, which was distributed all over the Mediterranean area, got split (Hrbek et al., 2002). The ancestral *Aphanius* population evolved an eastern and a western branch through vicariance (Hrbek and Meyer, 2003) during the Messinian salinity crisis, 6 Mya (Krijgsman et al., 1999), when the Mediterranean desiccated. The recent Anatolian species developed from the western branch (Hrbek and Meyer, 2003).

During the later Tethys Sea period the landmass, which formed modern-day Anatolia, was separated into a southern and a northern part (Hrbek et al., 2004). Thus, the colonization of Turkey by *Aphanius* was initialized from East to West, because water sheds were closed gradually by the Arabian plate and the Alanya massif, which slowly moved northwards (Quennell and Waldron in Hrbek et al. 2002). In consequence of the closing, many disconnected water bodies were formed, where separated populations developed specific characteristics. For that reason, Turkey is the hotspot for speciation in the genus *Aphanius* (Hrbek et al., 2002, Wildekamp et al., 1999). Nevertheless, all *Aphanius* species in Turkey are monophyletic (Hrbek and Meyer, 2003, Parker and Kornfield, 1995). All recent populations can be classified into six clades and *A. asquamatus* (Sözer, 1942) as a solitary endemic species in Lake Hacer in the east of Turkey. However, *A. anatoliae* (Leidenfrost, 1912) has the widest distribution of all Anatolian species and comprises different eco- and phenotypes (Hrbek et al. 2002, Hrbek and Meyer, 2003). Four *A.* cf. *anatoliae* clades, including the populations of the Lakes District, are mainly concentrated to the western and southern part of the land, whereas two *A. danfordii* (Boulenger, 1890) clades can be found in middle and northern Anatolia (Bardakci et al., 2004, Hrbek et al. 2002, Wildekamp et al., 1999). From the upper Sakarya River basin, in the northwest of Anatolia, a new *Aphanius* species, *A. villwocki* was described, which is a sister taxon to *A. anatoliae* and *danfordii* (Hrbek and Wildekamp, 2003).

Our research addresses the Lakes District, where a remarkably high number of *Aphanius* populations occurs. This Anatolian region encompasses Lakes Salda, Burdur and Aci, characterized as variable habitats with highly alkaline water parameters. Originally all these populations were described as subspecies of *A. anatoliae* (Akşiray, 1948; Wildekamp et al., 1999), however, no subspecies term will be used in this study, due to the recent isolation and the beginning process of speciation (species "*in statu nascendi*", Grimm, 1979; Villwock, 2004). Populations of *A.* cf. *anatoliae* and *A. splendens* are present in Lake Salda (Schulz-Mirbach et al., 2006), which contains a high amount of magnesium carbonate ($MgHCO_3$). A macro-population (Kosswig, 1956) of *A. sureyanus* is located in the brackish

Lake Burdur (Villwock, 1964). Lake Aci is a hypersaline sulfurous lake, surrounded by twelve tributaries, where diverse populations of *A. transgrediens* were seasonally separated (Grimm, 1979). According to Hrbek and Meyer (2003), only two tributaries were inhabited by *A. transgrediens* in 2002, due to the introduction of the competitive fish species *Gambusia affinis*. Nevertheless, no lake has an outflow system (Villwock, 1964). Several of these populations developed specific limnetic phenotypes, therefore, they were classified as distinct subspecies of *A.* cf. *anatoliae* (Grimm, 1980). Based on specific distinctions, which mainly encompass a high variability in the degree of squamation (Akşiray, 1948, Grimm, 1979) and successful cross-breeding experiments between all Lakes District populations (Villwock, 1964), the new genus *Anatolichthys* was erected to classify the populations of this region (Kosswig and Sözer, 1945). However, Villwock (1964) showed with his cross-breeding experiments and the resulting fertility in males that this genus was equal to the genus *Aphanius*. The number of scales and the number and shape of teeth seem to be an unstable trait for morphological studies within this genus (Villwock 1964, Wildekamp et al., 1999). The genetic differentiation of the Lakes District clade was estimated to date back to 7.5 ± 0.5 Mya (Hrbek and Meyer, 2003). However, during the Pleistocene, 2.5 Mya until 10.000 years ago, Lake Aci and Lake Burdur were still connected (Kosswig, 1953) through the Burdur basin (Louis, 1938), which includes Lake Salda, located farther south. Thus, in less than 20.000 years a separation of different populations emerged (Kosswig, 1953). *Aphanius splendens* in Lake Salda, *A. sureyanus* in Lake Burdur and *A. transgrediens* in Lake Aci are endemic species (Güçlü et al., 2007; Güçlü and Küçük, 2012).

Even today, new *Aphanius* populations can be discovered in Turkey. The Kaklik cave population was newly found by Prof. Dr. Füsun Erk'Akan (exact date missing) and firstly mentioned in Ulcay et al. (2012) and Güçlü et al. (2013) classified it as subpopulation of *A. anatoliae* from the Menderez River system. In this study the by now undescribed population in Kaklik was morphologically and genetically characterized and compared to *Aphanius* populations of the Lakes District and *A. maeandricus*, which was originally described from Menderez River (Akşiray, 1948). The populations in the Lakes District and the Menderez River formed two monophyletic *A. anatoliae* clades in Hrbek et al. (2002) and in Hrbek and Meyer (2003). Following questions were addressed to the data: How closely is the Kaklik population related to the populations of the Lakes District? How can the relationships between the other populations be characterized? A comparison of three genetic (mitochondrial, microsatellites and amplified fragment length polymorphism) DNA-fingerprinting techniques and a geometric morphometric method were used to reveal the relationship between the sampled populations. For mitochondrial analysis the non-coding D-loop region was chosen, due to its fast evolutionary rate (Upholt and Dawid, 1977), wherefore, this region was often used in phylogenetic studies.

Materials and Methods

Sampling and DNA extraction

We sampled in total 200 fish at four locations (40 samples per site) in the southwest of Turkey comprising five different populations: *Aphanius* cf. *anatoliae* (which will be simply called *A. anatoliae* hereafter) and *A. splendens* (co-occurring in Lake Salda), *A. sureyanus* (juvenile fish only; Lake Burdur), *A. transgrediens* (Lake Aci) and *A.* sp. *"Kaklik cave"* (Kaklik cave) (Table 1 and map in Figure 1). The fish were caught with seine nets at the beginning of October 2012 in course of a conservation project, realized by the Faruk Yalçin Zoo Istanbul, Turkey. The GPS-Data were taken with a Garmin GPSMAP 78s. Furthermore, each fish's left side was photographed for geometric morphometric analysis. For genetic samples small pieces (~ 20 mm^2) of the caudal fin were taken from all sampled specimens with dissecting scissors and were stored in absolute ethanol. Afterwards, all fish were immediately released at their native sampling location. Additionally, Dr. Jörg Freyhof supplied five fin samples of *Aphanius maeandricus* of Işıklı spring for comparison. All genomic DNA was extracted following a standard phenol-chloroform-extraction-protocol (Sambrook et al., 1989). Digestion was performed with Proteinase K.

Table 1: Collection sites with coordinates and specific sampling locations.

Sample site	Populations	Coordinates	Local sampling spot	[m] above sea level
Lake Salda	*Aphanius anatoliae*, *A. splendens*	N 37°31.816' E 29°39.462'	Both species were found at a shore in the southwest of the lake next to a stream outlet	1145
Lake Burdur	*A. sureyanus*	N 37°69.455' E 30°19.012'	Southeast coast; spot was located on the left side of a landing stage.	855
Lake Aci	*A. transgrediens*	N 37°82.450' E 29°93.972'	Tahtaköprü Spring (Grimm, 1979) on the southeast side; high number of *Gambusia* cf. *affinis*	833
Kaklik Cave	*A.* sp. "Kaklik cave"	N 37°51.365' E 029°23.166'	Artificial channel draining a small pond next to a parking area	514

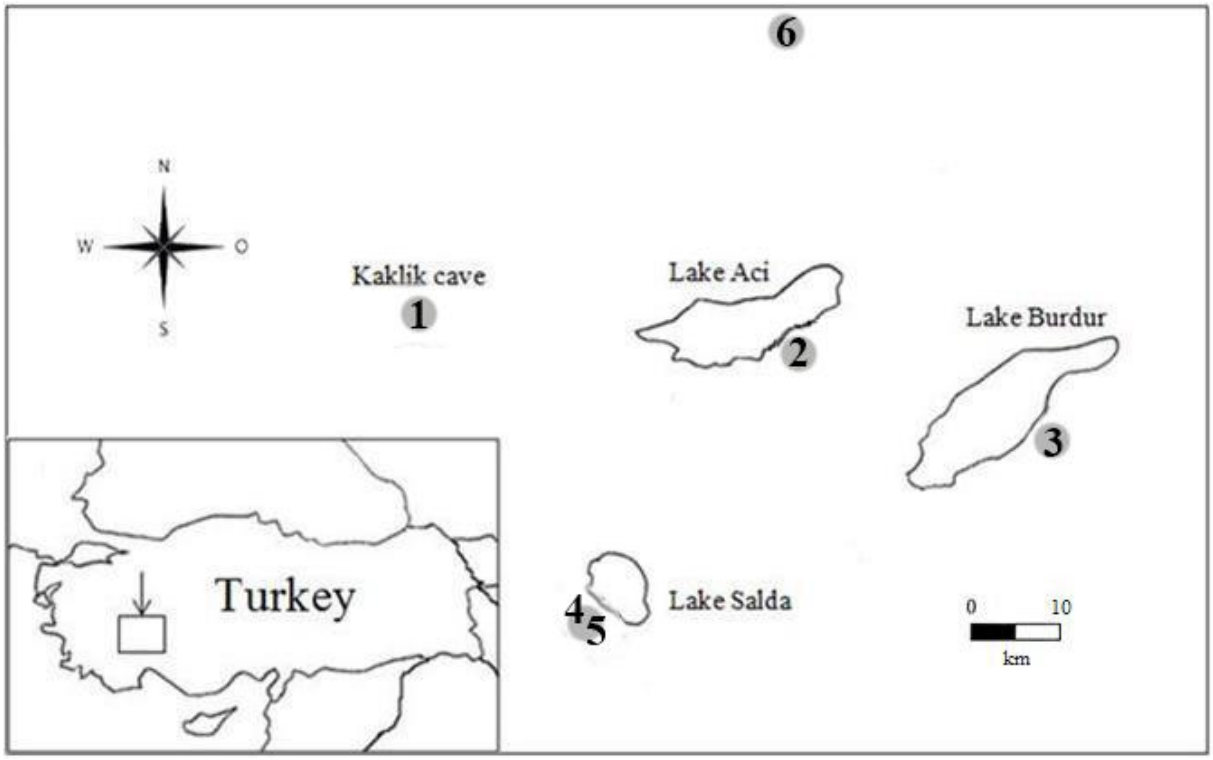

Figure 1: Sketch map of the sampling sites and their distances to each other in the southwest of Turkey. The symbols describe approximately the locations, where the fish were caught. 1 = *Aphanius* sp. "Kaklik cave", 2 = *A. transgrediens*, 3 = *A. suyreanus*, 4 = *A. anatoliae*, 5 = *A. splendens*, 6 = Işıklı (source of Menderez River; first describtion of *A. maeandricus* (Akşiray, 1948))

Microsatellite genotyping

All individuals were genotyped at six microsatellite loci, originally published in Babbucci et al. (2007) and Vogiatzi et al. (2009). Initially, the microsatellite analysis was conducted by using 14 loci, but only six of them were sufficiently amplified within this species complex. Polymerase chain reactions (PCR) were performed with some adjustments concerning the annealing temperature (see Table 10). We used reverse primers that were labelled with the fluorescent dyes FAM, HEX, TET and NED (different companies, see Table 10). All microsatellites were amplified in 10 µl reaction volumes composed of 10 ng genomic DNA, 0.2 mM of each dNTP, 1 µM of each forward and reverse primer, 0.5 U BioTaq Polymerase (Axon) and 1 µl of 10x NH_4 reaction buffer (Axon), at a final concentration of 1.5 mM $MgCl_2$ (Ringler et al., 2013). The PCR program was performed with an initial denaturation step at 95 °C for 8 min followed by 38 cycles beginning at 95 °C for 45 s, continuing at the specific annealing temperature (see Table 10) for 45 s and final elongation at 72 °C for 45 s. The different primer dyes and sizes of the PCR products, which were diluted first 1:25, enabled us to merge multiple loci into different sets. HiDi-Formamid (Applied Biosystems) and the internal size standard GS500 were added and the product was performed on an ABI 3130xl sequencer. Alleles were automatically binned using TANDEM (Matschiner and Salzburger, 2009). Data were analyzed using PeakScanner 1.0 (Applied Biosystems), CERVUS (Kalinowski et al., 2007), GenAlEx 6.501 (Peakall and Smouse,

2006, 2012) and FSTAT (Goudet, 2002) for F-statistics. A Principal Coordinate Analysis (PCoA) was performed in PAST 3.10 (Hammer et al., 2001). Dice was chosen as similarity index. Locus Af18 gave multiple peaks on the electropherogram, so that we assumed that its primer probably bind on at least two different loci within the genome. For that reason, we excluded Af18 from analysis.

D-loop

Additionally, we sequenced all individuals at the mitochondrial section "D-loop" (Lee et al., 1995 Table 2). To process this mitochondrial marker, the PCR protocol was performed with an initial denaturation at 94°C for five minutes and was followed by 35 cycles starting at 94°C for 45 sec, then shifting to 48°C for 45 sec and afterwards to 72°C for one minute for elongation. The lab protocol was similar to the microsatellites with the exception that all amounts of reagents were doubled to get a sufficient amount of PCR product. Sequencing was performed by Microsynth AG. Because of a sequencing problem, sequences were shortened to a length of 123 base pairs. Data analysis was conducted with BioEdit (Hall, 1999) and MEGA 5 (Tamura et al., 2011). Because of the similarity of sequences, only a subset of specimens, including one sample per population, which represented the most abundant sequence of each population, was used to construct a dendrogram. Further four out-groups were included from NCBI GenBank (*A. dispar*: U06052.1, *A. mento*: U06054.1, *A. fasciatus*: U06053.1 and *A. chantrei*: U06062.1; Parker and Kornfield, 1995). The mitochondrial sequence data were used to construct a statistical parsimony network in TCS (Clement et al., 2000) to estimate the genealogical relationships between mtDNA haplotypes of all included populations. Nucleotide and haplotype diversity was calculated by the use of DnaSP 5.10 (Librado and Rozas, 2009).

Table 2: Mitochondrial locus. T_a, annealing temperature;

Locus	Primer sequence	T_a (°C)
AE	F: TTCCACCTCTAACTCCCAAAGCTAG	48
	R: CCTGAAGTAGGAACCAGATC	

AFLP – Amplified Fragment Length Polymorphism

Quantity of DNA per sample was tested with a NanoDrop spectrophotometer 2000c (peqlab). For the AFLP-method (Vos et al., 1995) only 16 samples per population with the highest DNA amount (> 70 ng) were included, except for *A. maeandricus*, of which all specimens were examined. In addition, genetic samples of further four fish, fixated in 70% ethanol, from the Kaklik population of an earlier excursion in 2010 were included.

For digestion with the enzymes MseI (New England Biolabs) and EcoRI (Promega) and for ligation (T4-ligase; Promega) of specific double-stranded adapters, three µl of genomic DNA were used during a restriction-ligation-reaction at 37°C for three hours. Preselective PCR was performed with MseI + C and EcoRI + A primers (Eurofins MWG Operon) and for Selective PCR three primer pairs were utilized, which were marked with different fluorescent dyes (see Table 3).

Table 3: AFLP primer pairs with additional selective nucleotides and dyes for the Selective PCR.

EcoRI primer	Fluorescent dye	Company	MseI primer	Company
- AT	FAM	Life Technologies™	- CTC	Eurofins MWG Operon
- AA	NED	Life Technologies™	- CAG	Eurofins MWG Operon
- AC	VIC	Life Technologies™	- CAA	Eurofins MWG Operon

All reactions were performed on a GeneAmp PCR System 9700 thermal cycler (PE Applied Biosystems) following the protocol of Paun and Schönswetter (2012) with modifications (see supplementary). The PCR products of the three primer pairs were merged and sequencing was implemented with a MegaBACE 1000 sequencer.

The program DAx (Van Mierlo Software Consultancy; Eindhoven, the Netherlands) was used to create binning sheets for each dye. Due to alignment difficulties, which affected the locating of the size standard, the Lakes District data were scored separately from the Kaklik and the *A. maeandricus* populations (dataset A and B in results Table 10). Fragment peaks between 100 and 570 base pairs were taken into account. A number of 15 individuals, which were not calibrated correctly, was excluded. Further analyses were performed with an exported presence/absence matrix. An error rate for each dataset was calculated by dividing the number of errors between replicates (in dataset A 22 and in B two) and the original data by the total number of characters.

The neighbor-joining analysis in TreeCon (Van de Peer and De Wachter, 1994) was based on Nei and Li's (1979) mathematical model of genetic variance. Bootstrapping was used with 1000 replicates and the tree was rooted with an individual of *A. maeandricus*. Because of highly similar electropherograms within each population, two individuals per population were chosen and scored together to build a dendrogram. To estimate the gene diversity (Nei, 1973), which is the mean proportion of pairwise differences of individuals, an AFLP package (AFLP.dat, Ehrich, 2006) in R (RDC Team, 2005) was applied and an AMOVA (analysis of molecular variance, Excoffier et al., 1992) was conducted by using the program Arlequin 3.5 (Excoffier and Lischer, 2010). A Principal Coordinate Analysis (PCoA) was performed in PAST 3.10 with *A. anatoliae*, *splendens* and *sureyanus*.

Population structure was estimated by the program STRUCTURE (Pritchard et al., 2000). A burnin of 200.000 and 500.000 Markov-Chain-Monte-Carlo repeats were set and an admixture model was used with correlated allele frequencies among populations. The suggested number of populations K was preadjusted for testing for one to four groups by repeating each group test ten times. The best result was chosen depending on the highest likelihood.

Geometric Morphometrics

Pictures of all specimens' left side were taken on scaled paper. Further four fish from the Kaklik population (excursion 2010) were photographed and added, yielding a total sample size of 204 fish. To assess the differences in shape, a method using discrete fixed landmarks was applied (Bookstein, 1997). The software tpsDig2 (Rohlf, 2008) was used to set 19 landmarks following Herler et al. (2010) (Table 4). For data analysis, R in combination with the geomorph package (Adams and Otárola-Castillo, 2013) was applied. All specimens' landmarks were superimposed by a Generalized Procrustes Analysis (GPA).

Furthermore, a Principal Component Analysis (PCA) was applied in R and principal components one and two, two and three and three and four were correlated and plotted, also showing thin-plate splines grids for two samples with the maximal distance in variation in shape within the principal component of the horizontal axis of the plot. In addition, equal-frequency ellipses (Rau and Fassuliotis, 1970) were applied to the PCA plot to improve the visual distinction of each population by representing 95% of all individuals per population.

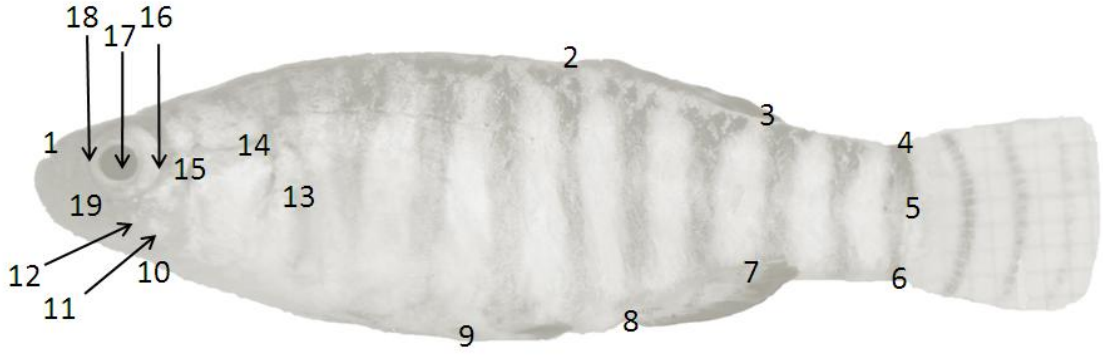

Figure 2: Setting of the 19 geometric morphometric landmarks.

Table 4: Description of each landmark (Herler et al., 2010).

No. Landmark	Description
1	Anterior tip of snout
2	Anterior insertion of dorsal fin
3	Posterior insertion of dorsal fin
4	Upper insertion of caudal fin
5	Midpoint of the origin of caudal fin
6	Lower insertion of caudal fin
7	Posterior insertion of anal fin
8	Anterior insertion of anal fin
9	Insertion of ventral fin
10	Anteroventral tip of pectoral girdle (cleithrum)
11	Ventral-most point of the border between interopercle and subopercle
12	The point where preopercle, interoperculum and subopercle meet
13	Upper insertion of pectoral fin
14	Dorsal origin of operculum
15	Dorsal end of preopercular groove
16	Posterior rim of orbit along the horizontal body axis
17	Centre of orbit
18	Anterior rim of orbit along the horizontal body axis
19	Posterior-most point of lips

Results

D-loop

The statistical parsimony network (Figure 3) showed three clusters: haplotypes of the Kaklik population clustered with *A. maeandricus* with a difference of two base pairs. These haplotypes were connected through eight steps (<90% connection certitude) with *A. transgrediens*. After further five base pairs (<90% certitude) *A. anatoliae, splendens* and *sureyanus* arranged into a group. Within this cluster, haplotypes of *A. splendens* and *sureyanus* divided into separated subgroups, mirroring Lake Salda and Burdur. A single haplotype of *A. anatoliae*, present in a high number of individuals, was similar to one haplotype of *A. sureyanus*, but other haplotypes were also found within the *A. splendens* subgroup.

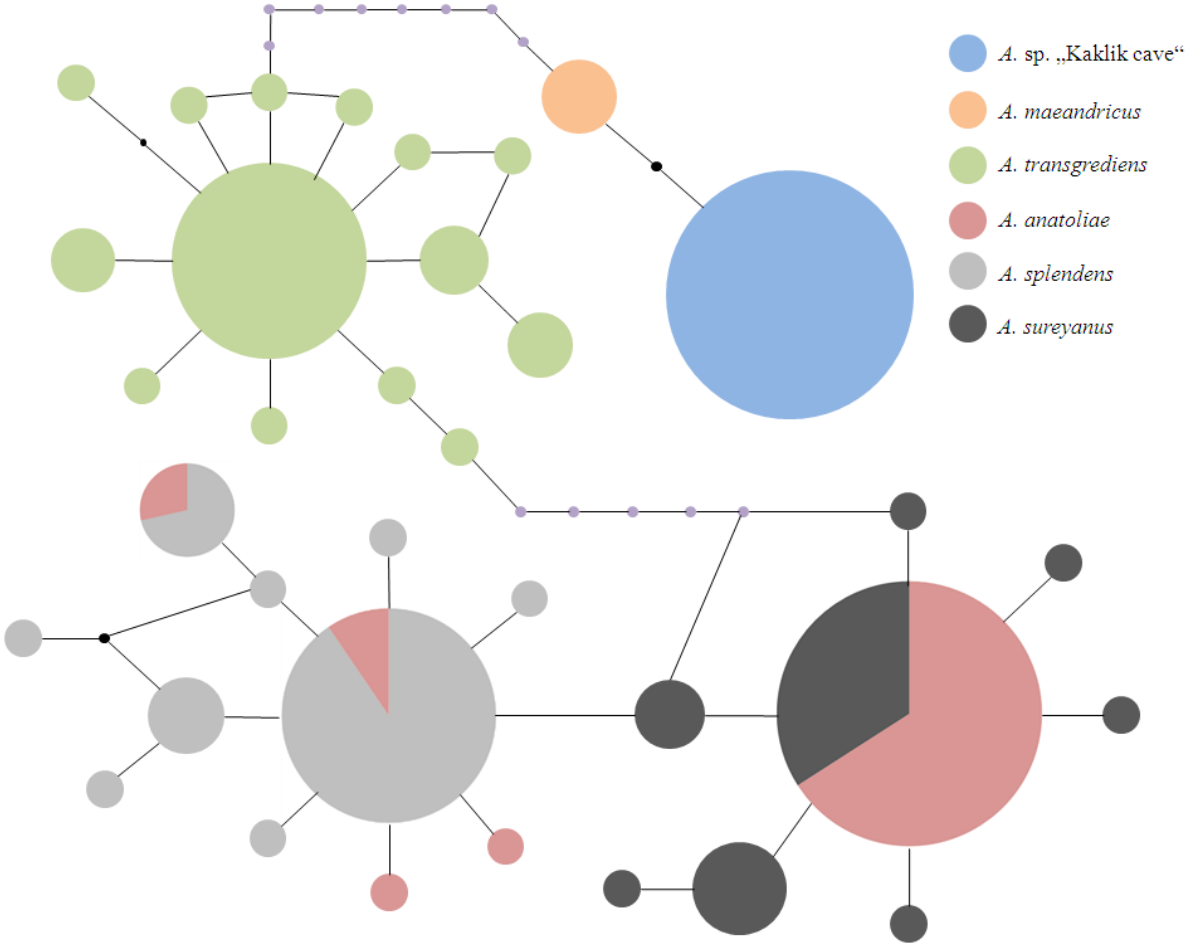

Figure 3: Haplotype network of mitochondrial D-loop dataset. Specific haplotypes are defined by circles, which sizes are related to the number of sequences included. The coloration refers to the populations listed in the figure's key. Violet connection steps stand for <90% connection certitude.

Among the Lakes District populations *A. sureyanus* was not diverse in its nucleotides, but varied in haplotypes, whereas nucleotide and haplotype diversity of *A. anatoliae* were low, but its haplotype variance was the highest. *Aphanius transgrediens* possessed the largest number of haplotypes (14), thus, the highest haplotype diversity as well. Furthermore, this population showed the greatest variance in nucleotides. The sequences of the Kaklik population and *A. maeandricus* were uniform (Table 5).

Table 5: Nucleotide and haplotype diversities of the mitochondrial dataset. ND = nucleotide diversity, V_{ND} = variance of nucleotide diversity, HD = haplotype diversity, V_{HD} = variance of haplotype diversity, N_H = number of haplotypes

	ND	V_{ND}	HD	V_{HD}	N_H
A. transgrediens	0.01462	0.0000173	0.753	0.00495	14
A. anatoliae	0.01028	0.0000105	0.314	0.00997	5
A. splendens	0.01646	0.0000030	0.679	0.00593	9
A. sureyanus	0.00789	0.0000014	0.702	0.00446	8
A. sp. "Kaklik cave"	0	0	0	0	1
A. maeandricus	0	0	0	0	1

AFLP

The AFLP dataset A (Table 6) produced 381 scoreable fragments, of which 205 were polymorphic (53.8%). The calculated error rate amounted to 0.77%. Fragment length ranged from 101 to 536 base pairs. In the dataset B 418 fragments were found, containing 149 polymorphic characters (35.6%) and the error rate was 0.36%. Fragment length encompassed 100 to 580 base pairs. The mean number of fragments per population was rounded to 275 in *A. transgrediens*, 256 in *A. sureyanus*, 257 in *A. anatoliae*, 258 in *A. splendens*, 342 in *A. maeandricus* and 339 in *A.* sp. "Kaklik cave".The highest gene diversity was found in *A. maeandricus* (N = 5, Table 6). In contrast, the Kaklik cave population exhibited the lowest gene diversity value of all populations. Both showed high numbers of fixed private alleles, indicating isolated populations. *Aphanius anatoliae* and *A. transgrediens* displayed higher gene diversity and proportion of variable markers. Moreover, both had fixed private fragments (2 and 24). In the contrary, the gene diversity of *A. splendens* and *sureyanus* was minor and they did not have fixed private fragments.

Table 6: AFLP gene diversity within the populations. D = dataset, N = number of individuals, P_{VM} = Proportion of variable markers, GD = gene diversity, CI = confidence interval, N_{PF} = number of private fragments, N_{FPF} = number of fixed private fragments.

D		N	P_{VM}	GD	CI	N_{PF}	N_{FPF}
A	*A. transgrediens*	12	0.17	0.048	0.037 - 0.061	48	24
	A. anatoliae	14	0.18	0.048	0.036 - 0.059	12	2
	A. splendens	12	0.16	0.038	0.029 - 0.050	10	0
	A. sureyanus	12	0.14	0.036	0.026 - 0.045	9	0
B	*A.* sp. "Kaklik cave"	19	0.07	0.013	0.008 - 0.019	32	20
	A. maeandricus	5	0.22	0.103	0.082 - 0.120	66	21

The distance based neighbor-joining trees of the D-loop dataset, including four out-groups (*A. mento*, *dispar*, *fasciatus* and *chantrei*) from GenBank, and the AFLP dataset (Figure 4) were similar. The AFLP tree had a strong bootstrap support at each node (100%), whereas the mitochondrial tree exhibited some uncertainties. The node between *A. transgrediens* and the other populations of the Lakes District had only a 65% bootstrap support. The node of *A.* sp. "Kaklik cave" and *A. maeandricus* was well supported.

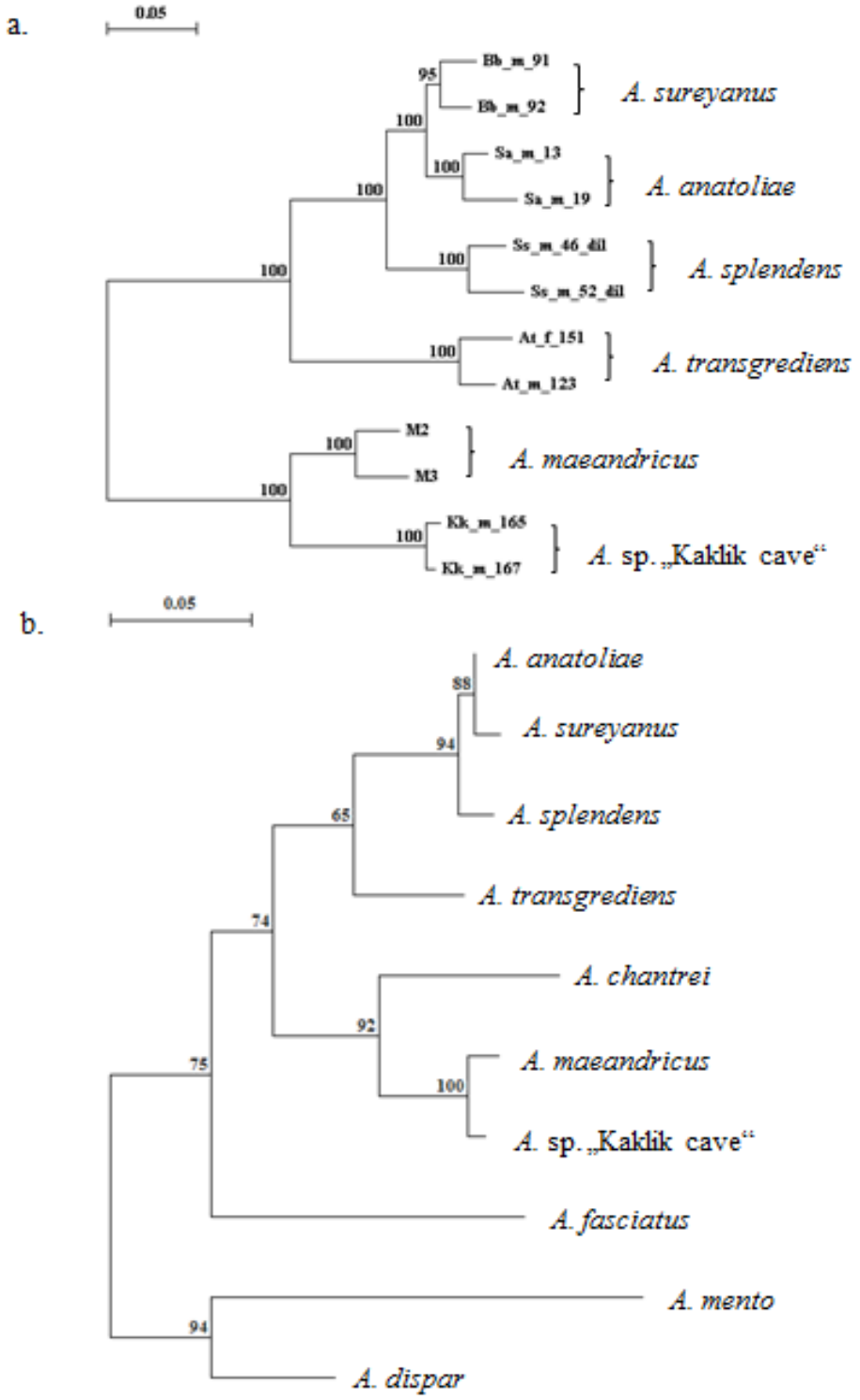

Figure 4: Distance based neighbor-joining tree of a. the AFLP dataset: two samples per population, rooted with *A. maeandricus* and b. the D-loop dataset: one sequence per population with four outgroups (*A. chantrei, A. fasciatus, A. mento* and *A. dispar*). Numbers above the branches show the bootstrap values higher than 50% (1000 replicates).

The analysis of molecular variance showed that both datasets displayed high F_{ST} values. In dataset B the among-populations F_{ST} value accounted for 0.84 (Table 7). The pairwise F_{ST} of dataset A (Table 8) exhibited a high differentiation between most populations. *Aphanius sureyanus* and *splendens*

represented the populations with the lowest F_{ST} value (F_{ST} = 0.1080), whereas *A. transgrediens* segregated (F_{ST} > 0.80).

Table 7: Analysis of molecular variance (AMOVA) of AFLP datasets A and B. A = *A. anatoliae, A. splendens, A. transgrediens* and *A. sureyanus*; B = *A. maeandricus* and *A.* sp. "Kaklik cave"

Dataset	Source of variation	df	Sum of squares	Variance components	Percentage of variation	F_{ST}*
A	Among populations	3	860.497	22.32032	72.95	0.73
	Within populations	46	380.643	8.274840	27.05	
B	Among populations	1	260.121	32.07406	83.80	0.84
	Within populations	22	136.421	6.200960	16.20	

*all F_{ST} values were significant with $p < 0.0001$

Table 8: Pairwise F_{ST} values of AFLP dataset A. ANA = *A. anatoliae*, SPL = *A. splendens*, SUR = *A. sureyanus*, TRA = *A. transgrediens*.

	SPL	SUR	TRA
ANA	0.4675	0.3369	0.8405
SPL		0.1080	0.8322
SUR			0.8089

The Principal Coordinates Analysis displayed in PCo 1 an unambiguous distinction of *A. anatoliae*. The clusters of *A. splendens* and *A. sureyanus* overlapped minimally in PCo 1 and 2 (Figure 5).

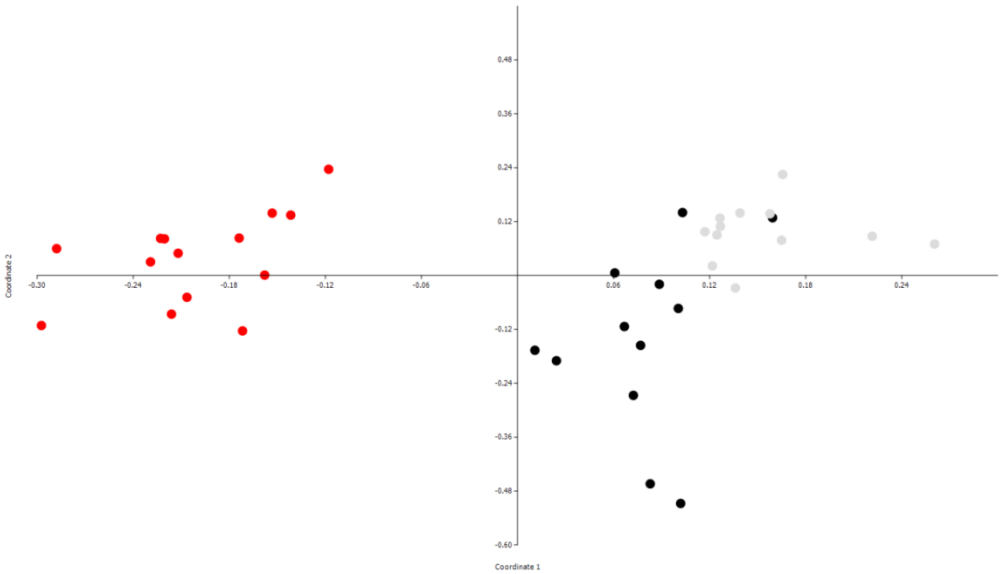

Figure 5: Principal Coordinates Analysis of AFLP data of *A. anatoliae* (red), *A. splendens* (grey) and *A. sureyanus* (black). PCo1 and 2 correlated.

The analysis for population structure among the dataset A in STRUCTURE resulted in two hypothetical groups as optimal solution (Figure 6), due to the lowest variability within the likelihood of all ten tests. In this case, only *A. transgrediens* formed its own group. On closer examination the K-equals-three-model showed a better resolution (Figure 7), where *A. sureyanus* and *A. splendens* clustered together. *Aphanius transgrediens* and *anatoliae* formed each a group. The program also assigned individuals of *A. sureyanus* to *A. anatoliae* to a mean extent of 12.23%.

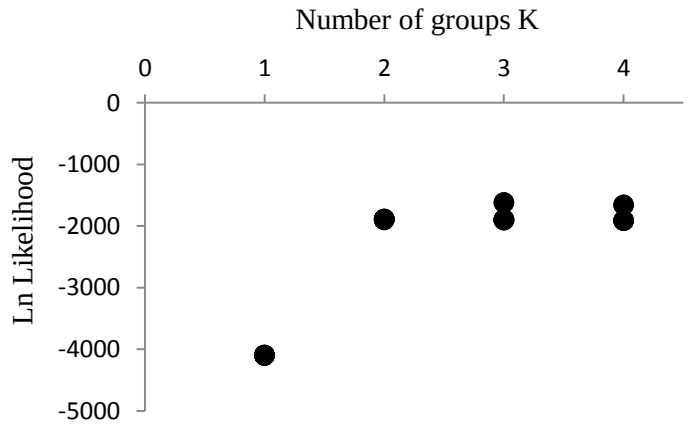

Figure 6: Likelihood Ln of every tested number of groups (K) of the AFLP dataset A.

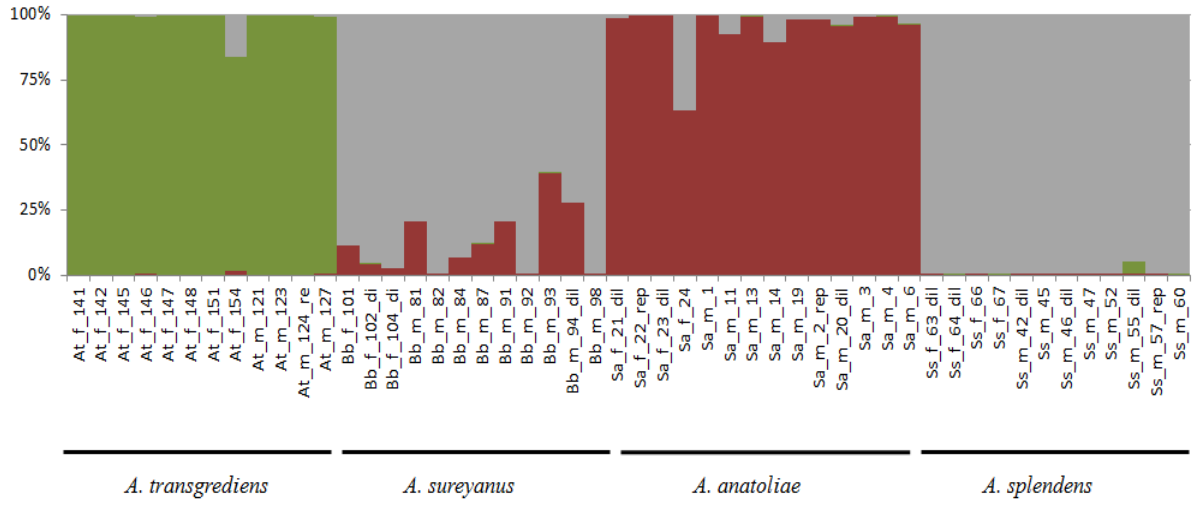

Figure 7: Population structure among AFLP dataset A. K (number of populations) = 3.

Microsatellites

The microsatellite data showed polymorphism in each population and the number of alleles ranges from five to 16 alleles with a mean number of 8.67. The microsatellite locus Af18 shows a higher observed heterozygosity than expected (0.934 to 0.750, Table 10), which was suggested to result from the binding behavior of this primer set, which appears to amplify more than one locus. Therefore, an overrepresentation of heterozygous alleles occurred and we excluded this locus from subsequent analyses. All other loci exhibited a lower observed heterozygosity than expected, which was indicative for a low genetic variability and inbreeding populations. In all loci null alleles occurred.

Linkage equilibrium for the remaining loci, which was based on 1000 permutations, was neither significant for five percent nominal level (Bonferroni adjusted p = 0.005), nor for one percent nominal level (Bonferroni adjusted p = 0.001) out of ten pairwise comparisons among all loci. The pairwise F_{ST} calculation showed that the populations of the Lakes District had the lowest F_{ST} values (Table 9). *Aphanius splendens* and *A. sureyanus* displayed the smallest genetic difference (F_{ST} = 0.0587, not significant: p = 0.036, adjusted p = 0.0033, 1500 permutations). The co-occurring species in Lake Salda, *A. anatoliae* and *splendens*, showed a low, but significant F_{ST} of 0.1319. However, *A.* sp. "Kaklik cave" and *A. maeandricus* had the highest F_{ST} values in comparison to the other populations. The F_{ST} between them was not significant (F_{ST} = 0.1084, p = 0.0667). Remaining F_{ST} results exhibited significance.

Table 9: Microsatellite FSTAT results for pairwise F_{ST}. ANA = *A. anatoliae*, SPL = *A. splendens*, SUR = *A. sureyanus*, TRA = *A. transgrediens*, KAK = *A.* sp. "Kaklik cave", M = *A. maeandricus*

	SPL	SUR	TRA	KAK	M
ANA	0.1319	0.2615	0.4020	0.3690	0.4749
SPL		0.0587	0.3658	0.4871	0.6298
SUR			0.3694	0.5961	0.7527
TRA				0.5445	0.5800
KAK					0.1084

The PCoA over all alleles of the microsatellite dataset resulted in clusters of populations as follows (Figure 8): the Kaklik population and *A. maeandricus* formed their own cluster, but individuals of *A. sureyanus*, *splendens* and *anatoliae* partially overlapped with both in PCo 1. On the contrary, *A. transgrediens* arranged as own group with intersection of the same mentioned three populations. *Aphanius sureyanus* was stronger clustered, whereas *A. splendens* and *A. anatoliae* were spread over the total axis of PCo 1. Only the first principal coordinate was informative, due to strong overlapping in all following PCos.

Table 10: Microsatellite loci. T_a annealing temperature, N_A number of alleles, H_O observed heterozygosity, H_E expected heterozygosity; Company: a = Thermo Scientific, b = Eurofins MWG Operon, c = Microsynth AG; Citation: 1 = Babbucci et al., 2006, 2 = Vogiatzi et al., 2009.

Locus	Primer sequence	Dye	Repeat unit	T_a (°C)	N_A	H_O	H_E	Comp	GenBank Accession no	Cit.
Af18	F: CCAATATACACATCTACACG	FAM	$(CA)_{14}$	48	16	0.934	0.750	a	DQ865159	1
	R: TTGTCTCTTTTCTTCTGCAG							b		
Af7	F: GGAAGCACACATTCAAAACC	HEX	$(CA)_{14}$	46	8	0.061	0.659	b	DQ865156	1
	R: TGTGAGGTCAGAAAGGGAGA							a		
Af8	F: TGCCCGAAGGTAACATTCTT	TET	$(TG)_4TT(TG)_6$	48	8	0.242	0.517	b	DQ865157	1
	R: ACCAAACTGCTCTCTACTCT							a		
Af9	F: CCCACATTCTTGTGTTGAAA	NED	$(TG)_{14}$	50	6	0.122	0.434	a	DQ865158	1
	R: GTGCATGCATATCAAACAAG							b		
Af20b	F: GAGGCTCACTAATCCACTCT	FAM	$(CA)_{11}$	46	9	0.117	0.279	a	DQ865161	1
	R: TCAATTACCAAAGCAGGGCT							b		
VL072	F: GACAGGTCGCCAGTCCAT	FAM	$(AC)_2(AG)_2(AC)_5$	58	5	0.058	0.232	c	FJ841871	2
	R: AGACATGACTATTAGGTTCACTGGTT							c		
Mean					8.67	0.256	0.478			

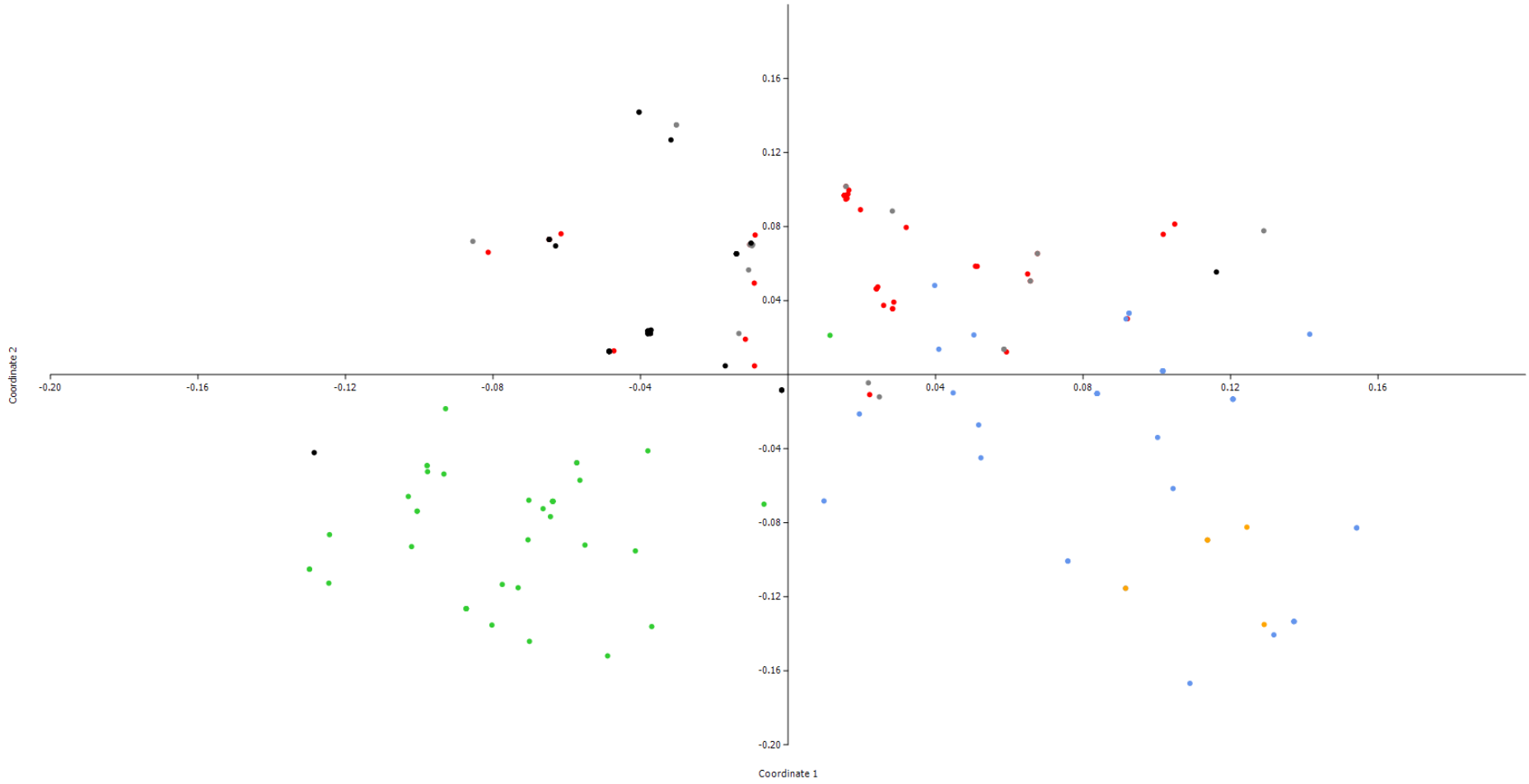

Figure 8: Principal Coordinates Analysis of microsatellites of *A. anatoliae* (red), *A. splendens* (grey), *A. sureyanus* (black), *A. transgrediens* (green), *A.* sp. "Kaklik cave" (blue), *A. maeandricus* (orange). PCo1 and 2 correlated.

The observed heterozygosity was lower than the expected heterozygosity in all populations (Table 11). In particular, *A. splendens*, *sureyanus* and *maeandricus* exhibited low observed heterozygosities (H_O = 0.086, 0.088 and 0.080). The last population also showed the highest F_{IS} value (F_{IS} = 0.698). Due to Shannon's information index, *A. anatoliae* and *transgrediens* were the most diverse populations (I = 0.648 and 0.619).

Table 11: Mean heterozygosity, F-statistics and polymorphism by population of the microsatellite data. N = mean sample size per locus, N_A = mean number of alleles, N_E = effective number of alleles, I = Shannon's information index, H_O = observed heterozygosity, H_E = expected heterozygosity, uH_E = unbiased expected heterozygosity, F_{IS} = inbreeding coefficient, %P = percentage of polymorphic loci

Pop	N	N_A	N_E	I	H_O	H_E	uH_E	F_{IS}	%P
ANA	35.8	3.4	1.710	0.648	0.128	0.358	0.364	0.652	80
SPL	34.2	3	1.472	0.458	0.086	0.238	0.241	0.644	80
SUR	33.2	3	1.186	0.313	0.088	0.148	0.151	0.418	100
TRA	33.8	4	1.661	0.619	0.173	0.324	0.329	0.477	100
KAK	35	2.2	1.430	0.423	0.150	0.266	0.269	0.447	100
M	5	1.8	1.359	0.361	0.080	0.220	0.244	0.698	60

Geometric morphometric

The General Procrustes Analysis (Figure 9) showed a high differentiation among the populations at certain landmarks. Especially the posterior body part of the fish differed strongly. Thus, individuals of the Kaklik population appeared to be much shorter, but higher than the other populations, whereas *A. splendens* was the most slender species.

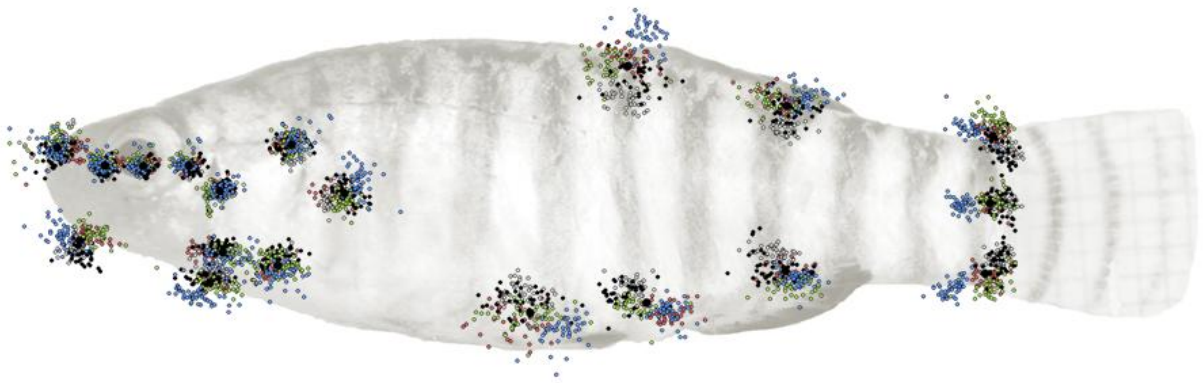

Figure 9: General Procrustes Analysis of geometric morphometric data with coloured data points. Grey = *A. splendens*, black = *A. sureyanus*, red = *A. anatoliae*, green = *A. transgrediens* and blue = *A.* sp. „Kaklik cave".

The variance of the first principal component exhibited the main difference between the Kaklik cave population and the remaining species (Figure 10). The thin-plate splines deformation grids showed a high difference in body length and height, so that *A.* sp. "Kaklik cave" segregated. In PC 1 *A. splendens* was the most slender population and almost only overlapped with *A. sureyanus*, which also overlapped with super-imposed *A. anatoliae* and *A. transgrediens*. The second principal component (Figure 11) primarily referred to the shape of the mouth region and head and identified *A. transgrediens* having a shorter, clinched anterior by tendency. The last informative principal component was the third (Figure 12), because in every further component all populations strongly overlapped. Here, *A. anatoliae* stood out, showing that the middle body area was tendentially higher and the head smaller compared to the other populations.

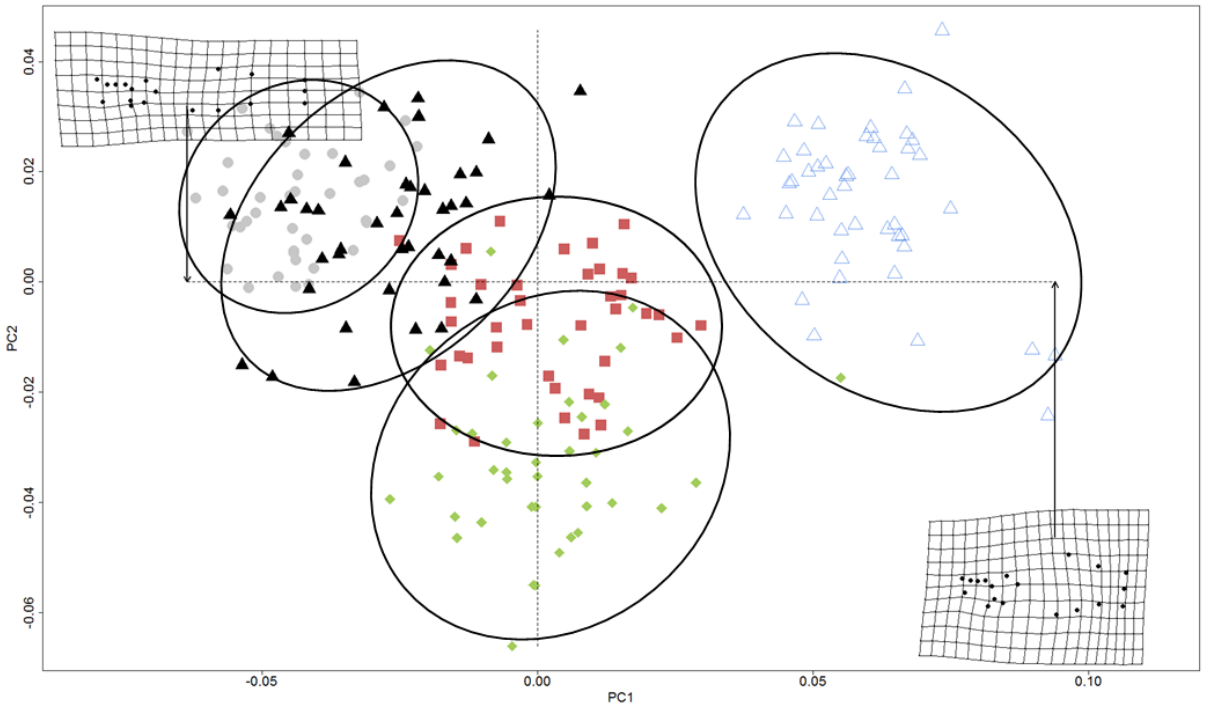

Figure 10: Principal components PC1 and PC2 of the geometric morphometric data correlated, showing the data points to each specimen. The two deformation grids exemplarily demonstrate the maximum distance in shape. Grey dots = *A. splendens*, black triangels = *A. sureyanus*, red squares = *A. anatoliae*, green rhombi = *A. transgrediens* and blue triangels with black frames = A. sp. „Kaklik cave". The equal-frequency ellipses show the mean distribution of data points per population.

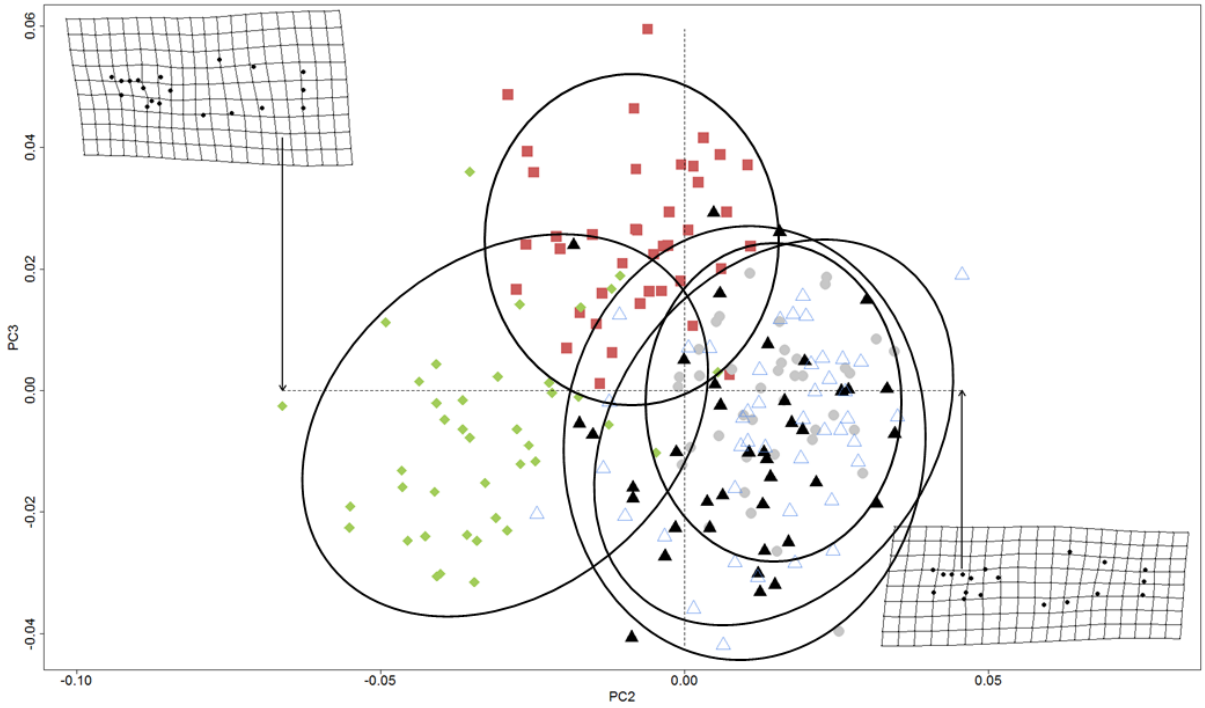

Figure 11: Principal components PC2 and PC3 of the geometric morphometric data correlated.

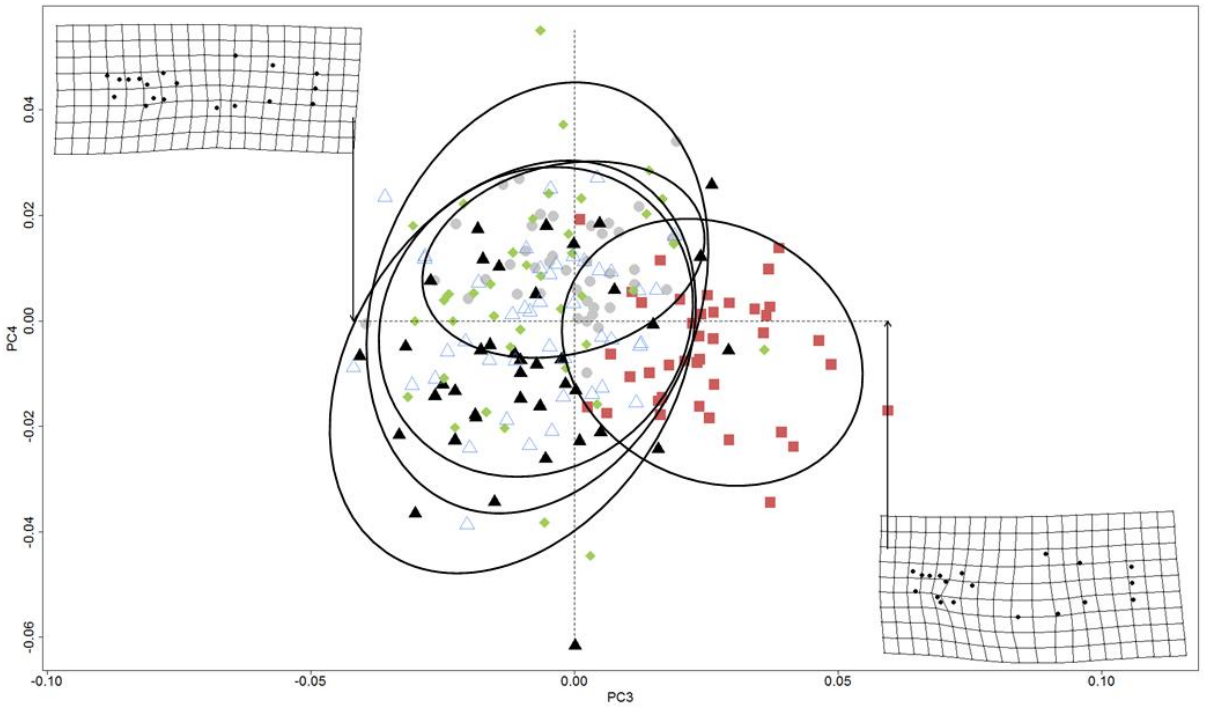

Figure 12: PC3 and 4 of the geometric morphometric data correlated.

Discussion

In our study several methods showed similar results: The newly found population in Kaklik clusters with *A. maeandricus* (except for the morphometrical dataset, where *A. maeandricus* was not included) and is clearly distinct from the other populations. *Aphanius transgrediens* differs from the populations of the Lakes District and *A. anatoliae* and *A. sureyanus* are closer related to each other than *A. splendens*, which shares the same habitat with *A. anatoliae*. However, earlier publications exhibited that the populations of the Lakes District were not clearly distinguished: in otoliths across-population morphologies existed (Schulz-Mirbach et al., 2006), crossbreeding experiments resulted in mostly fertile males (Villwock, 1964) and in the mitochondrial DNA data analysis all Lakes District populations formed a single clade in Hrbek et al. (2002). In our study, the microsatellite results showed that all populations tend to inbreeding, which could also be biased by the occurrence of null alleles within each locus.

The Kaklik population seems to be bottlenecked, due to its low genetic variability, suggesting a strong inbreeding situation, which can be explained by two possible events: first, the population went back to a natural colonization event or, second, humans set fish free for mosquito elimination. The genus *Aphanius* was often introduced to diverse habitats for the control of mosquito larvae (Blaustein and Byard, 1993; Fletcher et al., 1992; Yildirim and Karacuha, 2007). However, the Kaklik cave population is genetically and morphologically well separated from the Lakes District populations and clusters with *A. maeandricus*. Güçlü et al. (2013) classified *A.* sp. "Kaklik cave" as population of *A. anatoliae*. They also found one population of *A. fasciatus* and three other populations of *A. anatoliae*, including a population of Işıklı Spring-Çivril-Denizli from the Menderez River system, where our *A. maeandricus* samples probably originated from. The number of private alleles in AFLP dataset B could point to the fact that the Kaklik population was at the beginning of speciation. In this case, we got a first hint for putting *A.* sp. "Kaklik cave" into the state of subspecies of *A. maeandricus, anatoliae* respectively. On the contrary, the data are biased because of the small sample size of five individuals of the latter population. Further morphometric analysis should reveal the distance between these two groups to resolve the real relationship.

Aphanius maeandricus was originally described as a subspecies of *A. chantrei* by Akşiray (1948), who located its type specimens at Menderez River in the southeast of Turkey. Later on, Kosswig (1953) interpreted *A. chantrei* to be identical with *A. danfordii* from Elbistan, southeast Turkey. Wildekamp et al. (1999) compared the type specimens of these two species and concluded that *A. chantrei* was eventually a junior synonym of *A. danfordii*. In northern Anatolia, the populations at Kizilirmak were also classified as *A. chantrei* and were clearly separated from all western *Aphanius* populations, which were described as *A. anatoliae*. Cross-breeding attempts between both resulted in fertile females in F1, but mostly sterile males (Villwock, 1964). Eventually, *A. chantrei maeandricus* was synonymized with *A. anatoliae* (Wildekamp et al., 1999) and its populations in the Menderez River formed their

own *anatoliae*-clade in Hrbek et al. (2002). Our mitochondrial neighbor-joining tree clusters together the Kaklik population, the samples of *A. maeandricus* and the out-group *A. chantrei*. A BLAST-search in GenBank was conducted, where other mitochondrial *Aphanius* sequences, published by Hrbek et al (2002) and Hrbek and Meyer (2003), were compared to the 16S mtDNA *A. chantrei* sequence of Parker and Kornfield (1995). In GenBank *A. chantrei* mainly clustered with Hrbek's *A. anatoliae* clades, especially with clade V (Menderez River clade). Thus, the *chantrei* control region sequence used in this study has to be interpreted as a sequence of an *A. anatoliae* population. In consequence, *A. maeandricus* and *A.* sp. "Kaklik cave" likely class with clade V, west of the Lakes District, and both represent populations of *A. anatoliae*. The reproductive groups, characterized by reproductive barriers within several *A. anatoliae* populations found by Villwock (1964), correlated with the four *anatoliae* clades (Lake Tuz, Lakes District, Menderez River and Southern clade) in the study of Hrbek et al. (2002). Each distinct clade probably represents a real species, but additional testing is required (Hrbek et al., 2002; Hrbek and Wildekamp, 2003).

The habitat of *Aphanius transgrediens* is characterized by twelve freshwater tributaries, which drain Lake Aci. Each spring was inhabited by one population, but it depended on the water level, whether admixture was possible or not (see Akşiray and Villwock, 1962 and Grimm, 1979). In October 2012, only one inhabited spring at the southeastern shore of the lake was found, where a high number of *Gambusia* cf. *affinis* co-occurred (personal observation, C. Pichler). The presence of these Poeciliid fish threatens the recent *Aphanius* population, because of their high adaptability (Güçlü and Küçük, 2012). The mitochondrial and also the AFLP results, especially the number of haplotypes (14) and fixed private fragments (24) respectively, showed the genetic variability within the *A. transgrediens* population. According to the geographical pattern, an additional possibility to mutation for evolving private sequences was given. The AFLP data also suggest that *A. transgrediens* got earlier isolated to Lake Salda and Burdur, as the populations in these lakes got isolated from each other. On the other hand, the F_{ST} values of the AFLP dataset A show that *A. transgrediens* is somehow closer related to *A. sureyanus*, which emphasizes the proximity between these lakes and suggests that Lake Aci got latest separated from Lake Burdur and isolation by distance occurred. Until the Pleistocene Lake Aci and Lake Burdur were still connected (Kosswig, 1953), but divided in course of time.

The resolution of population structure within Lake Salda and Burdur is rather difficult. Originally, *A. splendens* was classified as *Anatolichthys splendens* (Kosswig and Sözer, 1945), inhabiting Lake Gölcük, east of Lake Burdur. This population went extinct caused by introduction of *Sander lucioperca* and pollution (Güçlü and Küçük, 2012). Later on, the *splendens* population in Lake Salda was described as *Anatolichthys splendens saldae* (Akşiray, 1955), but recently is classified as subspecies of *Aphanius anatoliae* as well as *A. sureyanus* (Wildekamp et al., 1999). No morphometric differences were found between *A. splendens* and *sureyanus* in this study, except for the more slender body in the former. The AFLP data exhibited a close relationship, due to the low F_{ST} value, whereas

the microsatellites showed the highest F_{ST} within the Lakes District between both. However, the microsatellite results should be compared with caution, due to the low allelic variability and the observed null alleles within the loci. Nevertheless, both populations appear closely related, because they also cluster together (also with other neighboring *A. anatoliae* populations) in Hrbek et al. (2002) and Hrbek and Meyer (2003).

Furthermore, specimens of *Aphanius anatoliae* were located in Lake Salda (Schulz-Mirbach et al., 2006). Despite the fact that the non-coding region, including the D-loop, is assumed to be the fastest evolving sequence in mitochondria (Upholt and Dawid, 1977), we found a shared mitochondrial haplotype between *A. anatoliae* from Lake Salda and *A. sureyanus* from Lake Burdur, even though they live in recently isolated habitats. The estimation of population structure also assigns most individuals of *A. sureyanus,* used in the AFLP analysis, to an extent of 12.23% to *A. anatoliae*, which also shows as only population fixed fragments and also the highest gene diversity. For these reasons, we suggest that ancestors of *A. anatoliae* could also be the ancestral population of Lake Burdur. On the other hand, regarding the PCo 1, the gene pool of *A. anatoliae* is distinct to *A. splendens* and *sureyanus*, which resemble in gene pools. In Lake Salda, syntopic *A. splendens* and *A. anatoliae* have distinct gene pools according to the AFLP data. Regarding the AFLP data both populations form own groups. Thus, species state should be discussed for *A. splendens*. However, in Schulz-Mirbach et al. (2006) was mentioned that the *A. anatoliae* population could be introduced into Lake Salda by egg-contaminated flues. Our results do not reject this assumption, even though shared haplotypes were found in *A. anatoliae* and *splendens,* indicating possible hybridizations. Nevertheless, reproductive barriers appear to exist, otherwise their gene pools would be closer related. Further investigations should compare the *A. anatoliae* population to neighboring *anatoliae* populations, to prove a possible introduction.

All three Lakes District populations (*A. transgrediens*, *splendens* and *sureyanus*) evolved characteristic mouth and jaw structures: *Aphanius transgrediens* showed a shorter head in our study, *A. splendens* has an angular lower jaw, which rises almost vertically to the mouth (Wildekamp et al., 1999), and *A. sureyanus* is similar to *splendens*, but its lower jaw is less angular. A modification in jaw morphology is described as second step of speciation, apart from occupying a new habitat and evolving specific sexual selection traits, in African cichlids, inhabiting the Lake Malawi (Kocher, 2004). On the other hand, a reduction in body scales was gradually found in all populations of the Lakes District, but *A. splendens* exhibits the highest degree of reduction and is almost nude (Wildekamp et al., 1999). Today, Lakes Aci, Burdur and Salda are geographically separated and their populations are endemic (Güçlü et al., 2007; Güçlü and Küçük, 2012). Due to the recent isolation, the populations are in the process of allopatric speciation, even though no cross-breeding barriers were found (Villwock, 1964). Therefore, the populations appear to be at the beginning of speciation (species "*in statu nascendi*", Grimm, 1979; Villwock, 2004). Our results show that the populations of

the Lakes District differ genetically from the *A. anatoliae* populations of the Menderez River clade in Hrbek et al. (2002) and that *A. splendens* is distinct to an *A. anatoliae* population, which probably was introduced into Lake Salda. We suggest that at least two of the four *anatoliae* clades in Hrbek et al. (2002), the Menderez River clade and the Lakes District clade, represent real species, but further testing is required to evaluate the definitive species state of the Lakes District populations, which probably comprise at least two species: *A. transgrediens* and *A. splendens*.

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Supplementary

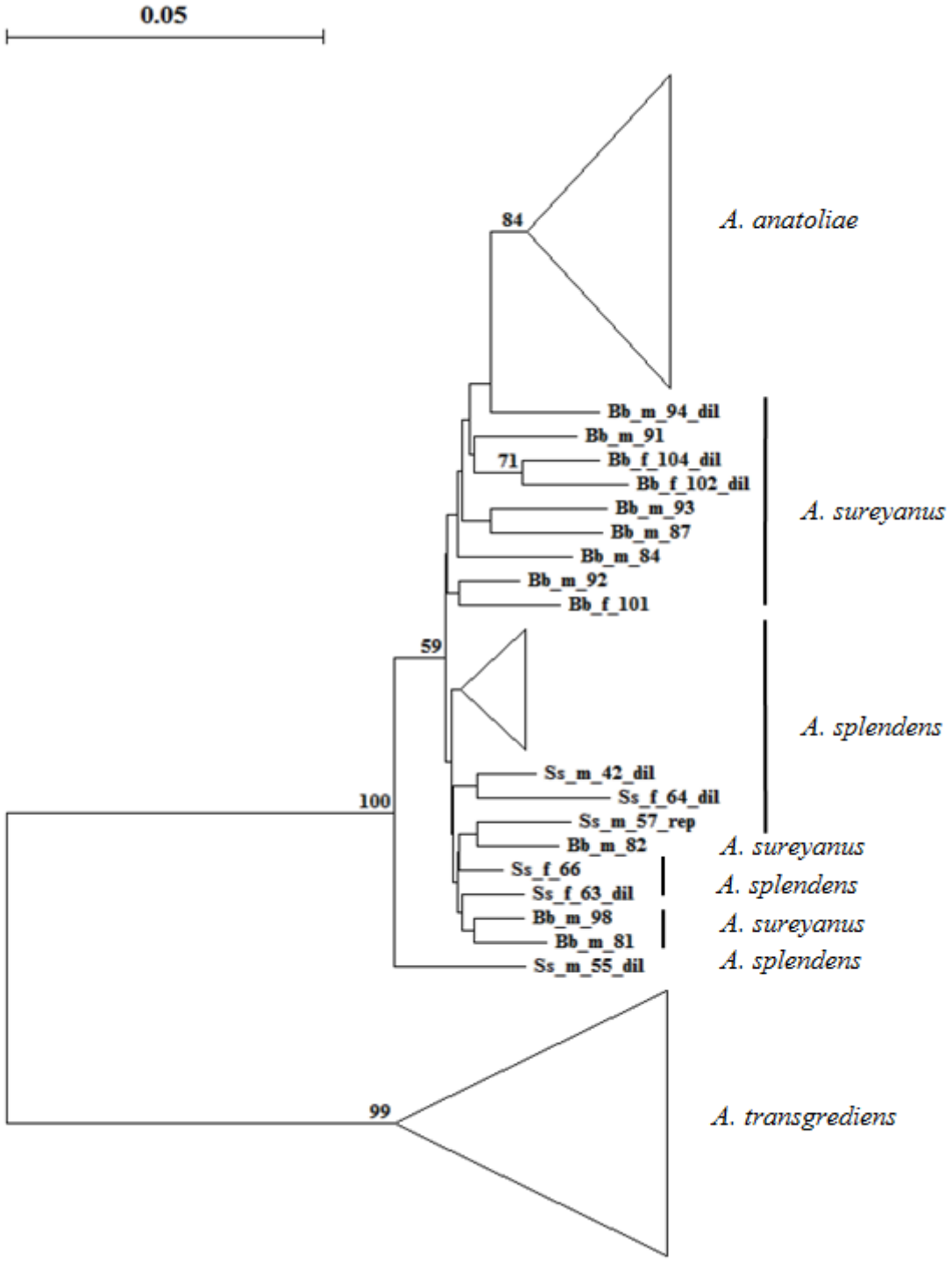
The PCR protocols of the Preselective and the Selective PCR (AFLP) were modified: the amount of Taq-polymerase was reduced to 10 percent in the former and 20 percent in the latter reaction.

Hold		Cycle		No. of Cycles
94°C 2 min	94°C 20 sec	66°C 30 sec	72°C 2 min	1
	94°C 20 sec	65°C 30 sec	72°C 2 min	1
	94°C 20 sec	64°C 30 sec	72°C 2 min	1
	94°C 20 sec	63°C 30 sec	72°C 2 min	1
	94°C 20 sec	62°C 30 sec	72°C 2 min	1
	94°C 20 sec	61°C 30 sec	72°C 2 min	1
	94°C 20 sec	60°C 30 sec	72°C 2 min	1
	94°C 20 sec	59°C 30 sec	72°C 2 min	1
	94°C 20 sec	58°C 30 sec	72°C 2 min	1
	94°C 20 sec	57°C 30 sec	72°C 2 min	1
	94°C 20 sec	56°C 30 sec	72°C 2 min	20
60°C 30 min				1
4°C Forever				1

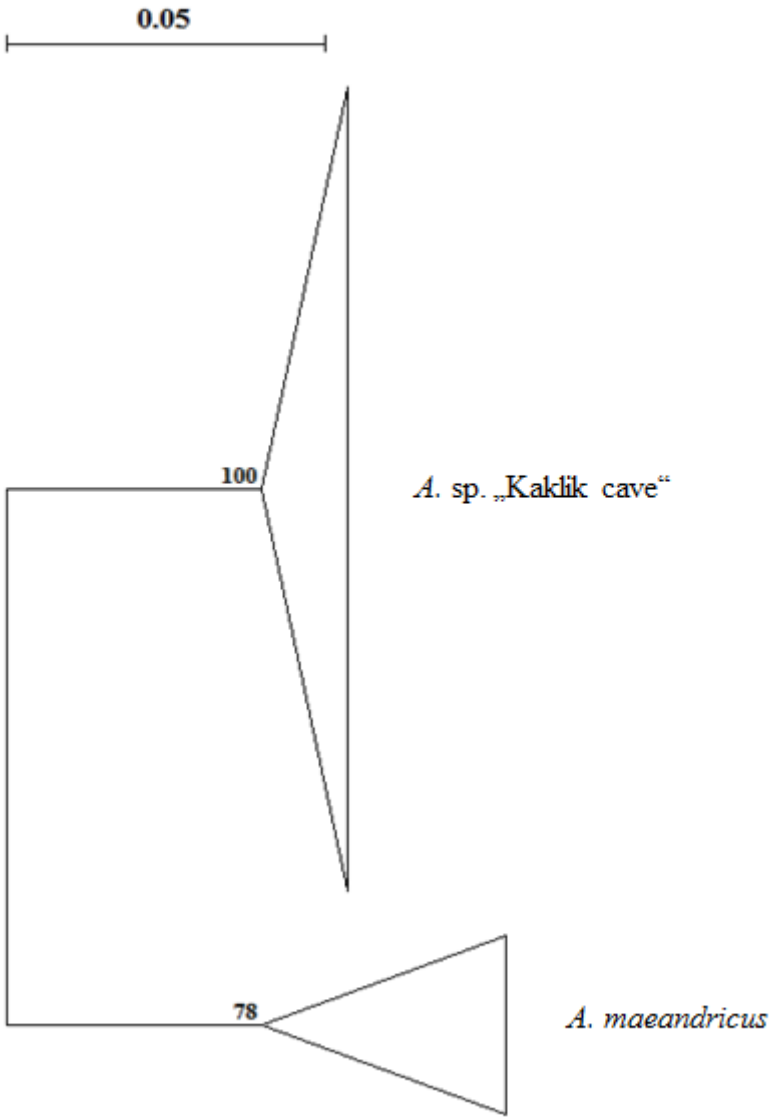
Table 12: Thermal cycler program for the Selective PCR

Neighbor-joining tree AFLP:

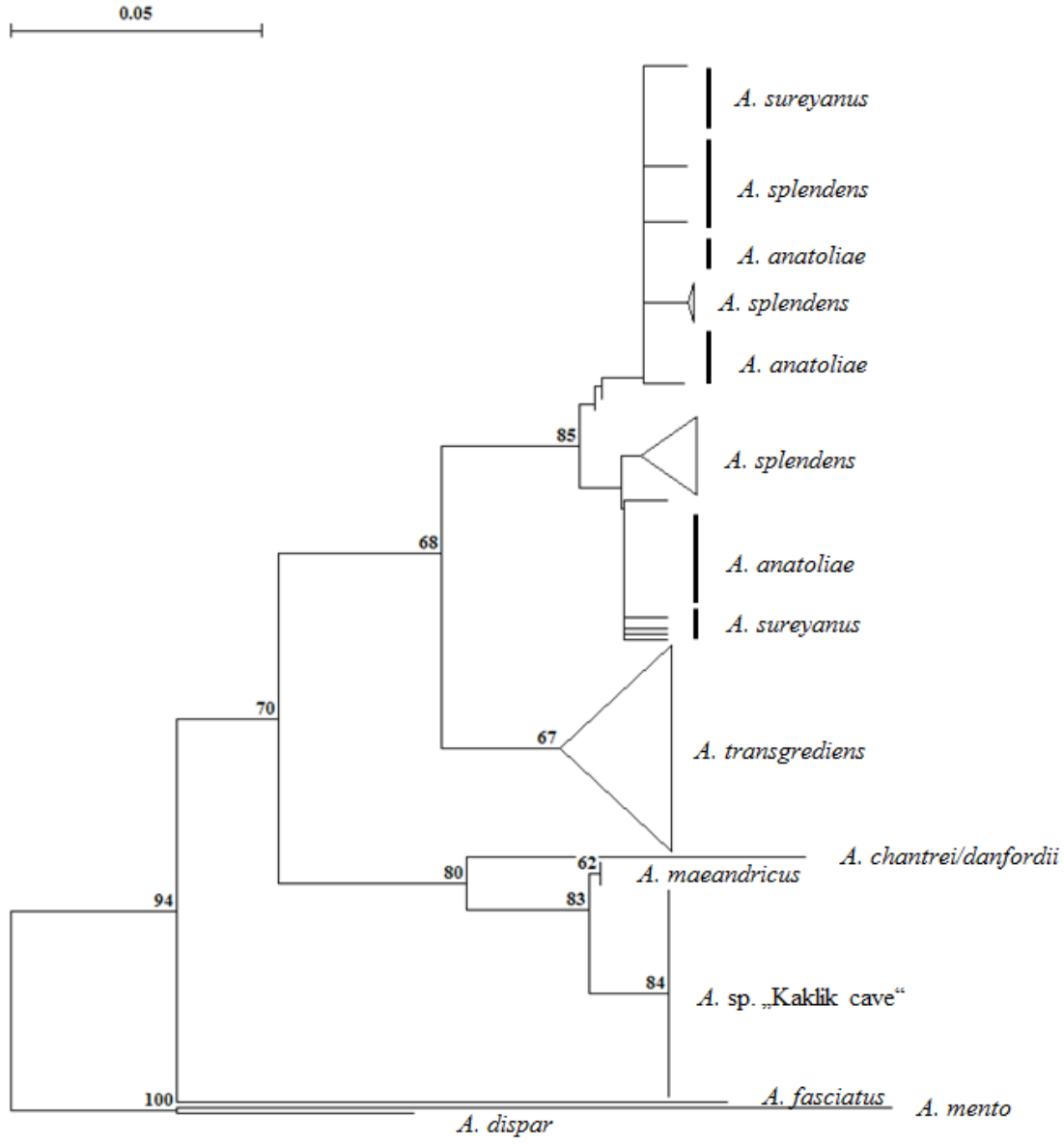
Dataset A:



Dataset B:



Neighbor-joining tree D-loop:



PCoA AFLP: Eigenvalues and variance

Table 13: Principal Coordinates Analysis AFLP: Eigenvalues, % variance and % cumulative variance.

PCo	Eigenvalues	Variance	Cumulative
1.	0,01645300	39,515	39,515
2.	0,00319580	7,676	47,191
3.	0,00271160	6,513	53,703
4.	0,00225400	5,414	59,117
5.	0,00187030	4,492	63,609
6.	0,00169600	4,073	67,682
7.	0,00136900	3,288	70,970
8.	0,00125880	3,023	73,993
9.	0,00109370	2,627	76,620
10.	0,00098690	2,370	78,991
11.	0,00091153	2,189	81,180
12.	0,00082111	1,972	83,152
13.	0,00070382	1,690	84,842
14.	0,00067912	1,631	86,473
15.	0,00054000	1,297	87,770
16.	0,00044607	1,071	88,842
17.	0,00035189	0,845	89,687
18.	0,00027082	0,650	90,337
19.	0,00022347	0,537	90,874
20.	0,00021118	0,507	91,381
21.	0,00012717	0,305	91,687
22.	0,00004385	0,105	91,792
23.	0,00003792	0,091	91,883
24.	0,00000000	0,000	91,883
25.	-0,00000107	-0,003	91,886
26.	-0,00002593	-0,062	91,948
27.	-0,00003915	-0,094	92,042
28.	-0,00006652	-0,160	92,202
29.	-0,00010894	-0,262	92,463
30.	-0,00014117	-0,339	92,802
31.	-0,00020501	-0,492	93,295
32.	-0,00025082	-0,602	93,897
33.	-0,00027973	-0,672	94,569
34.	-0,00031131	-0,748	95,317
35.	-0,00041113	-0,987	96,304
36.	-0,00044772	-1,075	97,380
37.	-0,00048157	-1,157	98,536
38.	-0,00060938	-1,464	100

PCoA Microsatellites: Eigenvalues and variance

Table 14: Principal Coordinates Analysis microsatilles: Eigenvalues, % variance and % cumulative variance.

PCo	Eigenvalues	Variance	Cumulative
1.	15,18900000	25,40500000	25,405
2.	7,00090000	11,70900000	37,114
3.	5,61610000	9,39320000	46,507
4.	5,05430000	8,45350000	54,961
5.	2,79630000	4,67700000	59,638
6.	2,06310000	3,45060000	63,088
7.	1,58830000	2,65660000	65,745
8.	1,02380000	1,71240000	67,457
9.	0,96177000	1,60860000	69,066
10.	0,67558000	1,12990000	70,196
11.	0,53203000	0,88985000	71,086
12.	0,47757000	0,79876000	71,884
13.	0,40791000	0,68225000	72,567
14.	0,32783000	0,54832000	73,115
15.	0,30078000	0,50307000	73,618
16.	0,25869000	0,43268000	74,051
17.	0,23122000	0,38672000	74,437
18.	0,22040000	0,36864000	74,806
19.	0,20130000	0,33668000	75,143
20.	0,16057000	0,26855000	75,411
21.	0,14198000	0,23746000	75,649
22.	0,12081000	0,20207000	75,851
23.	0,09645600	0,16133000	76,012
24.	0,04130900	0,06909200	76,081
25.	0,02410100	0,04031000	76,122
26.	0,01729500	0,02892600	76,151
27.	0,01559500	0,02608300	76,177
28.	0,01189100	0,01988900	76,196
29.	0,01060600	0,01774000	76,214
30.	0,00668830	0,01118700	76,225
31.	0,00080629	0,00134860	76,227
32.	0,00000000	0,00000000	76,227
33.	0,00000000	0,00000000	76,227
34.	0,00000000	0,00000000	76,227
35.	0,00000000	0,00000000	76,227
36.	0,00000000	0,00000000	76,227

PCA geometric morphometrics: Eigenvalues and variance

Table 15: Principal component analysis geometric morphometrics: Eigenvalues, % variance and % cumulative variance.

PC	Eigenvalues	Variance	Cumulative
1.	0.00145917	38.008	38.008
2.	0.00048298	12.581	50.589
3.	0.00036254	9.443	60.032
4.	0.00025439	6.626	66.659
5.	0.00024006	6.253	72.912
6.	0.00017006	4.430	77.342
7.	0.00015831	4.124	81.465
8.	0.00011423	2.975	84.441
9.	0.00010097	2.630	87.071
10.	0.00006785	1.767	88.838
11.	0.00005760	1.500	90.338
12.	0.00005094	1.327	91.665
13.	0.00004205	1.095	92.760
14.	0.00003888	1.013	93.773
15.	0.00003717	0.968	94.742
16.	0.00002836	0.739	95.480
17.	0.00002241	0.584	96.064
18.	0.00001994	0.519	96.583
19.	0.00001890	0.492	97.076
20.	0.00001818	0.474	97.549
21.	0.00001562	0.407	97.956
22.	0.00001227	0.320	98.276
23.	0.00001103	0.287	98.563
24.	0.00001020	0.266	98.829
25.	0.00000878	0.229	99.057
26.	0.00000697	0.182	99.239
27.	0.00000689	0.179	99.419
28.	0.00000575	0.150	99.568
29.	0.00000485	0.126	99.695
30.	0.00000404	0.105	99.800
31.	0.00000328	0.086	99.885
32.	0.00000215	0.056	99.942
33.	0.00000175	0.046	99.987
34.	0.00000049	0.013	100

Microsatellite allele frequency table:

Table 16: Microsatellite allele frequencies of each locus. ANA = *A. anatoliae*, SPL = *A. splendens*, SUR = *A. sureyanus*, TRA = *A. transgrediens*, KAK = *Ap.* Sp. "Kaklik cave", M = *A. maeandricus*. N horizontal = number of individuals per population, which show the according locus, N vertical = number of base pairs of each allele, F_{IS} = inbreeding coefficient.

	ANA	SPL	SUR	TRA	KAK	M
Locus: Af7						
N	38	40	37	38	39	5
118	0.013					
130				0.013		
134	0.013	0.038				
136	0.013		0.014	0.013		
138	0.158	0.313	0.135	0.092		
140	0.211	0.513	0.838	0.882		
142	0.592	0.138	0.014		0.744	0.800
144					0.256	0.200
F_{IS}	0.823	0.842	0.906	0.639	1.000	1.000
Locus: Af8						
N	39	39	36	36	35	5
165			0.014			
175	1.000	1.000	0.986		0.400	
180				0.056	0.600	1.000
185				0.014		
190				0.431		
195				0.389		
200				0.097		
205				0.014		
F_{IS}	NA	NA	0.000	-0.054	-0.176	NA
Locus: Af9						
N	38	38	38	38	40	5
142			0.013			
164				0.013		
170	0.039	0.039	0.039			
172	0.763	0.842	0.895	0.974	0.175	
174	0.171	0.118	0.053	0.013	0.825	1.000
176	0.026					
F_{IS}	0.532	0.626	0.337	-0.007	0.490	NA

Locus: Af20b

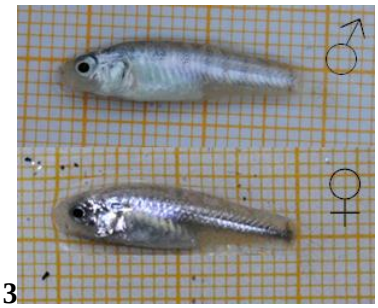
N	28	31	31	32	36	5
184						0.100
186		0.016				
196				0.031		0.200
198	0.625	0.952	0.935	0.719	0.986	0.700
200				0.250		
206			0.032			
208	0.107					
210		0.016	0.032		0.014	
212	0.268	0.016				
F_{IS}	0.404	-0.017	-0.034	1.000	0.000	0.238

Locus: VL072

N	36	23	24	25	25	5
93				0.020		
103	0.014	0.065	0.063			
105	0.167	0.043		0.040	0.040	0.200
107	0.819	0.891	0.938	0.840	0.920	0.800
111				0.100	0.040	
F_{IS}	0.910	0.365	0.365	0.864	1.000	1.000

1

2



3

4

5

6

Figures 1 to 6: Both genders of examined specimens displayed:

1 = *A.* cf. *anatoliae*

2 = *A. splendens*

3 = *A. sureyanus* (juveniles)

4 = *A. transgrediens*

5 = *A.* sp. „Kaklik cave“

6 = *A. maeandricus* (picture of a wild caught male from Dinar, Turkey: http://joerg-freyhof.de/; 03/20/2014)

Males and females in all populations differ by lateral stripes, spots respectively.

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