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Genetic relationships between Anatolian species and subspecies of *Aphanius* Nardo, 1827 (Pisces, Cyprinodontiformes) based on RAPD markers

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Anatolia is regarded as the center of diversification of *Aphanius* because 6 species and 4 subspecies of 14 *Aphanius* species described to date occur only in Anatolia. Random amplified polymorphic DNA (RAPD) markers were used to examine genetic relationship among *Aphanius* species and subspecies in Turkey. The average gene diversity between populations was 0.243 and only 38.78% of this diversity occurred within populations. Genetic variation between populations was in general congruent with the most recent classification of *Aphanius* based on morphological characteristics and of mitochondrial DNA sequence data. RAPD analysis has been useful in study of phylogenetic relationship of *Aphanius* species and subspecies from Anatolia.

Key words: *Aphanius*, RAPD, systematics, Turkey, speciation.

Introduction

The killifish genus *Aphanius* (Nardo, 1827) is regarded as a Tethyan relict (KOSSWIG, 1967; POR & DIMENTMAN, 1989) whose members are thought to have evolved from a common ancestor distributed around the periphery of the Tethys Sea. This hypothesis is supported by a recent molecular analysis of mitochondrial DNA genes of the genus (HRBEK & MEYER, 2003). Based on geological evidence, the genus is approximately 37 million years old, with the oldest fossils being approximately 30 million years (GAUDANT, 1982). The central Anatolian assemblage consisting of *A. anatoliae* (Leidenfrost, 1912), *A. danfordii* (Boulenger, 1890) and *A. villwocki* (Hrbek et Wildekamp, 2003) is approximately 14 million years old, and forms a sister clade to *A. asquamatus* (Sözer, 1942) from E Turkey. The timing of the diversification of this clade is concordant with the geological history of Turkey as a result of accretion of several geological blocks and microcontinents (HRBEK & MEYER, 2003). The other two species of Turkey are *A. fasciatus* (Valenciennes, 1821) and *A. mento* (Heckel, 1843). Based on mitochondrial DNA analysis, *A. fasciatus* forms a sister clade to the all the C Anatolian species of *Aphanius* including *A. asquamatus*, and *A. mento* is only distantly related to the other Turkish species (HRBEK & MEYER, 2003).

Aphanius exhibits a complex mosaic of phenotypic forms. It has a basic morphology found

in majority of know species (WILDEKAMP et al., 1999), but there are also very distinct limnetic body forms that show distinct adaptations to open water habitats. Despite its phenotypic diversity, *Aphanius* has limited number of distinct morphological and phenotypical characteristics that could be used in their classification and phylogeny. The most recent morphological revision done by WILDEKAMP et al. (1999) suggested that the main morphological characters used in classification of *Aphanius*, namely coloration, scalation and morphology of conical teeth, are taxonomically unstable, and thus of limited use in systematics and classification.

One of the earliest attempts at inferring the systematic relationships of C Anatolian *Aphanius* was the study of VILLWOCK (1964). In a series of hybridization experiments VILLWOCK (1964) studied premating and postmating isolating mechanism among numerous populations of C Anatolian *Aphanius*. Results of hybridization experiments indicated three reproductive groups, namely Kızılırmak, C Anatolia and SW Anatolia. VILLWOCK (1964) regarded Kızılırmak population as *A. chantrei* (Gaillard, 1895) (synonym of *A. danfordii*) and both C Anatolian and W Anatolian populations as *A. anatoliae*.

A mitochondrial DNA phylogeny of C Anatolian *Aphanius* (HRBEK et al., 2002) is congruent with VILLWOCK's (1964) hybridization results, as well as geological history of Anatolia. It resulted in the identification of several phylogenetically distinct clades of *A. anatoliae*, however, it could not resolve the phylogenetic relationships among these clades with confidence. Nevertheless, mtDNA might have some disadvantages in studies of population differentiation compared with nuclear markers. This is because of evidence that gene flow can occur across hybrid zones, in the absence of nuclear gene flow, in some animals (FERRIS et al., 1983; TEGELSTROM, 1987; WALLIS & ARNTZEN, 1989). In addition, as mtDNA normally evolves rapidly at the sequence level, homoplasy can be a substantial problem where distantly related taxa are compared (AVISE, 1994).

In this study, RAPD analysis was performed to clarify systematics and phylogenetic relationships within the genus *Aphanius* from Turkey. To date there are no studies done on genomic DNA of *Aphanius* that could be used for their systematic and phylogeny. Since there is no genomic sequence information of *Aphanius*, it is convenient to use RAPD analysis (WILLIAMS et al., 1990) for detection of nuclear DNA variation. There are also several other reasons why this technique may offer a more sensitive approach to this problem. As it was shown, RAPD markers may be useful for determination of taxonomic identity, establishment of systematic relationships and assessment of genetic differentiation of plants and animals (for reviews, see HADRYS et al., 1992; SMITH & WILLIAMS, 1994) including fishes (e.g. BARDAKCI & SKIBINSKI, 1994; BOROWSKY et al., 1995; NAISH et al., 1995). On the other hand, the suitability of RAPD data for phylogenetic inference is debatable. The obvious drawbacks are the persistence of artifactual non-reproducible bands, non-homology of amplifed products and high levels of intrapopulation polymorphism in some cases (SMITH et al., 1994; LAMBOY, 1994). Nevertheless, some authors advocate the application of cladistic methods for the analysis of RAPD markers emphasizing that resulting phylogenies are in agreement with those obtained on the basis of other types of data (e.g. BOROWSKY et al., 1995; RAAMSDONK et al., 1997). In addition, RAPD has advantages over the most other methods as it uses a smaller quantity of DNA and it is less expensive in terms of both cost and labour.

Material and methods

Fish samples

A total of eight populations representing species and subspecies from Turkey have been studied. Species and subspecies used in this study and their sites are given in Table 1, and indicated on a map (Fig. 1). Fish samples were collected and preserved in 95% ethyl alcohol.

DNA extraction and RAPD reaction

Approximately 50 mg muscle tissue from specimens was used for extraction of total genomic DNA. Muscle tissue was cut into small pieces in 500 μl STE buffer (0.1 M NaCl, 0.05 M Tris and 0.01 M EDTA, pH 8) and then incubated after adding 20 μl proteinase K (10 mg ml^{-1}) and 50 μl SDS (10%). Following incubation, DNA was extracted by a standard phenol-chloroform procedure and precipitated with absolute ethanol. Precipitated DNA was dissolved in distilled water and quantified at wavelength of 260 nm by a spectrophotometer.

RAPD reaction mixture (20 μl final volume) contained 100 μM each dATP, dCTP, dTTP and dGTP (Sigma), 1× *Taq* polymerase buffer (Sigma), 1.5 mM $MgCl_2$, 0.5 units of *Taq* DNA polymerase (Sigma), 5 pmol of primer and 50 ng of genomic DNA overlaid with the same volume of mineral oil to prevent evaporation. Enzymatic amplification was performed in a thermal cycler (Stuart, UK) for 40 cycles as follows: 30 sec at 95 °C for denaturation, 30 sec at 35 °C for annealing and 60 sec at 72 °C for extension.

RAPD primers were obtained from Operon Technologies Inc. (Alameda Calif., USA). The nucleotide

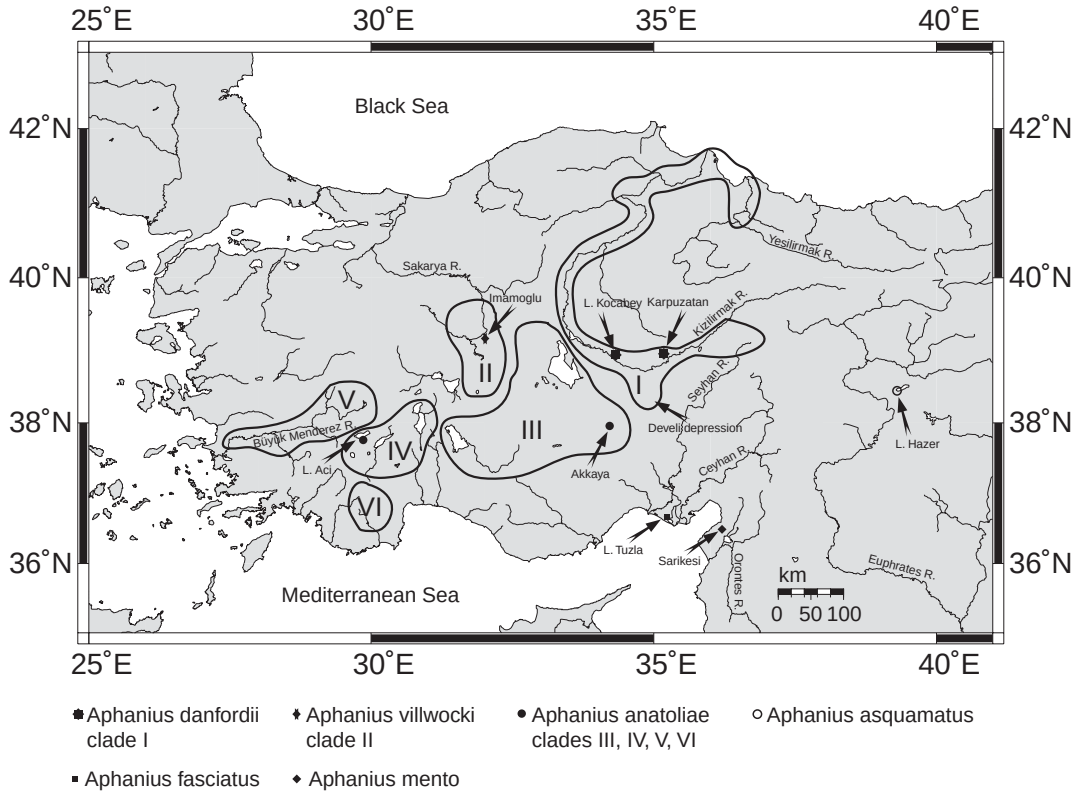

Fig. 1. Distribution map of the C Anatolian species complex of *Aphanius*. Approximate distribution ranges are from WILDEKAMP et al. (1999) and HRBEK et al. (2002). Symbols indicate collecting sites of the species and populations analyzed in this study.

Table 1. Species and subspecies used in this study.

Species and subspecies	Sites	Geographic co-ordinates
Aphanius asquamatus	Lake Hazer, Elazığ	38′27′46″ N, 39′17′59″ E
A. fasciatus	Lake Tuzla, Adana	36′40′55″ N, 35′05′02″ E
A. danfordii (1)	Karpuzatan by Kayseri	38′46′13″ N, 35′27′19″ E
A. danfordii (2)	Gözler, Kocabey, Kırşehir	38′59′15″ N, 34′06′58″ E
A. anatoliae anatoliae	Akkaya Dam near Niğde	37′55′50″ N, 34′36′38″ E
A. a. transgrediens	Lake Acı, Kırkpınar, Konya	37′49′21″ N, 29′56′04″ E
A. villwocki	Imamoğlu creek near Hacıfakılı, Polatlı	38′53′35″ N, 21′58′33″ E
A. mento	Sarıkesi, İskenderun	36′40′32″ N, 36′13′14″ E

sequence of each 10-mer primer used is: OPA05 (5'-AGGGGGCTTG-3'), OPA07 (5'-GAAACGGGTG-3'), OPA08 (5'-GTGACGTAGG-3'), OPA12 (5'-TCGGCGATAG-3'), OPA17 (5'-GACCGCTTGT-3'), OPC05 (5'GATGACCGCC-3').

Electrophoresis and staining

A mix of 10 μl of amplification products and 2 μl of loading dye (50% glycerol, 0.1 M EDTA, 1% SDS and 0.1% bromophenol blue) was separated on 8% vertical nondenaturing polyacrylamide gel 1 x TBE buffer (0.089 M Tris, 0.089 M Boric acid, 0.001 M disodium EDTA). Electrophoresis was carried out at 5 mA cm^{-1} for 5 h. A thermostatically controlled refrigerated circulator (Grant Instrument Limited, Cambridge) was used to maintain a constant temperature of 20 °C.

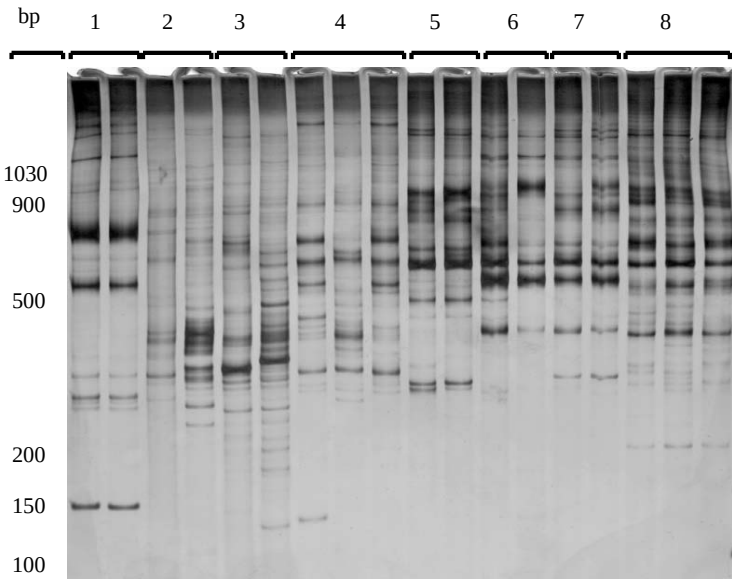

Fig. 2. RAPD electrophoretic pattern by using OPA07 primer on DNA from species and subspecies of *Aphanius* examined in this study. Lanes: 1 – *A. anatoliae anatoliae*; 2 – *A. danfordii* (from Kayseri); 3 – *A. danfordii* (from Kırşehir); 4 – *A. villwocki*; 5 – *A. anatoliae transgrediens*; 6 – *A. asquamatus*; 7 – *A. fasciatus*; 8 – *A. mento*.

Following the electrophoresis, gel were fixed with 10% ethanol and 0.5% acetic acid solution twice for 3 min, stained with 0.1 silver nitrate solution for 10 min, rinsed with distilled water, and then developed in alkaline solution (1.5% NaOH, 0.1% $NaBH_4$ and 0.15% CH_2O).

RAPD data analysis

Four individuals from each population were studied. The RAPD patterns within and between populations were compared. In order to overcome possible reproducibility problem of RAPD fragments and make comparison between individual RAPD banding patterns easier, electrophoresis of PCR products of all individuals from each population were carried out simultaneously. Fragments were assigned 1 if present or 0 if absent. RAPD data were analyzed using Popgene (version 1.31) computer program (Yang et al., 1999). For this analysis, it is assumed that the population is in Hardy-Weinberg equilibrium. Population divergence was examined by Nei's genetic identity and distance parameters (Nei, 1978) for all populations. Nei's genetic identity between populations X and Y with frequencies x_i and y_i of the ith allele at a particular locus is

$$I = \frac{J_{xy}}{\sqrt{\frac{2n_x\Sigma_i x_i^2 - 1}{2n_x - l}\ \frac{2n_z\Sigma_i y_i^2 - 1}{2n_y - l}}}$$

where J_{xy} is the arithmetic mean across loci of $\Sigma x_i y_i$ and n is the sample size. Genetic Distance (D) is $D = -\ln(I)$. Dendrograms based on Nei's genetic distances were constructed via the unweighed pair group method with arithmetic averages (UPGMA).

Shannon's index of phenotypic diversity (Lewontin, 1972) was used to proportion the diversity into within and between population components. Nei's gene diversity (Nei, 1973) and the proportion of polymorphic loci for each population were calculated.

Cladistic analysis should be based upon species (populations) rather than individuals, therefore data matrix for each individual was combined for each population as implemented in PAUP* (Phylogenetic Analysis Using Parsimony) (Swofford, 2001). Maximum parsimony-based phylogenetic relationships were estimated using PAUP* with exhaustive searches. Equal weight was given to all characters. Bootstrap resampling (Felsenstein, 1985) was applied to assess support for individual nodes using 2000 bootstrap replicates.

Results

Six primers were used to assess genetic relationships among populations representing *Aphanius* species and subspecies. All primers tested produced specific RAPD pattern consisting of 4 to 13 bands in each population studied. RAPD electrophoretic pattern by using OPA07 primer on DNA from species and subspecies of *Aphanius* examined in this study are given in Fig. 2. Nei's genetic variation and proportion of polymorphic loci within populations were computed. As shown in Table 2, *Aphanius danfordii* population from Lake Kocabey exhibits relatively high level of within population variability, but in contrast *Aphanius mento* was the population showing the lowest variability. The proportion of polymorphic loci detected for each population was also in agreement with results of gene variability for each population (Tab. 2). The proportion of polymorphic loci for

Table 2. Nei's (1973) gene diversity with standart deviations, number of polymorphic loci and proportion of polymorphic loci in populations examined.

Population	Gene diversity	No of polymorphic loci	Proportion of polymorphic loci (%)
Aphanius asquamatus	0.0574 ± 0.147	8	14.81
A. fasciatus	0.0509 ± 0.123	9	16.67
A. danfordii (1)	0.0547 ± 0.142	7	12.96
A. danfordii (2)	0.0929 ± 0.167	13	24.07
A. anatoliae anatoliae	0.0648 ± 0.144	10	18.52
A. a. transgrediens	0.0573 ± 0.130	10	18.52
A. villwocki	0.0544 ± 0.125	9	16.67
A. mento	0.0314 ± 0.1071	5	9.26

Table 3. The number of polymorphic loci, gene diversity and Shannon Index found for six primers for populations of *Aphanius* examined.

Primer	No of loci	Gene diversity	Shannon Index
OPA05	4	0.181	0.323
OPA07	13	0.244	0.379
OPA08	9	0.305	0.476
OPA12	8	0.266	0.419
OPA17	8	0.245	0.388
OPC05	12	0.212	0.341
Total	54	0.243	0.388

Table 4. Genetic identity values generated from Nei (1978) between populations of *Aphanius*.

Populations	1	2	3	4	5	6	7
1. *A. asquamatus*							
2. *A. a. anatoliae*	0.761						
3. *A. a. transgrediens*	0.765	0.858					
4. *A. fasciatus*	0.814	0.777	0.833				
5. *A. danfordii* (1)	0.660	0.787	0.855	0.727			
6. *A. danfordii* (2)	0.758	0.761	0.780	0.730	0.870		
7. *A. villwocki*	0.780	0.797	0.774	0.780	0.801	0.868	
8. *A. mento*	0.761	0.654	0.630	0.703	0.612	0.697	0.728

each population examined ranged from 9.26% for *A. mento* to 24.07% for *A. danfordii*.

The number of loci amplified by each primer and Nei's (1973) gene diversity is given in Table 3. Primer OPA07 and OPC05 produced respectively higher number of loci. However, the number of loci produced by OPA05 was as low as four. In addition to the number of loci, OPA08 detects the most and OPA05 the least variability among *Aphanius* populations. Shannon's index of phenotypic diversity was used to partition the diversity into within- and between-population components (Tab. 3). The results showed that average gene diversity between populations was 0.243. An examination of the proportion of diversity within populations and between populations indicates that, on average, most of the diversity within population occurs between *Aphanius* populations.

To complement the analysis based on phenotypic frequencies, Nei's estimate of genetic identity (Nei, 1978) was used to generate a genetic identity matrix (Tab. 4). The genetic identity values vary between 0.61 and 0.87 between populations. *Aphanius danfordii* population from Kay-

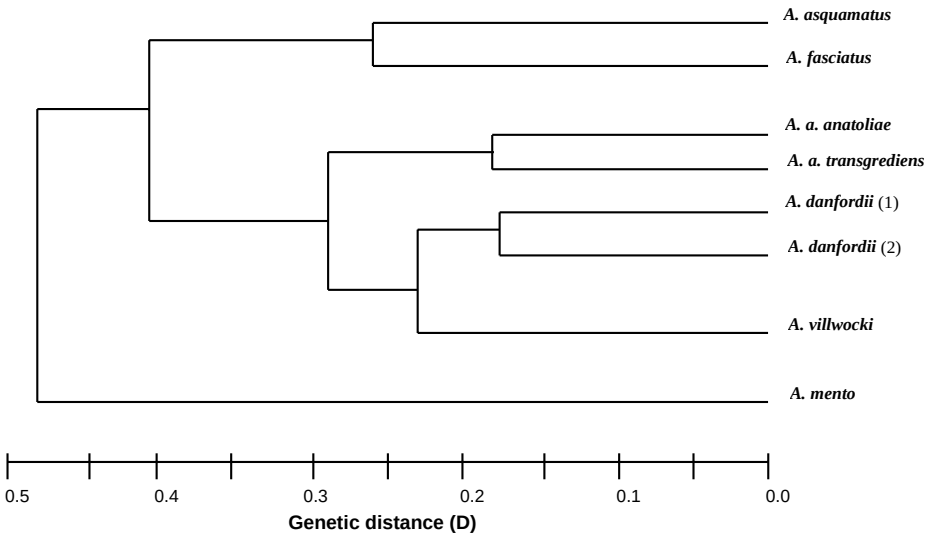

Fig. 3. UPGMA dendogram of species of *Aphanius* and subspecies of *A. anatoliae* based on values of genetic distance.

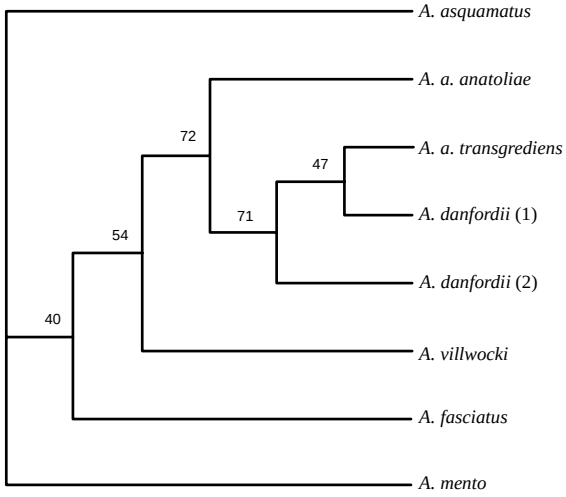

Fig. 4. Unrooted concensus tree generated by the parsimony analysis. The numbers above the branches are the values of bootstrap support (% of 2000 replicates).

seri and *A. danfordii* population from Kırşehir exhibit the highest similarity. On the other hand, *A. danfordii* population was least similar to *A. mento* population. A dendogram based on UPGMA cluster analysis using combined data of RAPD was used to show genetic relationships among populations of *Aphanius* (Fig. 3). Two populations of *A. a. anotoliae* clustered together and occurred as a sister taxa with *A. danfordii* populations.

Parsimony analysis was carried out in order to infer phylogenetic relationships among species and subspecies of *Aphanius*. Exhaustive topological search in PAUP (Swofford, 2001) produced eight equally parsimonius trees with the length of 40 steps. The concensus tree showed similar branching pattern with the UPGMA dendogram among the populations of *Aphanius* (Fig. 3). The bootstrap support for each branching was rather low. However, the standard cladistic indices for this tree were high (Consistancy Index = 0.825; Retention Index = 0.632) indicating low amount of homoplasy. Result of both distance-based and parsimony analyses showed that *A. mento* population is the most distinct from other species (Figs 3, 4).

Discussion

It has been suggested that the center of diversity of the killifish genus *Aphanius* (order: Cyprinodontiformes) is in Turkey (Wildekamp et al., 1999). They have been regarded as Tethyan relicts and therefore their distribution has been related with the closing of the Tethys Sea (e.g. Kosswig, 1967; Hrbek & Meyer, 2003). Of the 14 described species in the genus, six species and four subspecies occur in Turkey (Wildekamp et al., 1999; Hrbek et al., 2002).

Genetic variation among populations of *Aphanius* examined was higher than within population variability (Tab. 3). This result suggests that

there exists a limited gene flow among populations of *Aphanius* in question. This was an expected result as *Aphanius* fishes live in small water bodies such as small creeks, marshes and spring that undergoes seasonal fluctuations of temperatures and water levels. As a result of extreme conditions of their habitats, it is likely that many of the populations could periodically undergo dramatic reductions in population sizes. Periodic bottleneck would not only reduce intrapopulation variability, but would also lead to differentiation of populations as a result of genetic drift. In addition to natural bottlenecking event, a number of populations of *Aphanius* have drastically reduced census number or are now extinct. This appears to be largely due to agricultural pollution, pumping of water for irrigation, and the introduction of *Gambusia affinis* (Baird et Girard, 1853). For example, *A. danfordii* population from Karpuzatan lives in a swamp where most of its water evaporates during the summer leading to a substantial decrease in the population size as observed during field trips. Additionally it seems, it only forms a small percentage of the fish biomass, while the introduced *Gambusia affinis* predominates. On the other hand, small sample size for each population might not be sufficient to reveal actual genetic variation existing within populations.

Limited number of distinct morphological and phenotypical characteristics exists in Turkish *Aphanius* species, therefore there are a few diagnostic morphological differences among species. Classification of the genus *Aphanius* is based mainly on scalation, the number and shape of cross-bars and the characteristics of teeth, all of which are regarded as morphologically unstable characters (Wildekamp et al., 1999). Therefore molecular data are likely to be much better suited for the determination of phylogenetic relationships of C Anatolian *Aphanius* species. RAPD approach is suggested to be more useful in closely related populations (Smith & Williams, 1994; Borowsky et al., 1995).

Hrbek et al. (2002) have used mitochondrial DNA sequence information to study genetic relationship among populations of Anatolian *Aphanius*. We used RAPD markers to measure genomic DNA variation between populations representing six species of *Aphanius* including two subspecies of *A. a. anatoliae* and two populations of *A. danfordii*. Genetic variation between populations and among population relationships inferred from RAPD data was in general congruent with the recent classification by Wildekamp et al. (1999) and the results of Hrbek et al. (2002). Populations belonging to *A. anatoliae* subspecies, as well as *A. danfordii* from Kayseri and Kocabey Lake, were closely related. On the other hand, as can be seen from Fig. 3, *A. mento* from south of Turkey is the most distinct from the other species examined in this study. *A. villwocki* species is morphologically and phenotypically distinct from other C Anatolian species (Wildekamp et al., 1999) and appears to be reproductive isolated form of other C Anatolian clades (Villwock, 1964), however, its membership within the C Anatolian assemblage is strongly supported (Hrbek et al, 2002). Our RAPD analyses are well in agreement with mitochondrial DNA results in Hrbek et al. (2002).

Populations representing *A. danfordii* also showed close similarity with *A. villwocki*. Parsimony analysis showed a strong support for this relationship with bootstrap value of 72%. This relationship is quite usual and supported by mitochondrial DNA data (Hrbek & Meyer, 2003), and it is in agreement with the geological history of C Anatolia.

Based on our results, RAPD analysis has been useful in the study of genetic relationships of *Aphanius* populations representing species and subspecies from Anatolia. Electrophoretic patterns of RAPD products could radically be altered by small alterations in PCR parameters or quality and quantity of target DNA (e.g. Williams et al., 1993; Devos & Gale, 1992). Thus there may be reason to view with caution systematic conclusion based on the RAPD analysis alone. On the other hand, this approach is a good choice if there is no genomic DNA sequence information of the species of interest, funds are limited, or one needs to generate data rapidly.

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