

# Closing of the Tethys Sea and the phylogeny of Eurasian killifishes (Cyprinodontiformes: Cyprinodontidae)

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## Keywords:

*Aphanius* sp. complex;  
extinction;  
historical biogeography;  
Messinian Salinity Crisis;  
molecular phylogeny;  
mtDNA;  
Tethys Sea;  
vicariant speciation.

## Abstract

To test vicariant speciation hypotheses derived from geological evidence of the closing of the Tethys Sea, we reconstruct phylogenetic relationships of the predominantly fresh-water killifish genus *Aphanius* using 3263 aligned base pairs of mitochondrial DNA from samples representing 49 populations of 13 species. We use additional 11 cyprinodontid species as outgroup taxa. Genes analysed include those encoding the partial 12S and 16S ribosomal RNAs; transfer RNAs for valine, leucine, isoleucine, glutamine, methionine, tryptophan, alanine, asparagine, cysteine and tyrosine; and complete nicotinamide adenine dinucleotide dehydrogenase subunit I and II. Molecular substitution rate for this DNA region is estimated at of  $8.6 \pm 0.1 \times 10^{-9}$  substitutions base pair $^{-1}$  year $^{-1}$ , and is derived from a well dated transgression of the Red Sea into the Wadi Sirhan of Jordan 13 million years ago; an alternate substitution rate of  $1.1 \pm 0.2 \times 10^{-8}$  substitutions base pair $^{-1}$  year $^{-1}$  is estimated from fossil evidence. *Aphanius* forms two major clades which correspond to the former eastern and western Tethys Sea. Within the eastern clade Oligocene divergence into a fresh-water clade inhabiting the Arabian Peninsula and an euhaline clade inhabiting coastal area from Pakistan to Somalia is observed. Within the western Tethys Sea clade we observe a middle Oligocene divergence into Iberian Peninsula and Atlas Mountains, and Turkey and Iran sections. Within Turkey we observe a large amount of genetic differentiation correlated with late Miocene orogenic events. Based on concordance of patterns of phylogenetic relationships and area relationships derived from geological and fossil data, as well as temporal congruence of these patterns, we support a predominantly vicariant-based speciation hypothesis for the genus *Aphanius*. An exception to this pattern forms the main clade of *A. fasciatus*, an euhaline circum-Mediterranean species, which shows little genetic differentiation or population structuring, thus providing no support for the hypothesis of vicariant differentiation associated with the Messinian Salinity Crisis. The two phylogenetically deepest events were also likely driven by ecological changes associated with the closing of the Tethys Sea.

## Introduction

The closing of the Tethys Sea near the Oligocene / Miocene boundary had a major impact on the distribution of organismal diversity. For the first time since the break-up

of Pangea 200 million years ago (MYA) (Smith *et al.*, 1995), Gondwanan (Africa) elements became united with Laurasian (Eurasia) elements causing the now well documented faunal and floral exchanges among the regions (Brown & Lomolito, 1998). Severing of the marine seaway also terminated the exchange of tropical marine elements between the Atlantic and the Indian oceans. Much less studied, although likely to have had an equally important impact on the organismal diversity are the geological events resulting from the closing of the

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Tethys Sea. The formation and isolation of many new geological units likely resulted in the vicariant speciation of whole faunas and floras; however, only limited data testing this hypothesis exist, and most studies are of restricted geographical scope (e.g. Caccone *et al.*, 1997; Macey *et al.*, 1998; Penzo *et al.*, 1998; Veith *et al.*, 1998; Zardoya & Doadrio, 1999; Weisrock *et al.*, 2001).

It has been hypothesized (e.g. Kosswig, 1967) that aphaniiine killifishes are Tethyan relicts whose distribution can best be explained by the closing of the Tethys Sea. All 14 contemporary species, and 10 fossil relatives of the Cyprinodontiform genus *Aphanius* Nardo 1827 are widely distributed along the late period Tethys Sea coastlines. The present-day distribution includes coastal areas of the Mediterranean region, and coastal areas from the Gir peninsula of north-western India to north-eastern Somalia including the Red Sea and the Persian Gulf. Inland distribution is restricted primarily to the Mediterranean and Near Eastern orogenic belts, including Turkey and Iran. These areas include all of the regions impacted by the closing of the Tethys Sea. With this motivation we test the role of the closing of the Tethys Sea on the distribution and speciation in the killifish genus *Aphanius*.

### The study system – genus *Aphanius*

The genus *Aphanius* can be divided into two main ecological groups: species inhabiting brackish water and euhaline coastal environments, and species inhabiting fresh-water oases, and springs, creeks, marshes and lakes in landlocked basins. Of the two brackish water species, *A. fasciatus* occupies the central and western portions of the Mediterranean basin; however, several fresh-water isolates are known in, for example, Lake Bafa of Turkey, and north African oases. The second brackish water species, *A. dispar*, is present in the areas around the Red Sea, the Persian Gulf and portions of the Arabian Sea. Populations of *A. dispar* also occur in the south-eastern section of the Mediterranean basin, both in brackish water environments, as well as in Egyptian (together with *A. fasciatus*) and Arabian fresh-water oases, and springs of the hypersaline Dead Sea Valley. Ben-Tuvia (1966, 1971) suggested their presence in the extreme south-eastern part of the Mediterranean Sea as the result of a successful migration through the Suez Canal, whereas Kornfield & Nevo (1976) suggest a pre-Pleistocene dispersal through the Dead Sea Valley.

*Aphanius iberus* from the Iberian Peninsula and the western Maghreb inhabits both fresh-water as well as saline habitats. A second species, *A. apodus*, is found in the Tellian portion of the Atlas Mountains and is strictly fresh-water. The remaining nine other fresh-water species are found in Asia. *Aphanius mento* is found in the Tigris and Euphrates drainage basins, as well as in the Dead Sea Valley, the Orontes, Ceyhan and Seyhan drainages and few additional minor coastal drainages of

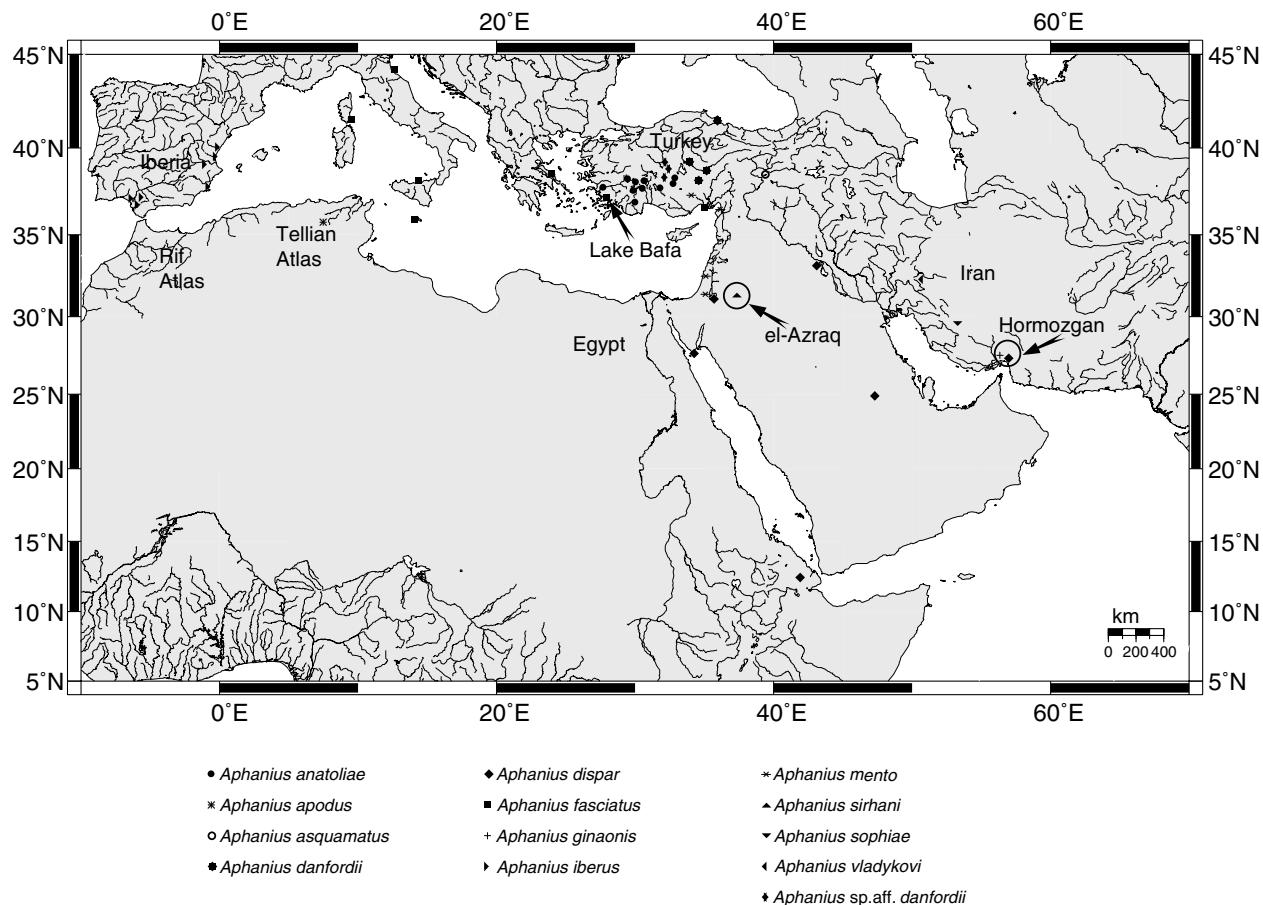
southern Turkey. The most diverse group of *Aphanius* species occurs in the mountainous regions of central Anatolia, Turkey, and the Iranian plateau. This region contains six species and three subspecies, and likely four additional undescribed species from Iran (Coad, 2000). In Central Anatolia some populations of *A. anatoliae* have adapted to such extreme environments as saline and bitter lakes. The recently described *Lebias stiassnyae* appears to be sympatric with *A. dispar* in hypersaline Lake Afdera, Ethiopia, and has been suggested to originate *in situ* (Getahun & Lazara, 2001). The generic names *Lebias* and *Aphanius* are synonyms, and debate whether *Lebias* is a junior synonym of *Aphanius*, or if *Aphanius* is a junior synonym of *Lebias* is ongoing (Lazara, 1995; Wildekamp *et al.*, 1999). *Lebias* has not been used since the beginning of this century, therefore we continue to use the traditional generic name *Aphanius*. Figure 1 shows the distribution and sampling localities of *Aphanius*. Additional information on the distribution and taxonomy of *Aphanius* may be found in the publications of Wildekamp and collaborators (Wildekamp, 1996; Wildekamp *et al.*, 1999), Lazara (1995), and Villwock and collaborators (Villwock, 1958, 1963, 1964, 1966; Scholl *et al.*, 1978; Villwock *et al.*, 1983).

### Tethyan and Mediterranean geology

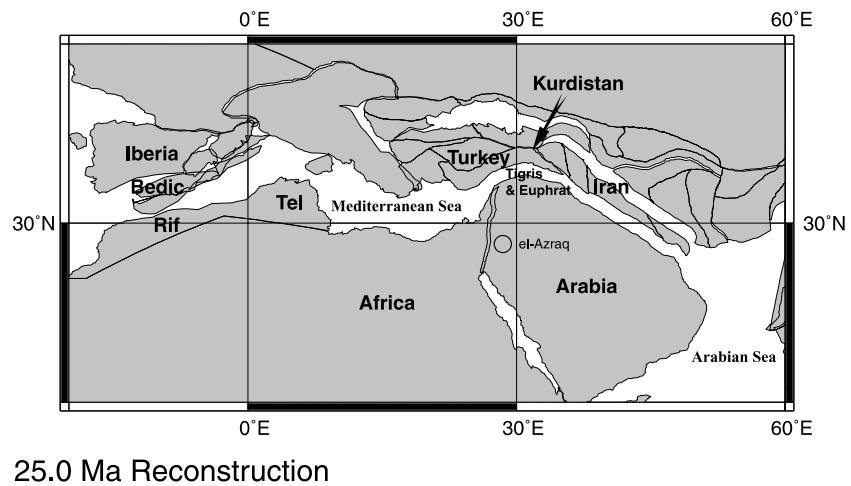
Based on geological evidence derived from the closing of the Tethys Sea, we propose a testable phylogenetic hypothesis for *Aphanius*, which may be generally applicable for other Tethyan relict faunas. The geology of the region is graphically summarized in Fig. 2. Assuming that the ancestral *Aphanius* species inhabited the coastal areas of the Tethys (Kosswig, 1967), final closing of the Tethys approximately 20 MYA (Steininger & Rögl, 1984; Dercourt *et al.*, 1986) would have separated the ancestral population into an eastern and a western group. At that same time the formation of the Red Sea was initiated (Coleman, 1993).

Within the eastern group, we hypothesize that the ancestor of *A. mento* invaded the northern margins of the Arabian plate, and then spread throughout the current and palaeo-drainage basins of the Tigris–Euphrates rivers, resulting in the divergence of *A. mento* from the other members of the eastern group. Thirteen million years ago (Bender, 1968), a major transgression of the Red Sea into the Wadi Sirhan basin of Jordan occurred. Villwock *et al.* (1983) hypothesized that this transgression resulted in the divergence of *A. sirhani* from the ancestor of *A. dispar* (Fig. 3).

The geology of the western Tethyan region is more complicated. The Atlas Mountains are composed of uplifted Tethys seafloor, and the Moroccan and Oran Mesetas (microcontinents) (Sengör *et al.*, 1988); they are divided into the Tellian and the Rif Atlas regions. The related Bedic system of southern Spain is entirely composed of uplifted Tethys seafloor. Compaction and



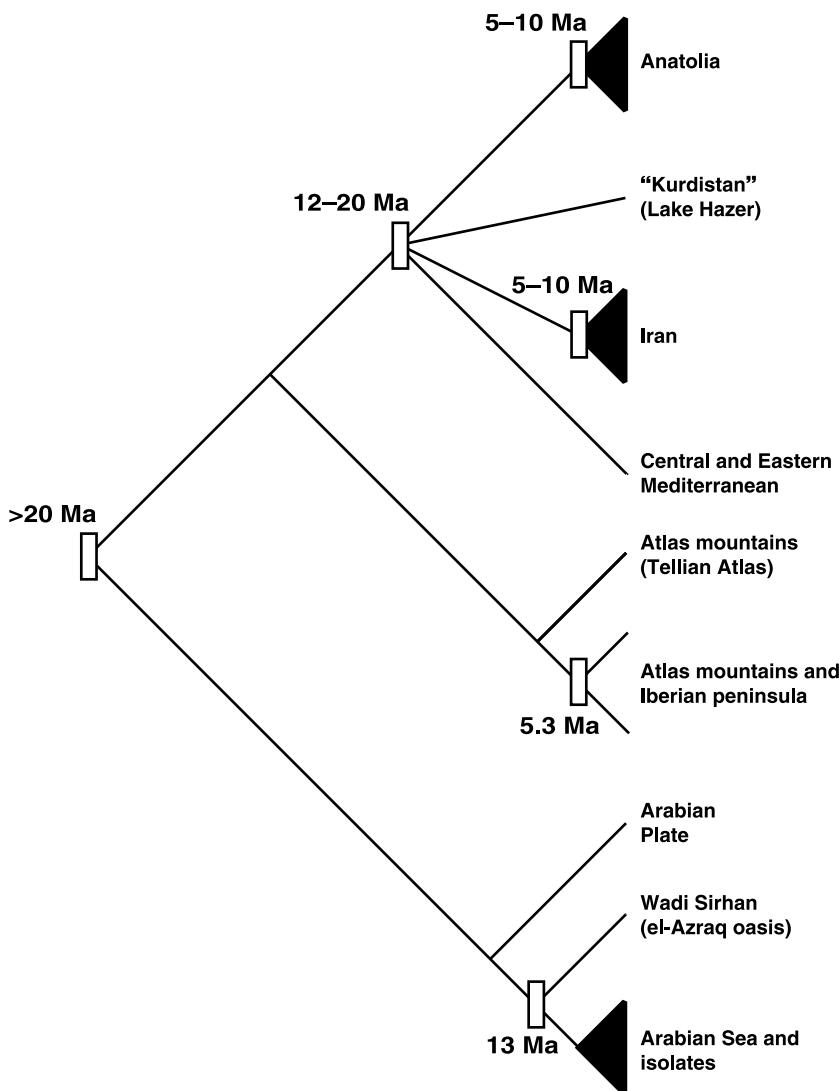
**Fig. 1** Distribution area of the killifishes of the genus *Aphanianus*. Localities included in this study are marked by species specific symbols.



**Fig. 2** A schematic map of the Mediterranean region 25 MYA. Blocks and geological regions discussed in the introduction are labelled except those in Turkey and Iran, where the whole region is labelled. Remnants of the Tethys Sea is the region between the Mediterranean and the Arabian Seas. Closing of the Tethys proceeded by northward movement of the African and Arabian plates, and simultaneous counter clockwise rotation of the Arabian plate.

orogeny of this region started in the middle Oligocene (~30 MYA), and in various stages continued until the early Pliocene (~8 MYA). Final emergence occurred with the closing of the Bedic (Garcés *et al.*, 1998) and Rif (Krijgsman *et al.*, 1999b) corridors in Spain and Morocco,

respectively. These events initiated the start of the Messinian salinity crisis 5.96 MYA (Krijgsman *et al.*, 1999a) during which faunal exchange between the Iberian Peninsula and the Atlas could occur (Steininger *et al.*, 1985). Based on this scenario, we hypothesize that



**Fig. 3** A hypothesis of area relationships among various portions derived from or formed by the closing of the former Tethys Sea at the Oligocene/Miocene boundary. Hypothesized dates of separation of areas from one another are based on available geological data listed in the text.

populations of *A. iberus* and *A. apodus* became isolated on different geological blocks from the brackish water ancestor similar to *A. fasciatus* sometimes after the late Oligocene, with *A. iberus* dispersing between the Iberian Peninsula and the Atlas Mountains after the beginning of the Messinian 5.96 MYA; however, all connections between north African and Iberian populations would have been severed by the formation of the Strait of Gibraltar at the end of the Messinian 5.33 MYA (Krijgsman *et al.*, 1999a) (Fig. 3).

The geological history of the Anatolian–Zagros mountain chain is also complex. This region is composed of several small plates, most significantly of the Sakarya continent, the Pondites, the Kirşehir, Menderez-Taurus and East Taurus blocks in Turkey, and the Iran, Lut, Helmand and Farah plates in Iran, and intervening uplifted regions such as ‘Kurdistan’, and the Lesser and Greater Caucasus mountains. These microplates were

consolidated and compressed into one landmass by the effects of the northward drifting Arabian Peninsula.

The conglomeration of central Anatolia began by the early middle Eocene (~50 MYA), although significant isolation of geological groups within Anatolia likely did not occur much before 10 MYA (Sengör & Yilmaz, 1981; Görür *et al.*, 1984). The killifish containing regions are the Kirşehir and the Menderez-Taurus blocks, and the Sakarya continent, with the Tuzgölü and Haymana basins trapped between them (Sengör & Yilmaz, 1981; Görür *et al.*, 1984). Especially, the Menderez-Taurus block has undergone significant folding and uplift during the last 5–10 MYA as a result of the acceleration of northward movement of the Arabian plate (Quennell, 1984; Steininger & Rögl, 1984). The Tuzgölü and the Haymana basins have acted as catchment areas of the Menderez-Taurus block and the Sakarya continent throughout the geological

evolution of the region (Görür *et al.*, 1984). It is unclear what should be the biogeographical relationships within Anatolia. Nevertheless, Anatolia is likely to be monophyletic and *Aphanius* populations inhabiting the four main regions are likely to be highly divergent from each other (Fig. 3).

The geology of Iran suggests rapid isolations of multiple areas from one another. Extensive indentation of the Arabian plate into the Iranian plate starting 10 MYA (Dercourt *et al.*, 1986) caused the uplift of the Zagros mountains at the southern edge of the Iranian plate. Continued north-eastern movement of the Arabian plate and a northerly movement of India resulted in additional mountain building by 5 MYA (Dercourt *et al.*, 1986) along the northern edge of the Iranian plateau as well as along the sutures of the Iranian, Lut, Helmand and Farah plates which compose present-day Iran. Based on this evidence, we hypothesize that the various *Aphanius* populations occurring in isolated basins of the Zagros, Little Caucasus and the Elburz mountains, and the intervening Iranian plateau form a monophyletic group; however, relationships among these populations are likely to approximate a star-like radiation (Fig. 3).

The middle Miocene also saw extensive mountain building in 'Kurdistan', the mountainous border areas of Turkey, Iran and Iraq, and culminated in the complete emersion of the Maden complex of south-eastern Turkey by 12 MYA (Aktas & Robertson, 1984; Dercourt *et al.*, 1986). Lake Hazer and its drainages which are nestled within the Maden complex therefore are estimated to have formed approximately 12 MYA, whereas the final separation of Anatolia from Iran began as early as the early to middle Miocene (~20 MYA) (Dercourt *et al.*, 1986). The relationship of the hypothesized brackish water ancestor similar to *A. fasciatus* to these three regions is difficult to predict.

Based on these data we hypothesize that the initial division within *Aphanius* separated the brackish water ancestor into the eastern and the western clades. Within each clade the brackish water ancestor gave rise to landlocked species, some of them speciating further. A complete area-relationship hypothesis derived from available geological data on the closing of the Tethys Sea is summarized in Fig. 3.

Neither molecular nor morphological phylogenies of *Aphanius* suitable for the study of Tethyan vicariance exist. A morphological phylogeny generated by Parenti (1981), is insufficiently detailed to test Tethyan hypotheses as it focuses on upper-level Cyprinodontiform relationships. The only available molecular data are those of Parker & Kornfield (1995); however, their study investigated the sister taxon relationship of the *Orestias* species from the South American Andes mountains and *Aphanius* species (Parenti, 1981), and lacks the species sampling necessary to test Tethyan vicariance hypotheses. A well supported phylogeny of the genus *Aphanius* is therefore warranted.

## Materials and methods

### Selection of ingroup and outgroup taxa

All species and locations used in this study are listed in Table 1. Whenever possible, more than one population per species and at least two individuals per population were included. The sample included morphologically diverse species (*A. iberus* – seven populations, *A. anatoliæ* – 12 populations, *A. danfordii* – four populations, *A. sp. aff. danfordii* – three populations, *A. mento* – four populations) and geographically diverse species (*A. fasciatus* – seven populations, *A. dispar* – six populations). Some species are only known from a single locality (*A. sirhani* – el-Azraq Oasis, *A. asquamatus* – Lake Hazer, *A. ginaonis* – Ginao Hot Springs), or are extinct in nature and only one aquarium population exists (*A. apodus* – Ain M'Lila, most likely extinct since 1989). *Aphanius sophiae* and *A. vladikovi* could only be obtained from the Kor River and Boldaji basins, Iran, respectively. The recently described *A. stiassnyae* could not be obtained for analyses, but geographically proximate *A. dispar* population was included in this study.

The phylogenetic relationships of the Cyprinodontiformes are in flux and thus the choice of the closest outgroups is not obvious. The outgroup genera and species were selected to give a good representation of observed morphological, taxonomical and geographical diversity (Parenti, 1981; Meyer & Lydeard, 1993; Costa, 1997). The Aplocheiloidei are clearly a sister group to all other Cyprinodontiformes (Parenti, 1981), and therefore were not included among the outgroups. The outgroups selected included genera from Europe (*Valencia*), North America (*Cyprinodon*, *Jordanella* and *Floridichthys*), Central America (*Oxyzygonectes*) and South America (*Orestias*, *Anableps* and *Jenynsia*).

### Laboratory protocols

Total genomic DNA was extracted from muscle tissue of the right caudal peduncle of specimens preserved in 95% ethanol. Muscle tissue was dissolved and digested with a Proteinase K/SDS solution, followed by phenol and chloroform extraction, the addition of 5 M NaCl followed by 70% ethanol precipitation of DNA product.

Polymerase chain reaction (PCR) amplification was performed on total genomic DNA. Negative controls were performed for all reactions. The temperature profile for the 30-cycle amplification reaction consisted of denaturation at 94 °C for 35 s, annealing at 50 °C for 35 s, and extension at 72 °C for 90 s per cycle. The resulting products were evaluated on a 1% agarose gel, and then purified with Qiagen spin-columns (Qiagen, Hilden, Germany). Amplified mitochondrial deoxyribonucleic acid (mtDNA) segments were sequenced from both the 5' and 3' ends. Sequencing reactions followed standard Perkin Elmer Big Dye sequencing protocol for double-stranded cycle

**Table 1** Species and localities included in this study. GenBank accession numbers are listed for the 16S – COI, and then the 12S–16S fragments, respectively.

Species	Population	GenBank no.
<i>Aphanius a. splendens</i>	Lake Salda, Turkey	AF449287, AF449349
<i>A. a. sureyanus</i>	Lake Burdur, Turkey	AF449288, AF449350
<i>A. a. transgrediens</i> (1)	Lake Acı (spring 6), Turkey	AF449289, AF449351
<i>A. a. transgrediens</i> (2)	Lake Acı (spring 1), Turkey	AF449290, AF449352
<i>A. a. anatoliae</i> (1)	Karapınar near Yeşilova, Turkey	AF449291, AF449353
<i>A. a. anatoliae</i> (2)	Lake Eğirdir at Karaot, Turkey	AF449292, AF449354
<i>A. a. anatoliae</i> (3)	Lake İslaklı near Beydilli, Turkey	AF449293, AF449355
<i>A. a. anatoliae</i> (4)	Karakuyu wetland near Dinar, Turkey	AF449294, AF449356
<i>A. a. anatoliae</i> (5)	Akçay near Nazılı, Turkey	AF449295, AF449357
<i>A. a. anatoliae</i> (6)	Konya, Turkey	AF449296, AF449358
<i>A. a. anatoliae</i> (7)	Thaliye Canal by Sulaması, Turkey	AF449297, AF449359
<i>A. a. anatoliae</i> (8)	Lake Beyşehir at Kanlibogaz, Turkey	AF449298, AF449360
<i>Aphanius danfordii</i> (1)	Sosyalı by Sultan Swamps, Turkey	AF449299, AF449361
<i>A. danfordii</i> (2)	Kızılırmak River delta, Turkey	AF449300, AF449362
<i>A. danfordii</i> (3)	Gözler by Kırşehir, Turkey	AF449301, AF449363
<i>A. danfordii</i> (4)	Karpuzatan by Kayseri, Turkey	AF449302, AF449364
<i>Aphanius</i> sp. aff. <i>danfordii</i> (1)	Pınarbaşı in upper Sakarya River, Turkey	AF449303, AF449365
<i>Aphanius</i> sp. aff. <i>danfordii</i> (2)	Lake Çavuşçu, Turkey	AF449304, AF449366
<i>Aphanius</i> sp. aff. <i>danfordii</i> (3)	Ahiler in upper Sakarya River, Turkey	AF449305, AF449367
<i>A. asquamatus</i>	Lake Hazer, Turkey	AF449306, AF449368
<i>A. fasciatus</i> (1)	Lake Bafa, Turkey	AF449307, AF449369
<i>A. fasciatus</i> (2)	Lake Prokopis, Greece	AF449308, AF449370
<i>A. fasciatus</i> (3)	Ravenna salina, Italy	AF449309, AF449371
<i>A. fasciatus</i> (4)	Lake Tuzla near Tarsus, Turkey	AF449310, AF449372
<i>A. fasciatus</i> (5)	Porto Vecchio, Corsica	AF449311, AF449373
<i>A. fasciatus</i> (6)	Marsascala, Malta	AF449312, AF449374
<i>A. fasciatus</i> (7)	Palermo, Sicily	AF449313, AF449375
<i>A. sophiae</i>	Lake Maharloo near Shiraz, Iran	AF449314, AF449376
<i>A. vladydoi</i>	Boldaji basin, Iran	AF449315, AF449377
<i>A. iberus</i> (1)	El Rioco, Spain	AF449316, AF449378
<i>A. iberus</i> (2)	Las Cabezas de San Juan, Spain	AF449317, AF449379
<i>A. iberus</i> (3)	Rio Roche, Spain	AF449318, AF449380
<i>A. iberus</i> (4)	Villena, Spain	AF449319, AF449381
<i>A. iberus</i> (5)	Valencia, Spain	AF449320, AF449382
<i>A. iberus</i> (6)	Mar Menor, Spain	AF449321, AF449383
<i>A. iberus</i> (7)	Rio Ter near Estartit, Spain	AF449322, AF449384
<i>A. apodus</i>	Aïn M'Lila, Algeria	AF449323, AF449385
<i>A. mento</i> (1)	Haifa, Israel	AF449324, AF449386
<i>A. mento</i> (2)	Ein Faska, Israel	AF449325, AF449387
<i>A. mento</i> (3)	Kızılca near Bor, Turkey	AF449326, AF449388
<i>A. mento</i> (4)	Lake Yeşilada near Samandag, Turkey	AF449327, AF449389
<i>A. sirhani</i>	el-Azraq Oasis, Wadi Sirhan, Jordan	AF449328, AF449390
<i>A. d. richardsoni</i>	Ein Faska, Israel	AF449329, AF449391
<i>A. d. dispar</i> (1)	Aïn Sokhna, Red Sea, Egypt	AF449330, AF449392
<i>A. d. dispar</i> (2)	Lake Assal, Afars depression, Djibouti	AF449331, AF449393
<i>A. d. dispar</i> (3)	Al Khari oasis, Saudi Arabia	AF449332, AF449394
<i>A. d. dispar</i> (4)	Faluja, Iraq	AF449333, AF449395
<i>A. d. dispar</i> (5)	Bandar-e Abbas, Hormozgan, Iran	AF449334, AF449396
<i>A. ginaonis</i>	Ginao Hot Springs, Hormozgan, Iran	AF449335, AF449397
<i>Valencia letourneuxi</i> (1)	Dhassia, Corfu	AF449336, AF449398
<i>V. letourneuxi</i> (2)	Igoumenitsa Thiamis, Greece	AF449337, AF449399
<i>V. hispanica</i> (1)	Marchal de Pego-Oliva, Spain	AF449338, AF449400
<i>V. hispanica</i> (2)	Albuixec, Spain	AF449339, AF449401
<i>Oxyzygonectes dovii</i>	Golfito, Panama	AF449340, AF449402
<i>Anableps anableps</i>	Demarara river, Georgetown, Guyana	AF449341, AF449403
<i>Jenynsia maculata</i>	San Carlos, Uruguay	AF449342, AF449404
<i>Jordanella floridae</i>	Lake Okefenokee, Florida, USA	AF449343, AF449405
<i>Cyprinodon variegatus</i>	Lake Ponchartrain, Louisiana, USA	AF449344, AF449406
<i>Floridichthys carpio</i>	Tampa Bay, Florida, USA	AF449345, AF449407
<i>Orestias agassizii</i>	Lake Titicaca, Bolivia	AF449346, AF449408
<i>O. gilsoni</i>	Lake Titicaca, Bolivia	AF449347, AF449409
<i>O. imparae</i>	Lake Titicaca, Bolivia	AF449348, AF449410

sequencing reactions. Sequences were determined on a Perkin Elmer ABI 3100 automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA). Amplification and sequencing primers were taken from the literature or designed specifically for this study (Table 2). The DNA sequences used in this study consisted of genes encoding part of 12S and 16S rRNA, complete sequences of ND1 and ND2, transfer RNAs for valine, leucine, isoleucine, glutamine, methionine, tryptophan, alanine, asparagine, cysteine and tyrosine, and the light-strand replication origin. The gene regions were selected to include both faster and slower evolving regions in order to maximize the likelihood of obtaining phylogenetic resolution of recent as well as more ancient cladogenic events.

## Data analysis

Orthologous protein-coding regions (ND1, ND2) were aligned manually and confirmed by translating DNA data into amino acid sequences with BioEdit (Hall, 1999). Alignments of ribosomal and transfer RNAs were constructed manually based on secondary structural models (Kumazawa & Nishida, 1993; Ortí *et al.*, 1996; Ortí & Meyer, 1997). All regions whose alignment is ambiguous were excluded from phylogenetic analyses. A total of 3263 alignable characters representing 49 populations of 13 ingroup and 11 outgroup species were scored; 1674 of these characters were variable and 1413 were parsimony informative. All sequences were tested for an anti-G bias characteristic of the mtDNA genes, but not of the nuclear genome, to confirm that we have collected genuine mtDNA data (Zhang & Hewitt, 1996).

Maximum parsimony-based phylogenetic relationships were estimated using the program PAUP\* (Swofford,

2001) with 100 heuristic searches using random additions of sequences and implementing the tree bisection and reconnection (TBR) algorithm. Equal weight was given to all characters. Bootstrap resampling (Felsenstein, 1985) was applied to assess support for individual nodes using 2000 bootstrap replicates with simple addition and TBR branch swapping. A Nexus file containing a constraint tree for each node of the maximum parsimony tree and directives for a heuristic search with 25 random additions and TBR branch swapping and 25 replicates was used to find the minimum lengths of alternative trees not satisfying each constraint. Bremer branch-support values (Bremer, 1994) were calculated by subtracting the length of the shortest tree from the shortest tree constrained not to include the branch being analysed.

Maximum likelihood-based phylogenetic relationships were estimated using the program PAUP\* (Swofford, 2001) and implementing a full heuristic search of the tree space. The simplest maximum likelihood model that best explains the data was estimated using the program MODELTEST (Posada & Crandall, 1998). Results of MODELTEST (Posada & Crandall, 1998) indicate that the HKY85 model (Hasegawa *et al.*, 1985) of evolution with rate heterogeneity, rates for variable sites assumed to follow the gamma distribution with shape parameter estimated by the maximum likelihood method, was the most appropriate model for this data set.

Distance-based phylogenetic relationships were estimated using the program TreeCon (Van de Peer & De Wachter, 1997). The Van de Peer and de Wachter model (Van de Peer *et al.*, 1996) of molecular evolution was used, as it allows among site rate variation and thus is comparable with the HKY85 model (Hasegawa *et al.*, 1985) with gamma distributed among site rate variation.

**Table 2** Primers used in this study. The primer designation corresponds to its 3' position in the human mitochondrial genome (Anderson *et al.*, 1981) by convention.

Location	Gene	Sequence	Source
L1090	12S	5'-AAACTGGGATTAGATACCCCACTA-3'	Hrbek & Larson (1999)
H1782	16S	5'-TTTCATCTTCCCTTGCGGTAC-3'	Hrbek & Larson (1999)
L3002	16S	5'-TACGACCTCGATGTTGGATCAGG-3'	This study
L3079	16S	5'-ACGTGATCTGAGTTTCAAGACCG-3'	This study
L3899	ND1	5'-GAAACAAACCGAGCCCCYTT-3'	This study
H4280	tRNA <sup>Ile</sup>	5'-ACTGTATCAAAGTGGYCCCT-3'	This study
L4299	tRNA <sup>Ile</sup>	5'-AAGGRCCACTTTGATAGAGT-3'	This study <sup>2</sup>
H4419	tRNA <sup>Met</sup>	5'-AAGCTTYGGGCCCATACC-3'	This study <sup>2</sup>
L4882a	ND2	5'-TGACAAAARCTAGCCCC-3'	Hrbek & Larson (1999)
L4882b	ND2	5'-TGACAAAARCTAGCACC-3'	Hrbek & Larson (1999)
H4980a	ND2	5'-CGSAGTTGTGTTGGTT-3'	Hrbek & Larson (1999)
H4980b	ND2	5'-CGSAGTTGTGTTGATT-3'	Hrbek & Larson (1999)
H5540a	tRNA <sup>Trp</sup>	5'-TTTAGRGCTTGAAAGGC-3'	Hrbek & Larson (1999)
L5554	tRNA <sup>Trp</sup>	5'-CCAAGRGCCTTCAAGCCCT-3'	This study <sup>2</sup>
H5934	CO1	5'-AGRGTGCCAATGCTTTGTGRTT-3'	Macey <i>et al.</i> (1997)

H and L designates the heavy and the light strand, respectively. Positions with mixed bases are labelled with standard mixed base codes: R = G or A; Y = C or T; S = C or G.

<sup>1</sup>Modified from Kessing *et al.* (1989).

<sup>2</sup>Modified from Hrbek & Larson (1999).

Support for individual branches was estimated using 2000 bootstrap replicates.

All sequence data have been deposited in GenBank under numbers AF449287–AF449348 and AF449349–AF449410 (Table 1). A file with the aligned data is available at directly from the first author, or at [www.treebase.org](http://www.treebase.org). Additional information including data are available at [www.cyprinodontiformes.org](http://www.cyprinodontiformes.org).

### Tests of vicariant hypotheses

Although patterns of phylogenetic relationships may appear to be congruent with patterns of vicariant events, concluding that vicariant processes were responsible for the formation of the phylogenetic patterns may be misleading. Vicariant explanations are valid only if temporal congruence between vicariant events and cladogenesis can be established.

To test the temporal congruence between cladogenic and vicariant events, we performed a rate constancy test (two-cluster test) using the program LINTREE (Takezaki *et al.*, 1995) under the HKY85 plus gamma (Hasegawa *et al.*, 1985) distance option. Those taxa that showed significantly different substitution rates at the 0.01 level were excluded from further analyses. Maximum likelihood distance matrix assuming clock-like behaviour of the data was then calculated in PAUP\* (Swofford, 2001) and used to estimate the timing of divergence between all populations. The best dated vicariant event, the separation of *A. sirhani* from *A. dispar* 13 MYA was used as a basis of calibration of other cladogenic events. The opening of the Strait of Gibraltar, the other very well supported vicariant event, could not be used, as it appears that all Moroccan and Algerian populations of *A. iberus* are extinct.

## Results

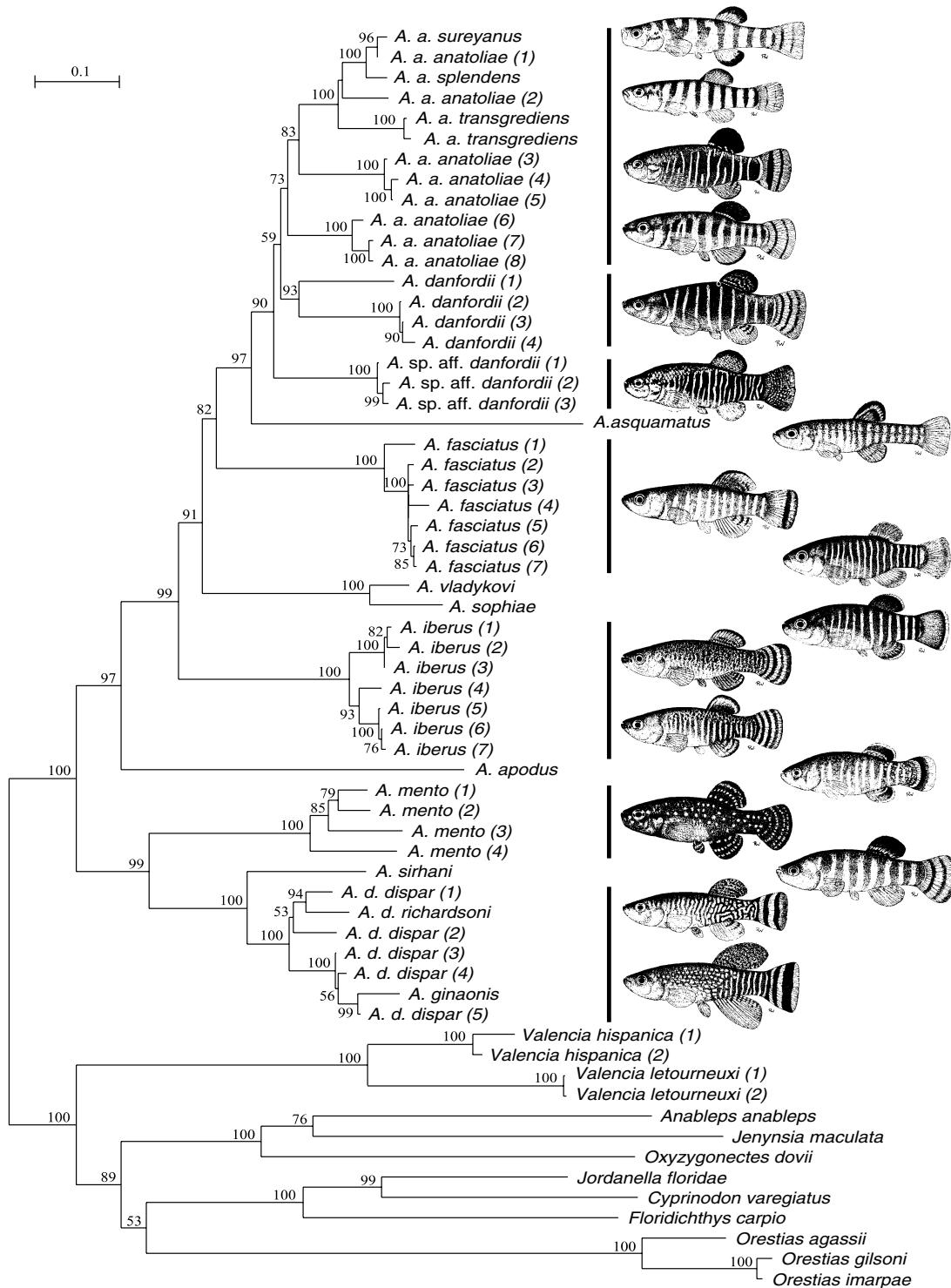
### *Aphanius* phylogeny

The data were analysed using maximum parsimony, maximum likelihood and neighbour-joining. The resulting phylogenies were largely congruent, although major differences occur in the placement of *A. apodus*. In neighbour-joining and maximum likelihood analyses, *A. apodus* forms a sister taxon to all *Aphanius* species but the *A. mento*, *A. sirhani*, *A. dispar* and *A. ginaonis* clade. Both topologies are strongly supported by high bootstrap values (Figs 4 and 5). In the maximum parsimony phylogeny, *A. apodus* forms the sister taxon to all other *Aphanius* species (Fig. 6); this topology is not significantly different (Templeton test,  $P > 0.05$ ; Shimodaira-Hasegawa test,  $P > 0.05$ ) from the alternate topology suggested by the neighbour-joining and maximum likelihood analyses. We believe that the unstable position of *A. apodus* results from its relatively fast rate of molecular evolution relative to other *Aphanius* species.

All phylogenetic methods reconstruct the same order of phylogenetic relationships among the remaining *Aphanius* species with the exception of the central Anatolian clade. Maximum parsimony and minimum evolution reconstruct *A. sp. aff. danfordii* as the sister group to other central Anatolian clades, whereas maximum likelihood places *A. danfordii* as the sister group to other central Anatolian clades. All methods of reconstruction show low support among the main clades of *A. anatoliae*, *A. danfordii* and *A. sp. aff. danfordii*. The relationships and branching order among other *Aphanius* species are generally well supported by all methods of phylogenetic reconstruction. We observe two main clades containing *A. mento*, *A. sirhani*, *A. dispar* and *A. ginaonis*, i.e. fishes of the eastern clade and a western clade containing all other species. *Aphanius sirhani* forms the sister group to *A. dispar* including *A. ginaonis*. Although morphologically distinct, *A. ginaonis* is most closely related to a geographically proximate *A. dispar* population, and is deeply nested within the *A. dispar* clade. The Dead Sea Valley subspecies of *A. dispar*, *A. d. richardsoni*, is the sister group to geographically proximate *A. dispar* population from the Sinai Peninsula, and is also nested within the *A. dispar* clade. *Aphanius dispar* is thus a paraphyletic taxon. The western clade contains the remaining species of *Aphanius*. All species are monophyletic. After the initial divergence of *A. apodus*, *A. iberus* diverged first from the remaining members of the western clade, followed by the *A. vladykovi*/ *A. sophiae* clade, both from inland Iran. *Aphanius fasciatus*, the brackish water species of the western clade, is a sister group to all species from Turkey, including *A. asquamatus* from 'Kurdistan', eastern Turkey. The central Anatolian clade contains six deeply divergent lineages comprising two described species (*A. danfordii* and *A. anatoliae*) and one undescribed species (*A. sp. aff. danfordii*). *Aphanius danfordii* contains two main lineages and *A. anatoliae* contains three main lineages. The three limnetic subspecies of *A. anatoliae*, *A. a. sureyanus*, *A. a. splendens* and *A. a. transgrediens* are part of the Lakes District clade, but have evolved independently (Templeton test,  $P < 0.0001$ ; Shimodaira-Hasegawa test,  $P < 0.000$ ). Members of the six main lineages show partial or full reproductive isolation (Villwock, 1964).

### Dating the divergence of *Aphanius* species and populations

Dating past evolutionary events has been problematic both from theoretical and empirical perspectives. Observed pair-wise sequence divergences are assumed to result from accumulation of mutations since the time of final separation of the study populations retaining little or no ancestral polymorphisms. Yet even clearly separated populations following independent evolutionary trajectories can experience occasional gene flow, at least in the early stages of separation (Avise, 1994; Templeton, 1994). Any initial gene flow and shared polymorphism



**Fig. 4** Minimum evolution estimate of the phylogenetic relationships of the genus *Aphanius*; ME = 2.4132. Values above branches indicate bootstrap values based on 2000 replicates. Numbers after specific names refer to geographical locations listed in Table 1 from which the said specimens originate. Drawing portrays representative members of given populations or species.

therefore reduces the observed pair-wise sequence divergence to a minimum estimator of divergence age, whereas geographical structuring overestimates the age of the hypothesized vicariant event.

Mutation rates are traditionally calibrated using geology and fossil records. In the case of *Aphanius* a fossil record does exist. Sauvage (1874) described *Prolebias*, an *Aphanius*-like cyprinodont, from the middle Oligocene of Europe and Priem (1908) described middle Miocene *Aphanius* fossils from the Lake Orumiye basin in north-western Iran. Other Oligocene and Miocene fossils of *Aphanius*-like fishes have been described from Spain, France and Switzerland (e.g. Gaudant, 1978; Reichenbacher & Weidmann 1992; Gaudant, 1993). Based on these fossils, the minimum estimate for the age of the genus *Aphanius* is 30 million years. However, as fossils give a minimum estimate of the age of a clade, using fossils as calibration points can potentially underestimate the divergence times of nested clades. We therefore use the well-dated Red Sea transgression into the Wadi Sirhan (el-Azraq oasis) of Jordan 13 MYA (Bender, 1968) as a calibration point. This transgression is hypothesized to have resulted in the differentiation of the common ancestor into the modern-day species *A. sirhani* and *A. dispar* (Villwock *et al.*, 1983). At  $22.46 \pm 0.26\%$  maximum likelihood pair-wise sequence divergence between *A. sirhani* and *A. dispar* (all populations were included in the rate calibration), we estimate a substitution rate of  $8.6 \pm 0.1 \times 10^{-9}$  substitutions base pair $^{-1}$  year $^{-1}$ . This estimate is comparable with the substitution rate of  $7.4 \times 10^{-9}$  substitutions base pair $^{-1}$  year $^{-1}$  derived for the same gene regions of nonannual rivulid killifishes (Hrbek & Larson, 1999) and to the widely accepted mtDNA substitution rate of  $7 \times 10^{-9}$  substitutions base pair $^{-1}$  year $^{-1}$  for poikilotherm vertebrates (Martin & Palumbi, 1993). The well-dated separation of the Iberian Peninsula and the African Atlas (Krijgsman *et al.*, 1999a) could not be used as a point of calibration as it appears that all North African population of *A. iberus* are extinct. Estimates of divergence dates for main cladogenic events affecting the genus *Aphanius* are listed in Table 3a. For comparison, an estimate of divergence dates based on fossil data calibration ( $\mu = 1.1 \pm 0.2 \times 10^{-8}$  substitutions base pair $^{-1}$  year $^{-1}$ ) is listed in Table 3b.

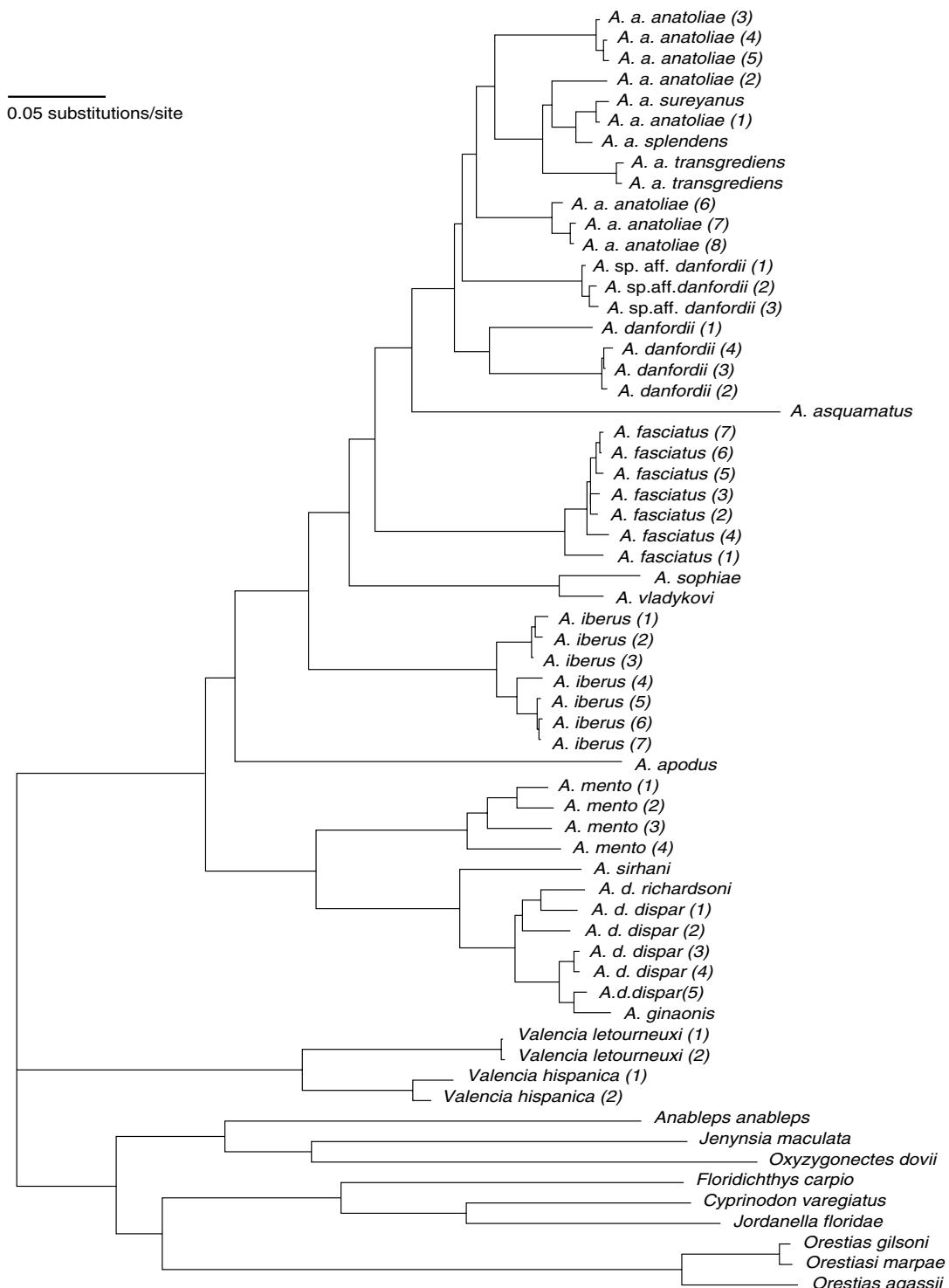
## Discussion

Killifishes of the genus *Aphanius* have a late period Tethys Sea coastline distribution. Two of the species inhabit estuarine and environments and the rest of the species are landlocked in oases or intermontane basins; one species is found in both types of habitat. Based on the presented data, it appears that vicariant orogenetic events associated with the closing of the Tethys Sea are a likely cause of population and species differentiation in the genus *Aphanius*. Not only is there general agreement of the *Aphanius* phylogeny with the hypothesized area

relationship cladogram, but there is also temporal congruence of many of the geological and divergence events (Table 3).

The divergence estimates derived from the molecular clock calibrated on the separation of the Wadi Sirhan from the Red Sea (Table 3a) tend to give divergence estimates which precede the geologically derived hypotheses of divergence, especially for older events. This is to be expected if the species undergoing vicariant speciation is genetically highly divergent. This appears to be the case for many of the species examined in this study. In contrast, divergence estimates based on the molecular clock calibrated from fossil data (Table 3b) tend to fit better the older divergence dates. However, it should be noted that fossil data tend to underestimate actual divergences. In our case, the molecular evolutionary rate obtained from the fossil data is also higher than normally observed, and some of the best dated and previously supported vicariance events are not well supported by the fossil calibrated data. We, therefore, consider the geologically derived estimates of molecular evolutionary rate to be the upper-bound estimate, and closer to the true molecular evolutionary rate, with the fossil derived estimates forming the lower-bound divergence estimates.

The oldest division within *Aphanius* exists between the eastern and western clades (Fig. 7). This division is estimated to be about  $37.34 \pm 6.99$  million years old. The fossil data suggest a 30 million year minimum estimate for the age of the genus *Aphanius*. As the final closing of the Tethys did not occur till approximately 20 MYA (Dercourt *et al.*, 1986), there must have already been a significant amount of differentiation between populations inhabiting eastern and western portions of the Tethys Sea at the time of its final closing, or significant barriers to gene flow must have preceded the final closing. With the exception of *A. fasciatus*, all species show a great deal of population differentiation, including the two species inhabiting coastal brackish water environments, *A. dispar* and *A. iberus*; the two species show a maximal  $7.18 \pm 1.17$  and  $4.72 \pm 0.10$  million year divergence among populations, respectively. Significant geographical differentiation in the ancestor appears to be a real possibility. The final closing of the Tethys was also preceded by the formation of a hypersaline, periodically desiccating seaway (Dercourt *et al.*, 1986) which could prevent contact between eastern and western portions of the Tethys Sea. This scenario is supported by evidence from eels of the genus *Anguilla*, marine as well as fresh-water species, that diverged into eastern and western groups 30–45 MYA (Aoyama & Tsukamoto, 1997; Tsukamoto & Aoyama, 1998), preceding the final closing of the Tethys by a comparable amount of time as *Aphanius* killifishes. Thus although the older division is much older than the final closing of the Tethys Sea, it appears that the ecological changes preceding the final closure of the Tethys Sea have



**Fig. 5** Maximum likelihood estimate of the phylogenetic relationships of the genus *Aphanius* based on TBR rearrangements of the best maximum likelihood topology. For the HKY85 model transitions/transversion ratio was calculated at 3.424, the shape parameter alpha equals 0.758 and the proportion of invariable sites is 0.388;  $-\ln = 40872.707$ . Numbers after specific names refer to geographical locations listed in Table 1 from which the said specimens originate.

resulted in the divergence of the two main clades of *Aphanius*.

### ***Aphanius apodus***

An interesting aspect of the phylogeny is the position of *A. apodus*. In the maximum parsimony phylogeny, *A. apodus* is a sister group to all other species of *Aphanius* (Fig. 6). These topologies are not significantly different (Table 4) from the minimum evolution or the maximum likelihood topology where *A. apodus* is the sister taxon to the western clade of *Aphanius* (Fig. 4), and are likely a result of long-branch attraction between *A. apodus* and the relatively faster evolving outgroups. By correcting for the long-branch attraction artefact through the use of an appropriate evolutionary model we should recover the correct topology. Maximum parsimony is sensitive to the long-branch attraction artefact as it assumes evolutionary rate homogeneity (Huelsenbeck & Hillis, 1993). The gamma corrected molecular evolutionary model of Van de Peer *et al.* (1996) in the minimum evolution analysis, and the gamma corrected HKY85 model of Hasegawa *et al.* (1985) in the maximum likelihood analysis, places *A. apodus* at what we believe is the most likely phylogenetic position, i.e. the earliest branching member of the western *Aphanius* clade (Figs 3 and 4).

### **The western clade**

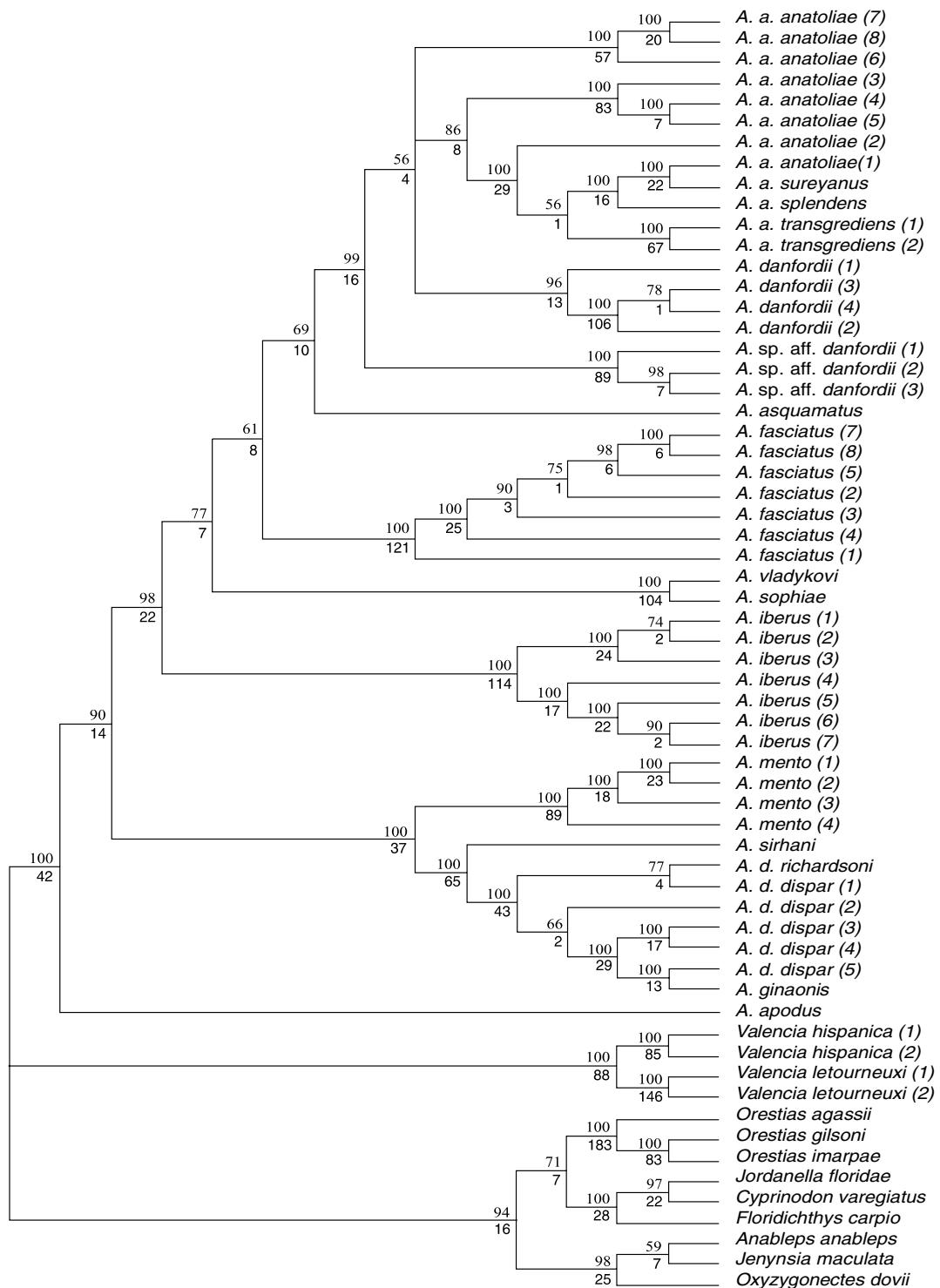
The lack of sister group relationship of *A. apodus* and *A. iberus* supports the hypothesis that the present-day Atlas Mountains has been colonized on two separate occasions. The best explanation appears to be that the Tel Atlas has been colonized by the ancestor of *A. apodus*, whereas the Rif Atlas has been colonized by the ancestor of *A. iberus*. The Tel and the Rif Atlas have in general different floras as well. With the closing of the Riffian and the Bedic gaps (Garcés *et al.*, 1998; Krijgsman *et al.*, 1999b) shortly before the onset of the Messinian saltwater crisis 5.96 MYA (Krijgsman *et al.*, 1999a), the ancestor of *A. iberus* was able to colonize lower elevations of the Tellian Atlas as well as the Iberian Peninsula with additional areas north to the Camargue (Rhône delta) of France. Opening of the Strait of Gibraltar at the end of the Messinian is hypothesized to have separated the Iberian and Maghrebian populations. This hypothesis is supported by data from European cyprinids (Zardoya & Doadrio, 1998; Zardoya & Doadrio, 1999). Unfortunately we could not test this hypothesis on *A. iberus* as all historically known populations of *A. iberus* from the Maghreb are presumed to be extinct (van der Zee & van Kessel, 1988; van der Zee & Vonk, 1991; Wildekamp, 1996). However, the Iberian populations of *A. iberus* show a deep phylogenetic split between populations of the Guadalquivir drainage (west of Gibraltar), and populations of the Balearic coast (east of Gibraltar). Opening of the Strait

of Gibraltar at the end of the Messinian 5.33 MYA (Krijgsman *et al.*, 1999a), and the resulting strong current, is likely to have acted as a strong barrier to gene flow between the Guadalquivir and Balearic coast populations similarly as it has formed a strong barrier to gene flow between the Iberian Peninsula and the Maghreb. Our estimate of  $4.72 \pm 0.10$  million year divergence is in good agreement with the timing of the opening of the Strait of Gibraltar, and is supported by the recent work of Perdices *et al.* (2001) who also estimated a 5 million-year divergence between the Guadalquivir and Balearic populations of *A. iberus*<sup>1</sup>.

### **Anatolian *Aphanius* species**

The genus *Aphanius* has a centre of diversity in Anatolia (Wildekamp *et al.*, 1999), with the greatest amount of morphological and genetic diversity existing in the *A. anatoliae* species complex (Fig. 4). In the central-western portion of its distribution, *A. anatoliae* is found predominantly in small isolated intermontane basins, the so-called Lakes District, in the north-western portion it is found in the Büyüük Menderez River drainage, whereas in the eastern portion of its distribution, it is found in creeks and springs of the Tuzgölü plain. In the Lakes District region, numerous populations also inhabit large lakes. In lakes with high salt content, such as the Lake Salda, Burdur, Gölçük and Aci, *A. anatoliae* is the only native fish present. In these lakes *A. anatoliae* has evolved distinct limnetic (open-water dwelling) phenotypes which have been regarded as distinct subspecies (Grimm, 1980). The limnetic subspecies differ from each other morphologically, do not form a monophyletic group, and also are deeply nested within the nominal subspecies of *A. anatoliae* (Fig. 4; Table 4), pointing to their independent origins. In other large lakes that contain native fishes in addition to *A. anatoliae*, *A. anatoliae* are found in reedy shore habitats and possess non-limnetic phenotypes. The separation among the three main groups of *A. anatoliae* is estimated to be maximally  $11.79 \pm 0.52$  million years, whereas differentiation within the Lakes District group is estimated to be  $7.48 \pm 0.49$  million years (Table 3a). The observed degree of intraspecific differentiation is unusual within fish species (McCune & Lovejoy, 1998); however, our results are supported by geological data (Table 3a) and the works of Villwock (1958, 1964, 1966) which show various degrees of reproductive isolation among the three main groups of *A. anatoliae* and also *A. danfordii*. *Aphanius danfordii* is found in the Kızılırmak River drainages, in the lower reaches of the Yeşilırmak River, and in the Sultan swamps of the Develi depression. It is

<sup>1</sup>During the typesetting of this manuscript, the Guadalquivir populations of *A. iberus* have been described as a new species *A. baeticus* [Doadrio, I., J. A. Carmona & C. Fernandez-Delgado 2002. Morphometric study of the Iberian *Aphanius* (Actinopterygii, Cyprinodontiformes), with description of a new species. *Folia Zoologica* 51: 67–79].



**Fig. 6** Maximum parsimony estimate of the phylogenetic relationships of the genus *Aphanius*, TL = 8472, CI = 0.342. Values above branches indicate bootstrap values based on 2000 replicates. Values below branches refer to Bremer branch-support values. Numbers after specific names refer to geographical locations listed in Table 1 from which the said specimens originate.

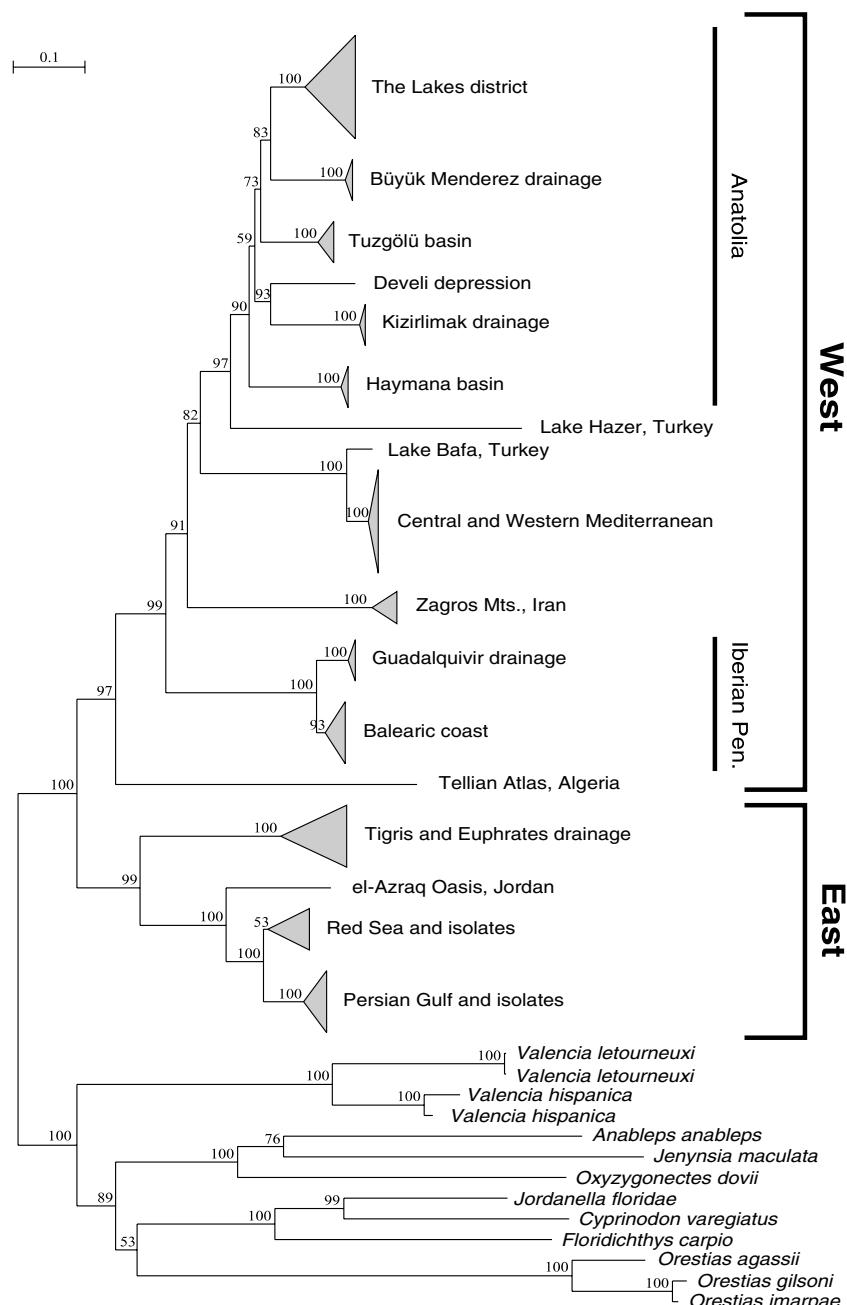
**Table 3** (a) Estimates of time of divergence of *Aphanius* populations and species based on maximum likelihood estimate of distances, implementing the HKY85 model + gamma model of molecular evolution. The calibration point used was the 13 MYA divergence of *A. sirhani* from *A. dispar* and a maximum likelihood pair-wise divergence estimate between the two taxa was estimated at  $22.46 \pm 0.26$  per cent, resulting in a mutation rate estimate of  $8.6 \pm 0.1 \times 10^{-9}$  substitutions per base pair per year. (b) For comparison, a second calibration point used was the 30-million-year-old fossil of *Prolebias* (Sauvage, 1874) assumed to represent the deepest split within *Aphanius*. This calibration resulted in a mutation rate estimate of  $1.1 \pm 0.2 \times 10^{-8}$  substitutions per base pair per year.

	<i>d</i>	$\mu = 8.6 \times 10^{-9}$ (MYA)	95% CI range (MYA)	Geology (MYA)
<b>(a) Maximum-likelihood results – geological calibration</b>				
<i>Geology predicted events</i>				
<i>Aphanius fasciatus</i> diversification	2.15	$1.25 \pm 0.36$	1.08–1.41	~5
Lake Bafa vs. <i>A. fasciatus</i> *	6.86	$3.99 \pm 0.05$	3.89–4.07	~5
<i>A. anatoliae</i> Lakes diversification*	12.18	$7.48 \pm 0.49$	6.12–8.04	5–10
Anatolian diversification*	23.82	$14.07 \pm 1.63$	10.86–17.28	~10
Iran vs. Mediterranean	45.62	$26.52 \pm 3.19$	20.27–32.78	12–20
<i>A. sophiae</i> vs. <i>A. vladkovi</i> *	11.76	$6.84 \pm 0.00$	–	5–10
<i>A. sirhani</i> vs. <i>A. dispar</i>	22.46	(calibration event)	–	13
East vs. West*	64.23	$37.34 \pm 6.99$	23.64–51.04	>20
<i>Other events</i>				
<i>A. anatoliae</i> diversification	20.29	$11.79 \pm 0.52$	10.77–12.82	–
<i>A. fasciatus</i> vs. Anatolia	40.06	$23.29 \pm 3.17$	17.07–29.51	–
Iberian Peninsula	8.12	$4.72 \pm 0.10$	4.53–4.92	–
Iberia vs. Mediterranean	45.74	$26.59 \pm 2.61$	21.47–31.72	–
<i>A. mento</i> vs. <i>A. dispar</i>	46.21	$26.87 \pm 1.31$	24.30–29.44	–
<i>A. dispar</i> diversification	12.36	$7.18 \pm 1.17$	4.90–9.47	–
	<i>d</i>	$\mu = 1.1 \times 10^{-8}$ (MYA)	95% CI range (MYA)	Geology (MYA)
<b>(b) Maximum-likelihood results – fossil calibration</b>				
<i>Geology predicted events</i>				
<i>A. fasciatus</i> diversification	2.15	$0.98 \pm 0.28$	0.85–1.10	~5
Lake Bafa vs. <i>A. fasciatus</i>	6.86	$3.20 \pm 0.04$	3.13–3.28	~5
<i>A. anatoliae</i> Lakes diversification*	12.18	$5.69 \pm 0.39$	4.92–6.49	5–10
Anatolian diversification*	23.82	$11.13 \pm 1.81$	7.59–14.67	~10
Iran vs. Mediterranean*	45.62	$21.32 \pm 2.57$	16.29–26.35	12–20
<i>A. sophiae</i> vs. <i>A. vladkovi</i> *	11.76	$5.50 \pm 0.00$	–	5–10
<i>A. sirhani</i> vs. <i>A. dispar</i>	22.46	$10.49 \pm 0.12$	10.26–10.73	13
East vs. West*	64.23	$30.01 \pm 5.62$	19.00–41.03	>20
<i>Other events</i>				
<i>A. anatoliae</i> diversification	20.29	$9.48 \pm 0.42$	8.66–10.30	–
<i>A. fasciatus</i> vs. Anatolia	40.06	$18.72 \pm 2.55$	13.72–23.72	–
Iberian Peninsula	8.12	$3.80 \pm 0.08$	3.64–3.95	–
Iberia vs. Mediterranean	45.74	$21.37 \pm 2.10$	17.26–25.49	–
<i>A. mento</i> vs. <i>A. dispar</i>	46.21	$21.60 \pm 1.05$	19.53–23.66	–
<i>A. dispar</i> diversification	12.36	$5.77 \pm 0.94$	3.94–7.61	–

\*A hypothesized vicariance event based on the congruence between molecular and geological data. (*d* = maximum-likelihood distance;  $\mu$  = mutation rate, divergence times predicted based on this mutation rate; 95% CI range = 95% confidence range of divergence times; Geology = divergence times predicted from geological data).

Comparisons predicted by geology: (1) *A. fasciatus* diversification – diversification of the main group of *A. fasciatus* not including the landlocked population of Lake Bafa. (2) Lake Bafa vs. *A. fasciatus* – Divergence of landlocked *A. fasciatus* of Lake Bafa from other Mediterranean populations of *A. fasciatus*. (3) *A. anatoliae* Lakes diversification – Diversification of the Lakes District clade, south-western section of the Taurus-Menderez block. (4) Anatolia diversification – Beginning of diversification of all central Anatolian species. (5) Iran vs. Mediterranean – Divergence of the Mediterranean including Turkey from Iran. (6) *A. sophiae* vs. *A. vladkovi* – Divergence of common ancestor of *A. sophiae* and *A. vladkovi* – no SE or 95% CI range exists, as only one population per species could be analysed. (7) *A. sirhani* vs. *A. dispar* – Divergence of the common ancestor of *A. sirhani* and *A. dispar*. (8) East vs. West – Divergence of the common ancestor of the eastern and western clades of *Aphanius*. Other cladogenic events for which geology makes no prediction: (9) *A. anatoliae* diversification – Diversification of all clades of *A. anatoliae*. (10) *A. fasciatus* vs. Anatolia – Divergence of common ancestor of all Anatolian species and the ancestor of *A. fasciatus*. (11) Iberian Peninsula – Divergence of *A. iberus* populations Atlantic and Mediterranean drainages of the Iberian Peninsula. (12) Iberia vs. all – Divergence of *A. iberus* from its sister clade. (13) *A. mento* vs. *A. dispar* – Divergence of the common ancestor of *A. mento* and *A. dispar*. (14) *A. dispar* diversification – Diversification of the *A. dispar* clade.

Comparisons not included: Diversification of *A. asquamatus* and *A. apodus* from their sister clades because of their significantly fast rate of molecular evolution.



**Fig. 7** A simplified phylogeny of area relationships where *Aphanius* occurs. The figure was generated by replacing names of species on the minimum evolution phylogeny by names of areas and then collapsing monophyletic areas. Values above branches indicate bootstrap values based on 2000 replicates.

separated from *A. anatoliae* by a volcanic range at the boundary of the Tuzgölü basin and the Kirşehir block (Sengör & Yilmaz, 1981), and forms a sister group to *A. anatoliae*. A species identified as *Aphanius* sp. aff. *danfordii* (Wildekamp *et al.*, 1999) is found in the upper reaches of the Sakarya river and the Haymana basin. It is

morphologically and phenotypically distinct, forms the sister group to the other central Anatolian species (Figs 4 and 5), and appears to be reproductive isolated from other central Anatolian clades (K. Valkenburg, pers. comm.). The age of this central Anatolian radiation is estimated at  $14.07 \pm 1.63$  million years (Table 3a).

**Table 4** (a) Tests of phylogenetic hypotheses conducted on the most parsimonious tree. Previous morphological hypotheses, and the monophyly of various taxonomic units are tested against the most parsimonious pruned tree derived from the molecular data. Comparisons are as follows: (1) The molecular data set is constrained such that *Aphanius anatoliae sensu lato* is monophyletic, i.e. does not include *A. danfordii*. (2) The molecular data set is constrained such that *Aphanius anatoliae sensu stricto* is monophyletic, i.e. does not include *A. a. splendens*, *A. a. sureyanus* and *A. a. transgrediens*. (3) The molecular data set is constrained such that *A. a. splendens*, *A. a. sureyanus* and *A. a. transgrediens* form a clade. (4) The molecular data set is constrained such that *Aphanius dispar sensu lato* is monophyletic, i.e. it does not include *A. ginaonis*. (5) The molecular data set is constrained such that *Aphanius dispar sensu stricto* is monophyletic, i.e. it does not include *A. ginaonis* and *A. d. richardsoni*. (6) The molecular data set is constrained such that *Aphanius apodus* forms a sister group to the Eastern clade of *Aphanius* as suggested by ME (Fig. 4) and ML (Fig. 5) analyses. (Length = minimum number of character changes for the topology being analysed;  $T_s$  = critical value of the Wilcoxon signed rank test;  $N$  = number of characters requiring different minimum numbers of changes on the topologies being compared;  $z$  = normal approximation for the Wilcoxon signed rank test). (b) Tests of phylogenetic hypotheses conducted on the maximum likelihood tree. (1) The molecular data set is constrained such that *A. a. splendens*, *A. a. sureyanus* and *A. a. transgrediens* form a clade. (2) The molecular data set is constrained such that *Aphanius dispar sensu lato* is monophyletic, i.e. it does not include *A. ginaonis*. (3) The molecular data set is constrained such that *Aphanius dispar sensu stricto* is monophyletic, i.e. it does not include *A. ginaonis* and *A. d. richardsoni*. (Diff -ln L = -ln likelihood difference between best and the alternate topology being analysed).

Tree	Length	$T_s$	$n$	$z$	$P^*$
<i>(a) Templeton (Wilcoxon signed rank) test (Templeton, 1983)</i>					
MP tree	8472	(best)			
(1) <i>Aphanius anatoliae</i> s. l. monophyly	8473	90.0	19	0.2294	0.8185
(2) <i>A. anatoliae</i> s. s. monophyly	8572	1837.5	148	8.0579	<0.0001†
(3) <i>A. anatoliae</i> ssp. monophyly	8514	1034.0	46	6.1926	<0.0001†
(4) <i>A. dispar</i> s. l. monophyly	8508	949.0	81	3.7910	0.0002†
(5) <i>A. dispar</i> s. s. monophyly	8548	943.0	110	7.2463	<0.0001†
(6) <i>A. apodus</i> in Eastern clade	8488	837.5	66	1.9596	0.0528
<i>(b) Kishino-Hasegawa (1989) and Shimodaira-Hasegawa (1999)</i>					
ML tree	40872.64637	(best)		KH-test	SH-test
(1) <i>A. anatoliae</i> ssp. monophyly	40999.35347	126.70710		$P_{\ddagger}^*$	$P_{\ddagger}^*$
(2) <i>A. dispar</i> s. l. monophyly	40981.15466	108.50829		<0.0001\$	0.000\$
(3) <i>A. dispar</i> s. s. monophyly	41133.52919	260.88282		<0.0001\$	0.000\$

\*Probability of getting a more extreme  $T$ -value under the null hypothesis of no difference between the two trees (two-tailed test).

†Significant at  $P < 0.05$ .

‡ $P$  – Probability of getting a more extreme  $T$ -value under the null hypothesis of no difference between the two trees (KH two-tailed test, SH one-tailed test).

\$Significant at  $P < 0.05$ .

The five areas of distribution, the Lakes District, the Büyükk Menderez drainage, the Tuzgölü basin, the Haymana basin and the Kızılırmak drainage, are monophyletic (Figs 4 and 7) and monophyly of the groups are well supported. *Aphanius danfordii* from the Develi depression also form a monophyletic group (T. Hrbek, unpubl. res.). However, these six *Aphanius* clades of Anatolia have rapidly diverged from one another between 11 and 17 MYA (Table 3) which can be observed by relative lack of phylogenetic resolution among these six clades (Figs 4–6). Only the sister group relationship of *A. danfordii* from the Develi depression and the Kızılırmak drainage is well supported; still the divergence between these clades is ancient, and is estimated at  $12.25 \pm 0.08$  million years. Additionally, large amount of among-population within-clade genetic differentiation exists, suggesting prolonged isolation of individual populations in particular those of the Lakes

District. Relatively little genetic differentiation (<1% sequence divergence) is observed among populations found in the Tuzgölü basin, which is consistent with data that the basin has been flooded during the Pleistocene (Roberts *et al.*, 1979). In contrast to the Lakes District populations, low genetic differentiation among populations within the Büyükk-Menderez and the Kızılırmak drainages suggests that gene flow can play a significant role in structuring of *Aphanius* populations, however, the ecological opportunity, here the possibility to migrate along rivers, must exist.

*Aphanius asquamatus* from Lake Hazer not only possesses a very distinct morphology and appears to be pelagic with annual vertical migrations in the lake but also forms a unique mtDNA lineage. Parenti (1981) even resurrected the generic name *Kosswigichthys* Sözer 1942 for *A. asquamatus*, and included the three limnetic subspecies *A. a. splendens*, *A. a. transgrediens* and *A. a. burburdicus*

(= *A. a. sureyanus*) of *A. anatoliae* into this genus. Following the accepted principles of phylogenetic systematics, the placement of *A. asquamatus* or the three limnetic subspecies into the genus *Kosswigichthys* is not supported by our data. *Aphanius asquamatus* is a truly unique species both in morphology as well as in ecology, however, its ecology and morphology is derived independently of the other three limnetic *A. anatoliae* subspecies. Due to a relatively rapid rate of molecular evolution observed in this taxon (Fig. 4), no temporal estimate of its divergence from its Anatolian sister group could be made. However, the divergence of *A. asquamatus* is bounded by the timing of separation of *A. fasciatus* and the Anatolian clade  $23.29 \pm 3.17$  million years, and the diversification of the Anatolian clade  $14.07 \pm 1.63$  million years (Table 3a).

### Circum-Mediterranean species

The western brackish water species, *A. fasciatus*, has a large distribution throughout the central and eastern Mediterranean, including several landlocked populations. Despite its wide distribution and the potential to have been subjected to Mediterranean vicariance events, genetic differentiation among these populations is relatively small with an average of 1.6% sequence divergence among populations. The widely dispersed populations are most likely connected through periodic migration events that prevent local differentiation. The only moderately differentiated population included in this study is *A. fasciatus* from Lake Bafa in Turkey (Fig. 1) with an average 6.86% sequence divergence from other *A. fasciatus* populations. This corresponds to a  $3.99 \pm 0.05$  million year separation of the Lake Bafa *A. fasciatus* from other Mediterranean populations (Table 3a). Geographically, Lake Bafa is separated by a moderate-sized mountain range from the Mediterranean sea; we hypothesize it and its *A. fasciatus* population most likely originated as a result of the folding of the Menderez Taurus block in the last 5 million years (Quennell, 1984). Together with *A. iberus*, *A. fasciatus* is the only species that could have been affected by the Messinian Saltwater Crisis. If the Messinian Saltwater Crisis played a role in the structuring of *A. fasciatus* populations, subsequent gene flow erased all traces of this vicariant event.

### Eastern Clade

Genetic differentiation in the other brackish water species, *A. dispar* of the eastern group, is much more extensive, suggesting an average  $7.18 \pm 1.17$  million year divergence. Two major clades, composed of the Persian Gulf and the Red Sea populations are delineated from this mtDNA data set (Figs 4 and 7). The Red Sea clade contains the *A. dispar richardsoni* subspecies from the Dead Sea Valley which was connected to the Red Sea during the late Pliocene (Coleman, 1993). The degree of

genetic differentiation, 7.3% sequence divergence equaling to 4.2 million years, between *A. dispar* populations occurring on the Sinai Peninsula and *A. dispar richardsoni* corresponds to its hypothesized late Pliocene separation (Kornfield & Nevo, 1976). Populations of *A. dispar* from the Afars basin of Djibouti also belong to the Red Sea clade. The Persian Gulf clade includes *A. dispar* from Saudi Arabia, Iraq and Iran, and includes *A. ginaonis*. *Aphanius ginaonis* is the sister taxon to a geographically close *A. dispar* population from Hormozgan, Iran (Fig. 1). Our data strongly reject the monophyly of *A. dispar* (Table 4) and suggest that it does not constitute a species in terms of the phylogenetic species concept.

Perhaps most surprising is the early divergence of *A. mento* from the ancestor of the *A. dispar* clade estimated at  $26.87 \pm 1.31$  MYA. We speculate that the ancestor of *A. mento* invaded the northern perimeters of the Arabian plate, the present-day Tigris and Euphrates Valley, as it was indenting into Laurasia at some time after the separation of the eastern and western clades of *Aphanius*, but before the complete closing of the Tethys Sea. Eventually, it spread through the present-day Tigris and Euphrates Valley into the Dead Sea Valley, the Orontes drainage as well as several river deltas of southern Turkey, experiencing a substantial amount of among-population genetic differentiation (Table 3). Despite that, this study potentially is missing the deepest phylogenetic separation by not including populations from the Tigris and Euphrates deltas, the present-day Iraq. The level of differentiation is still great, indicating nearly a 10-million-year divergence within the *A. mento* clade. The closing of the Tethys Sea was associated with extreme ecological changes at the present-day Tigris and Euphrates Valley (Dercourt *et al.*, 1986). We hypothesize that this region has become ecologically unavailable to the ancestral *A. dispar*-like population before the final closing of the Tethys Sea, but at the same time it may have provided an ecological opportunity into which a new species, in this case the ancestor of *A. mento*, could diversify, thus explaining the early divergence of *A. mento* and the *A. dispar*-like clades, as well as the eastern clade of *Aphanius*. However, additional data are needed to test this hypothesis.

### Conclusion

The geological events of the former Tethys region appear to have played a major role in the diversification of the genus *Aphanius*, however, ecological factors are likely to have played a role as well. In general, we observe a well supported spatial and temporal divergence pattern, thus supporting the hypothesis of primarily vicariant-based speciation for the genus *Aphanius*. The generality of the proposed hypothesis (Fig. 3) will require additional testing with groups showing similar distribution, such as melanopsid snails (Glaubrecht, 1993). The Mediterranean region and the Near East are also biological

hot-spots, containing numerous species many of which are threatened with extinction (Myers *et al.*, 2000). *Aphanius* are a particularly sad but unrecognized example. To our knowledge, all Maghreb populations of *A. iberus* and *A. apodus* are extinct in nature (van der Zee & Vonk, 1991). *Aphanius iberus* populations in Spain are protected, yet highly endangered (Planelles Gomis, 1999) as their habitat lacks formal protection. The habitat of *A. sirhani* in the el-Azraq oasis of Jordan was seriously degraded in 1988 and only three individuals could be located to start a captive breeding programme (K. Valkenburg, pers. comm.). The current state of this species is unknown. Populations in the Dead Sea Valley are similarly threatened (Goren & Ortal, 1999). Numerous Turkish populations, including the type species of *A. a. splendens* from Lake Gölcük, are extinct (Wildekamp *et al.*, 1999); the unique *A. asquamatus* from Lake Hazer nearly went extinct as a result of an unsuccessful attempt to drill Lake Hazer for irrigation purposes in the late 1980s (Wildekamp *et al.*, 1999); and in the last 2 years *A. a. transgrediens* from the Lake Aci springs have been extirpated by introduced *Gambusia affinis* in all but two of the 12 springs in which they occur (T. Hrbek pers. obs., April 2002). The situation of Iranian populations is not encouraging (Coad, 2000).

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