

Dressing down: convergent reduction of the mental disc in *Garra* (Teleostei: Cyprinidae) in the Middle East

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Abstract In the Middle East, species of *Garra* are believed to have invaded the area in two independent waves from the Indo-Malayan biogeographic region. This hypothesis is based on the structure of the mental disc, a unique specialization of the lower lip, which is believed to be an adaptation to fast-flowing waters.

While several species have such a mental disc, others completely lack a mental disc, being adapted to slow-moving water or to subterranean life. In this study, the phylogenetic relationships of Middle Eastern *Garra* species, including 16 described and 4 undescribed species, were analysed using mitochondrial cytochrome *c* oxidase I sequences. The results are concordant with traditional hypotheses on two invasion events; however, these invasion events are independent from the presence, absence or shape of the mental disc. We postulate

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convergent reduction of the mental disc in 5–6 independent lineages of *Garra* in the Middle East.

Keywords *COI* · Cyprinidae · Labeonin · Mental disc reduction · Middle East

Introduction

Biogeographical realms and their subunits, such as ecoregions, capture the uneven distribution of species and communities on Earth more accurately than do units based on gross biophysical features, such as rainfall, temperature (Holdridge, 1967; Walter & Box, 1976; Schultz, 1995; Bailey & Ropes, 1998), or vegetation structure (Ellenberg & Mueller-Dombois, 1969; De Laubenfels, 1975; Schmidthüsen, 1976). The overwhelming importance of biogeography and the role of tectonic and climatic changes on the distribution of biodiversity have long been appreciated by biologists (Wallace, 1876; Shermer, 2002). The re-connection of formerly separated continents by tectonic plate movements led to large-scale exchanges of species. Such exchanges occurred after the crash of the Indian subcontinent into Asia in the Paleogene (around 50 MYA; Karanth, 2006), the connection of Africa to Asia by the rotation of the Arabian plate in the late Miocene (Hedges, 2001), and the connection of South America to North America in the Pliocene (Marshall, 1988).

The Western Palearctic (Europe and the Middle East) became connected to the Asian continent as soon as the early Oligocene period when the Turgai Strait dried, allowing East Asian freshwater fishes to move west (Bănărescu, 1992; Briggs, 1995; Rögl, 1999). The process of freshwater fish faunal exchange between Europe and Siberia lasted until the very recent post-glacial past (Durand et al., 2003). In contrast, the Indo-Malayan or Oriental biogeographical realm was, and still is, isolated from Europe and northern Asia, most likely due to mountain ranges including the Himalayas uplifted by the northward-pushing Indian plate. As a consequence, none of the species of primary freshwater fishes found in the Indus River occur further west than Baluchistan in the Iran-Pakistan border area (Talwar, 1991). From the great diversity of Asian freshwater fishes, only a few genera are hypothesized to have entered the Palearctic region, namely some lineages of families Bagridae, Cyprinidae, Mastacembelidae,

Nemacheilidae, and Sisoridae. All of these lineages have species that are endemic to the Middle East, most in the Persian Gulf basin, which is adjacent to the Indo-Malayan biogeographical realm. This region has been proposed to be a transition zone between the Palearctic and the Indo-Malayan realms (Coad, 2010).

One of the freshwater fish genera believed to have invaded the Middle East from the Indo-Malayan biogeographical realm is the cyprinid genus *Garra* Hamilton, 1822 (Menon, 1964). Menon (1964) proposed two independent “waves” of migration from a hypothetical area of origin in Yunnan (southern China) to the Middle East, with one of the waves also reaching Africa (Tang et al., 2009; Yang & Mayden, 2010). Menon’s (1964) hypothesis of two invasion events is based on morphological differences in the mental disc among two groups of *Garra* species in the Middle East. The mental disc is an expansion of the lower lip in *Garra* and some related genera such as *Discocheilus* Zhang, 1997; *Discogobio* Lin, 1931; and *Placocheilus* Wu, 1977 (Zhang, 2005). Species of the *Garra variabilis* (Heckel, 1843) group have a small, poorly differentiated mental disc, which Menon (1964) interpreted as an ancestral character state, while species of the *Garra rufa* (Heckel, 1843) group have a large, highly differentiated mental disc, a character state that he interpreted as a derived state. However, Menon did not comment on the fact that no species with the presumed ancestral character state occurred in Yunnan, the proposed area of origin of the genus (Chu & Province, 1989). Only recently, Zheng et al. (2012) postulated that the mental disc in labeonin cyprinids has independently become reduced in several lineages. This view gains additional support by checking the position of the respective character states within phylogenetic tree reconstructions presented by Geiger et al. (2014) and Yang et al. (2012). All species lacking a mental disc found to be nested within *Garra* by Zheng et al. (2012), Yang et al. (2012), Geiger et al. (2014) had been described as belonging to different genera, although the presence of the mental disc is the diagnostic character state for the genus (Menon, 1964). In the Middle East, labeonin cyprinid species without a mental disc that formerly had been placed in the genera *Hemigrammocapoeta* Pellegrin, 1927, *Crossocheilus* Kuhl & van Hasselt, 1823, *Gonorynchus* McClelland, 1838, *Iranocypris* Bruun & Kaiser, 1944 or *Tylognathus* Heckel, 1843 (Hashemzadeh Segherloo et al., 2012; Farashi et al., 2014; Behrens-Chapuis et al., 2015) have later been moved to the genus *Garra* (Hamidan et al.,

2014). Also, Yang et al. (2012) found *G. variabilis*, a species with a small mental disc, to be nested among other species of the genus having a large and fully developed mental disc. Based on this, Yang et al. (2012) questioned the interpretation of a small and less structured mental disc as the ancestral character state in *Garra* species. The hypothesis that species of *Garra* having a small, or even absent mental disc might represent a derived rather than an ancestral character state is inconsistent with Menon's (1964) hypothesis and leads to different biogeographical conclusions.

In this context, our main objective was to assess the phylogenetic relationships of all *Garra* species in the Middle East, to test the hypothesis that a small or absent mental disc has independently evolved multiple times in *Garra*, and that species without a mental disc nest within species groups having a normally developed mental disc. To do so, we analysed a full set of species of *Garra* species found in the Middle East, excluding those from the Arabian Peninsula, which were recently studied by Hamidan et al. (2014). A second objective was to shed light on the potential ecological drivers for the reduction of the mental disc in species of *Garra*. Many species of *Garra* are highly rheophilic, inhabiting rapids and fast-flowing rivers (our own field data). The mental disc in *Garra* is a structure which is interpreted as an adhesive structure allowing fish to hold a stationary position in high water velocities (Zhang, 2005; Zhou et al., 2005). Thus, it is plausible that species with a reduced mental disc inhabit different habitats than species with a fully developed mental disc. If the mental disc functions as an adhesive structure, stagnophilic species should lack or show a reduced mental disc. To test this hypothesis, we compared general habitat information collected through our own fieldwork in the Middle East.

Materials and methods

Fish specimens ($n = 150$) of 16 described and 4 undescribed species were collected by electrofishing (Supplementary Table 1). The taxonomy of all species studied here follows strictly the latest version of the Catalogue of Fishes (Eschmeyer et al., 2016). Fish were killed by over-anaesthesia using clove powder, chlorobutanol or tricaine methanesulfonate (MS222). The right pectoral fin was clipped and fixed in 96% ethanol for subsequent DNA extraction and genetic

analyses. Voucher specimens were preserved in 5% formaldehyde, and stored in 70% ethanol.

DNA extraction was performed using the Chelex 100 method (Estoup et al., 1996) or NucleoSpin[®] Tissue kits (Macherey & Nagel GmbH). Either the complete mtDNA cytochrome *c* oxidase subunit I (*COI*) gene was amplified using primers *FCOI20* (5'-AACCTCTGTCTTCGGGGCTA-3') and *RCOI20* (5'-AGTGGTTATGYGGCTGGCTT-3') (Hashemzadeh Segherloo et al., 2012), or the 5' end of *COI* was amplified using primers FishF2_t1 (5'-TGTA AAC GACGGCCAGTCGACTAATCATAAAGATATCG GCAC-3'), FishR2_t1 (5-CAGGAAACAGCTAT GACTTCAGGGTGACCGAAGAATCAGAA-3'), VF2_t1 (5'-TGTA AAC GACGGCCAGTCAAC CAACCACAAAGACATTGGCAC-3'), and FR1d_t1 (5'-CAGGAAACAGCTATGACACCTCAGGGTGT CCGAARAAYCARAA-3') (Ivanova et al., 2007). Polymerase chain reaction (PCR) conditions were as follows: a 25- μ l final reaction volume containing 2.5 μ l of 10X *Taq* polymerase buffer, 0.5 μ l of (50 mM) MgCl₂, 0.5 μ l of (10 mM) dNTPs, 0.5 μ l (10 μ m) of each primer, 0.5 μ l of *Taq* polymerase (5u μ l⁻¹), 2 μ l of total DNA, and 18 μ l of H₂O. Amplification cycles were as follows: denaturation for 10 min at 94°C; 35 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min; and a final extension for 10 min at 72°C. The 5' end of the *COI* gene was sequenced in both direction using primer *FCOI20* on an ABI-3130xl DNA sequencer using the manufacturer's protocol (Life Technologies, www.lifetechnologies.com), or at MacroGen Europe Laboratories with the forward sequencing primer M13F (5' GTAAAACGACGGCCAGT) and reverse sequencing primer M13R-pUC (5' CAGGAAACAGCTATGAC).

DNA sequence data were aligned and processed using ClustalX (Thompson et al., 1997) and MEGA6 (Tamura et al., 2013). To determine the haplotype groups and the related frequencies, a parsimony network was constructed using TCS 1.21 (Thompson et al., 1997) with parsimony probability of 90%; haplotype frequencies were extracted manually from the resulting groups. All generated sequences were deposited in the NCBI GenBank database (Supplementary Table 1).

The *COI* haplotype sequences generated were compared to published cyprinid sequences following BLAST searches (Altschul et al., 1997), identifying similar sequences for use in phylogenetic analysis (Table 1). Kimura two-parameter (K2P) distances

Table 1 List of DNA sequences included from the NCBI GenBank database and associated accession numbers

Species	Accession no.	Species	Accession no.
<i>Bangana</i> sp.	JX074149.1	<i>Garra lamta</i>	JX074158.1
<i>Cirrhinus mrigala</i> (Hamilton, 1822)	GU195083.1	<i>Garra longipinnis</i> Banister & Clarke, 1977	KM214701.1
<i>Garra barreimiae</i> Fowler & Steinitz, 1956	KM214783.1	<i>Garra mullya</i> (Sykes, 1839)	JX074155.1
<i>Garra bicornuta</i> Narayan Rao, 1920	JX074156.1	<i>Garra nasuta</i> (McClelland, 1838)	JX074219.1
<i>Garra bourreti</i> (Pellegrin, 1928)	JQ864601.1	<i>Garra nasuta</i>	JX074219.1
<i>Garra congoensis</i> Poll, 1959	HM418168.1	<i>Garra orientalis</i> Nichols, 1925	GU086602.1
<i>Garra cyrano</i> Kottelat, 2000	JX074214.1	<i>Garra orientalis</i>	JQ864603.1
<i>Garra cyrano</i>	JX074214.1	<i>Garra orientalis</i>	HM536884.1
<i>Garra dembeensis</i> (Rüppell, 1835)	KT192819.1	<i>Garra ornate</i> (Nichols & Griscom, 1917)	JX074202.1
<i>Garra dembeensis</i>	KF929909.1	<i>Garra sahilia</i> Krupp, 1983	KM214682.1
<i>Garra flavatra</i> Kullander & Fang, 2004	JF915607.1	<i>Garra salweenica</i> Hora & Mukerji, 1934	KM610651.1
<i>Garra gotyla</i> (Gray, 1830)	FJ459493.1	<i>Garra tengchongensis</i> Zhang & Chen, 2002	JQ864607.1
<i>Garra gotyla</i>	JF915613.1	<i>Garra turcica</i> Karaman, 1971	KM214696.1
<i>Garra gravelyi</i> (Annandale, 1919)	JF915614.1	<i>Garra waterloti</i> (Pellegrin, 1935)	JX074212.1
<i>Garra hughi</i> Silas, 1955	HQ219117.1	<i>Garra waterloti</i>	JX074212.1
<i>Garra jordanica</i>	KJ553525.1	<i>Labeo bata</i> (Hamilton, 1822)	KC757216.1
<i>Garra kempfi</i> Hora, 1921	JX074161.1	<i>Labeo chrysophekadion</i> (Bleeker, 1849)	AP011199.1
<i>Garra lamta</i> (Hamilton, 1822)	JX074157.1	<i>Labeo rohita</i> (Hamilton, 1822)	JN412817.1

(Kimura, 1980) were computed at the intra- and interspecific levels using MEGA6 (Tamura et al., 2013).

For the phylogenetic analyses, the sequences were treated in two different ways. In the first approach, no evolutionary difference among the codon positions was assumed and hence all codon positions were exposed to regular non-partitioned phylogenetic analysis. In the second approach, the sequences were exposed to codon partitioning and analysed as different partitions to test for possible differences in phylogenetic inferences.

For the non-partitioned phylogenetic analysis, the best-fit model of base substitution was selected based on the Akaike information criterion (AIC) and Bayesian information criterion (BIC) using jModeltest 2.1.8 (Darriba et al., 2012). Bayesian inference was used to analyse the sequences with MrBayes 3.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The MCMC search was run for 5,000,000 generations, sampling the Markov chain every 1000 generations. The first 25% (1,250) of the resulting trees were discarded as burn-in. The Maximum Likelihood analysis was performed using PhyML program (Guindon et al., 2010). In order to analyse the codon positions under different partitioning schemes, the best schemes for both the Bayesian and Maximum likelihood

inferences of phylogeny were selected using PartitionFinder (Lanfear et al., 2012). Five different combinations of codon positions were checked using BIC criterion, and the best combination of model/s for each partitioning scheme was selected among the models. The Bayesian phylogenetic analysis was performed for 2,600,000 generations, sampling the Markov chain every 100 generations, with the first 25% of the resulting trees discarded as burn-in. Codon-partitioned Maximum Likelihood analysis was performed using RAxML (Stamatakis, 2006) with 1,000 bootstrap replicates as branch support. Five labeonin species—including *Labeo rohita*, *Cirrhinus mrigala*, *Labeo bata*, *Bangana* sp., and *Labeo chrysophekadion*—were used as out-group species based on other studies of labeonin phylogeny (Yang et al., 2012) and our preliminary analysis of data. In addition to the sequences produced in this study, sequences from 28 species from GenBank (NCBI) were included in the analysis (Table 1). Substitution saturation was checked using the substitution test (Xia et al., 2003; Xia & Lemey, 2009) implemented in DAMBE software (Xia, 2013).

The molecular clock test was performed by comparing the ML value for the given topology with and without the molecular clock constraints under the

Tamura-Nei (1993) model as implemented in MEGA 6 (Tamura et al., 2013). The null hypothesis of equal evolutionary rate throughout the tree was rejected ($P < 0.05$). Hence, to check for the divergence times among the Middle Eastern and Afro-Asian species of the genus, a molecular clock was calibrated using BEAST v 1.7 (Drummond et al., 2012). The time of the most recent common ancestor of the Middle Eastern *G. rufa* and its African sister clade has been estimated to be around 8–10 Ma (the possible connection period between the Africa and Asia via the Red Sea, Tang et al., 2009) and 23.4 Ma for the divergence of *Labeo* Cuvier, 1816 from *Garra* (Tang et al., 2009). The analyses were performed using the SRD06 model of sequence evolution, the relaxed uncorrelated lognormal (UCLN) molecular clock model, a Yule prior set on the tree, and other priors left as default (Tsigenopoulos et al., 2010). The analysis was performed for 10^7 generations. Parameters were logged every 1,000 generations, and the first 10% was discarded as burn-in. Because our molecular clock could not be calibrated with fossils, time estimates must be interpreted cautiously.

Results

COI sequence variation

Among the 150 individuals sequenced, 60 haplotypes were recovered. A total of 232 bp of the 652 bp were variable, and of these 199 sites were parsimony informative. The base composition over all sites was on average 29.2% (T), 26.2% (C), 26.1% (A), and 18.4% (G). There was no significant saturation in codon positions (ISS < ISSC; $P < 0.01$). The mean interspecies K2P distances ranged from 1.31% between *Garra mondica* Sayyadzadeh, Esmaceli & Freyhof, 2015 and *G. sp.* (Kol) to 19.75% between *Garra kemali* (Hankó, 1925) and *Garra ghorensis* Krupp, 1982 (mean \pm SD; 9.55 ± 4.84). The mean intraspecies genetic distances ranged between 0.1% in *Garra thyphlops* (Bruun & Kaiser, 1944) and 2.05% in *Garra gymnothorax* Berg, 1949 (mean \pm SD; 0.33 ± 0.48). The mean between-group K2P distance between the two major phylogenetic groupings (Clade I and Clade II, Fig. 1) was 15.9%. The mean within-group distances among the members of the clade I and clade II were 8.4 and 5.4%, respectively.

Phylogenetic analysis

The best mutational model based on AIC and BIC values was the GTR+ Γ +I model with an α value of 1.49. Phylogenetic trees reconstructed by Bayesian and Maximum Likelihood methods resulted in similar topologies. Under the codon partitioning approach, the best partitioning scheme for both the Bayesian inference of phylogeny and Maximum Likelihood methods was the scheme in which each codon position considered as a separate partition. The best mutational models to be used for Bayesian inference of phylogeny were SYM+I+G (for the 1st codon position), F81+I (for the 2nd codon position), and GTR+G (for the 3rd codon position). For the codon-partitioned Maximum Likelihood analysis of phylogeny, the 3-partitioned scheme was selected as the best scheme, but only one model (GTR+G) was used. The phylogenetic trees resulting from different approaches and models were mostly similar in topology, and the only observable difference between the non-partitioning and partitioning approaches was changes in support values related to each approach. Under the Bayesian method, in some cases, the posterior probability values were lower in the phylograms reconstructed via the codon partitioning approach, and for the Maximum Likelihood method the bootstrap support values were improved in some cases. Here the phylogram reconstructed via the codon partitioning approach is presented; in cases where the support values or posterior probabilities were different between the partitioned and non-partitioned analyses, we indicate that in the text as npBS and npBI.

In the phylogram (Fig. 1), the Middle Eastern species of *Garra* form two major clades that are called here clade I (BS: 58; npBS: 59, npBI: 0.95) and clade II (BS: 95; npBS: 89, npBI: 1.00). In our analysis, the African *Garra* clade is the sister to clade II (BS: 76; npBS: 65; npBI: 0.96). Clade I is composed of *Garra rossica* (Nikolskii, 1900), *G. kemali*, *Garra mendere-sensis* (Küçük, Bayçelebi, Güçlü & Gülle, 2015), and *G. varibilis*. Within clade II, there are a number of sub-clades, including sub-clades A (*Garra culiciphaga* (Pellegrin, 1927); BS: 99; BI: 1.00; npBS:96), B (*Garra lorestanensis* Mousavi-Sabet & Eagderi, 2016, *Garra thyphlops* (Bruun & Kaiser, 1944), *G. gymnothorax*; BS: 68; BI: 0.98; npBS:55; npBI:0.96), C (*Garra jordanica* Hamidan, Geiger & Freyhof, 2014 and *G. ghorensis*; BS: 93; BI: 0.99), D (*G. sp.*

Nahralkabir and Orontes; BS: 78), E (*Garra persica* Berg, 1914; BS: 84; BI: 0.99), F (*Garra widdowsoni* (Trewavas, 1955), *Garra turcica*, *Garra elegans* (Günther, 1868), *G. mondica*, *G. sp. Tigris*, *G. sp. Kol*; BS: 70; npBS:64), and G (*G. rufa*; BS: 54; npBS:58), with *Garra festai* (Tortonese, 1939) as the sister group of other sub-clades of clade II.

Molecular clock calibration

Based on the molecular clock calibration applied here, the mean divergence rate (substitution per site in million years) over all taxa included in the analysis was 0.008 ± 0.00007 (mean \pm SE). According to the molecular clock calibration, clade I probably diverged from some Asian species of *Garra* about 12 Ma (95% HPD: 8.09–16.75), and from the other group that includes clade II around 15.7 Ma (95% HPD: 12.07–19.90). The divergence of *G. rossica* from other members of clade I probably occurred around 10 Ma (95% HPD: 6.46–14.36). The divergence of *G. variabilis*

from *G. kemali* and *G. menderesensis* occurred around 5.5 Ma (95% HPD: 2.96–8.46). The divergence of clade II from the Asian and African *Garra* clades probably occurred around 10.5 Ma (95% HPD: 8.81–12.59) and 9.3 Ma (95% HPD: 8.21–10.47), respectively.

Discussion

Subdivisions and dispersal of *Garra* clades in the Middle East

Middle Eastern *Garra* forms two major clades supported with low to high bootstrap values of 58–95% (npBS: 59–89). The two clades concord with the two-invasion-wave hypothesis proposed by Menon (1964). Menon (1964) even postulated that *G. variabilis* and *G. rossica* are closely related as both have a small and poorly structured mental disc, a result confirmed by our study, although with low likelihood support (BS: 58; npBS: 59) (Fig. 1). In addition to *G.*

Fig. 1 Bayesian phylogram reconstructed based on the 5' end of the mitochondrial *COI* gene using a codon partitioning approach. The values on the branches are the maximum likelihood bootstrap values and Bayesian posterior probability values (after slashes) calculated as node supports for the Bayesian phylogram (higher than 0.95). *Green branches* = no mental disc, *blue branches* = reduced mental disc, and *black branches* = fully developed mental disc. The *triangle* and *circles* denote the African and non-Middle Eastern/Asian haplotypes, respectively. The codes presented after the names are unique voucher IDs containing the species name, main river basin, country of origin of the specimen, and the unique voucher code for the specimens used in this study. The out-groups are not shown

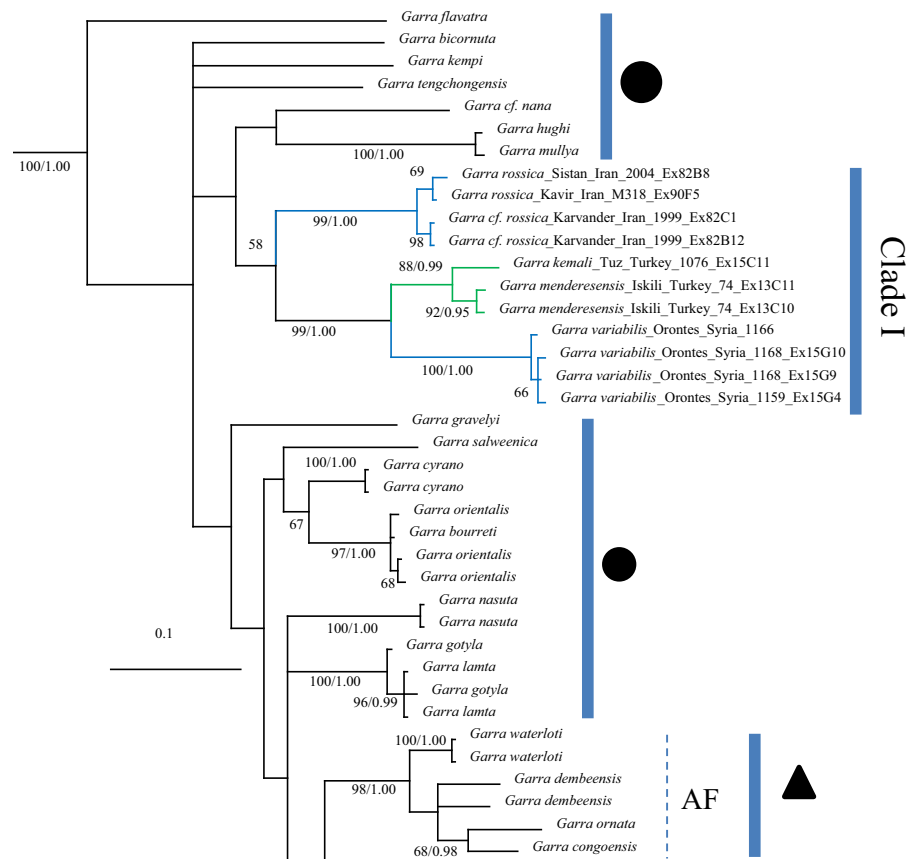
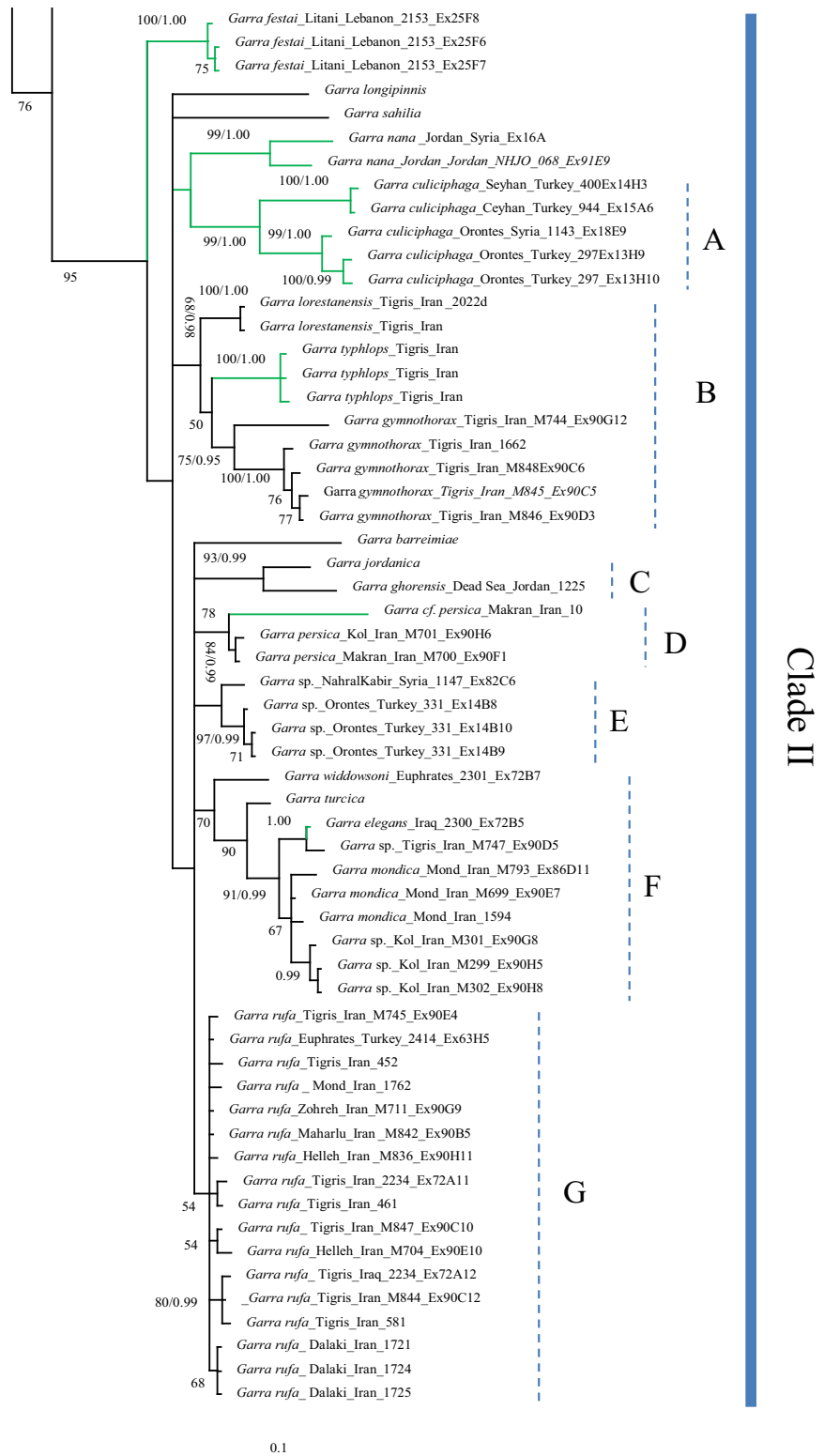


Fig. 1 continued



variabilis and *G. rossica*, *G. kemali* and *G. mendere-sensis* that lack a mental disc are nested in this clade. The inclusion of these species in the *G. variabilis* clade also is supported by the phylogeny reconstructed based on the rhodopsin gene (Behrens-Chapuis et al., 2015).

G. kemali and *G. mendrensensis* are endemic to lakes Tuz and Iskili in central Anatolia, and *G. variabilis* is distributed in the Orontes and Tigris–Euphrates systems. According to the geographic proximity of the Anatolian lakes and the Orontes system, the species may have their origin in the Orontes system. The molecular clock calibrated based on the dates proposed in Tang et al. (2009) suggests that the divergence of *G. kemali* and *G. mendere-sensis* from *G. variabilis* occurred around 5.5 Ma (95% HPD: 2.96–8.46). This period is concordant with the Mesenian salinity crisis of the Mediterranean Sea, during which confluence of the freshwater habitats may have occurred. Based on the molecular clock, the divergence between *G. kemali* of the Lake Tuz and *G. mendere-sensis* of Lake Iskili would have been occurred around 2 Ma (95% HPD: 0.90–4.10), which may be related to local-scale geodynamics in central Anatolia. It should be noted that these molecular clock dates may not be precise, since they are based only on *COI* sequences and geological data. As mentioned above, no *Garra* fossil data with relevant extant species exist from the Middle East to be used for molecular calibration. The only fossil data in the region are reported from Armenia (Vasilyan & Carnevale, 2013) where there is no extant *Garra* species and so no molecular data for clock calibration. The relationship of the clades I and II is not supported (BS < 50). The African species are sister to clade II (BS: 76; npBS: 65; npBI: 0.96). This inference is concordant with the Asia-to-Africa movement of the species of *Garra* proposed by Tang et al. (2009) and Yang et al. (2012). This relationship can be supported by the land bridge created between Africa and Asia around 8–10 Ma when sea levels decreased to about 60 m below the current level and connection of Asia to Africa became possible via the Red Sea (Tang et al., 2009).

Based on the *COI* phylogeny, seven sub-clades can be defined within clade II (Fig. 1). Sub-clade A includes *G. culiciphaga*, a species lacking the mental disc, of the Seyhan, Ceyhan and Orontes River drainages, all located in the northeastern Mediterranean basin. The approximate time of their

divergence is around 4 Ma, which may be related to river confluences that provided the possibility of invasion from the Orontes drainage to the Seyhan/Ceyhan drainages. However, one must keep in mind that the molecular clock calibrated for the *Garra* species may not be precise.

Sub-clade B includes *G. gymnothorax*, *G. typhlops*, and *G. lorestanensi*. *Garra typhlops* lacks the mental disc, but the other two species have a fully developed mental disc. These three species inhabit the Karun and Karkheh river drainages in the Iranian Tigris catchment. They show a high level of mtDNA divergence that may be due to the habitat isolation of *G. lorestanensi* and *G. typhlops* from *G. gymnothorax*. Based on our data, *G. lorestanensi* and *G. typhlops*, two sympatric subterranean species, probably resulted from two different colonization events of the subterranean habitat. This justification also can be the case for *G. widdowsoni* of sub-clade F, which is a subterranean species diverged from its surface-dwelling relatives. The members of sub-clade C, including *G. jordanica* and *G. ghorensis*—both with well-developed mental disc—occur in the Dead Sea basin and show close relationships to one another.

The non-disc-bearing *G. cf. persica* also nests among members of the sub-clade D, which includes the disc-bearing *G. persica* from the Persian Gulf basin. *Garra cf. persica* is highly diverged from *G. persica* and inhabits the Kesh River (Makran Basin) draining to the Sea of Oman. This pronounced level of divergence has resulted from the historic isolation of the basins in the Persian Gulf region and the Sea of Oman, a region which was not as strongly affected as the Persian Gulf during the last ice age. Hence, any interchange of the freshwater ichthyofauna between the two basins should relate to earlier periods.

Sub-clade F includes *G. widdowsoni* (Euphrates), *G. turcica* (Ceyhan drainage), *G. elegans* (Tigris), *G. sp.* (Tigris), *G. mondica* (Mond), and *G. sp.* (Kol). All the members of this sub-clade except *G. elegans* have a fully developed mental disc. Colonization events for members of this sub-clade, spanning the region from the Tigris to the Mond and Kol Basins in the Persian Gulf region, may have occurred during the last ice age via the watershed confluence events in the Persian Gulf. The members of this sub-clade in the Mond Basin were recently described as *Garra mondica* (Sayyadzadeh et al., 2015). The centre of origin of sub-clade F based on the haplotype network produced

using the present haplotypes cannot be resolved (Fig. 2), since none of the available haplotypes show to be ancestral. This question may be resolved via more intensive sampling to obtain more ancestral haplotypes.

Sub-clade G includes populations of *G. rufa*, all found in the Persian Gulf basin. Their close

relationship may indicate recent dispersal of the sub-clade during the last ice age (18,000 years bp). At that time, the Persian Gulf was dry, and the smaller Iranian rivers, including the Mond, were part of the greater Tigris drainage (Fagan, 2014). Hence, colonization events among the respective rivers were possible. According to the haplotype network, it seems that

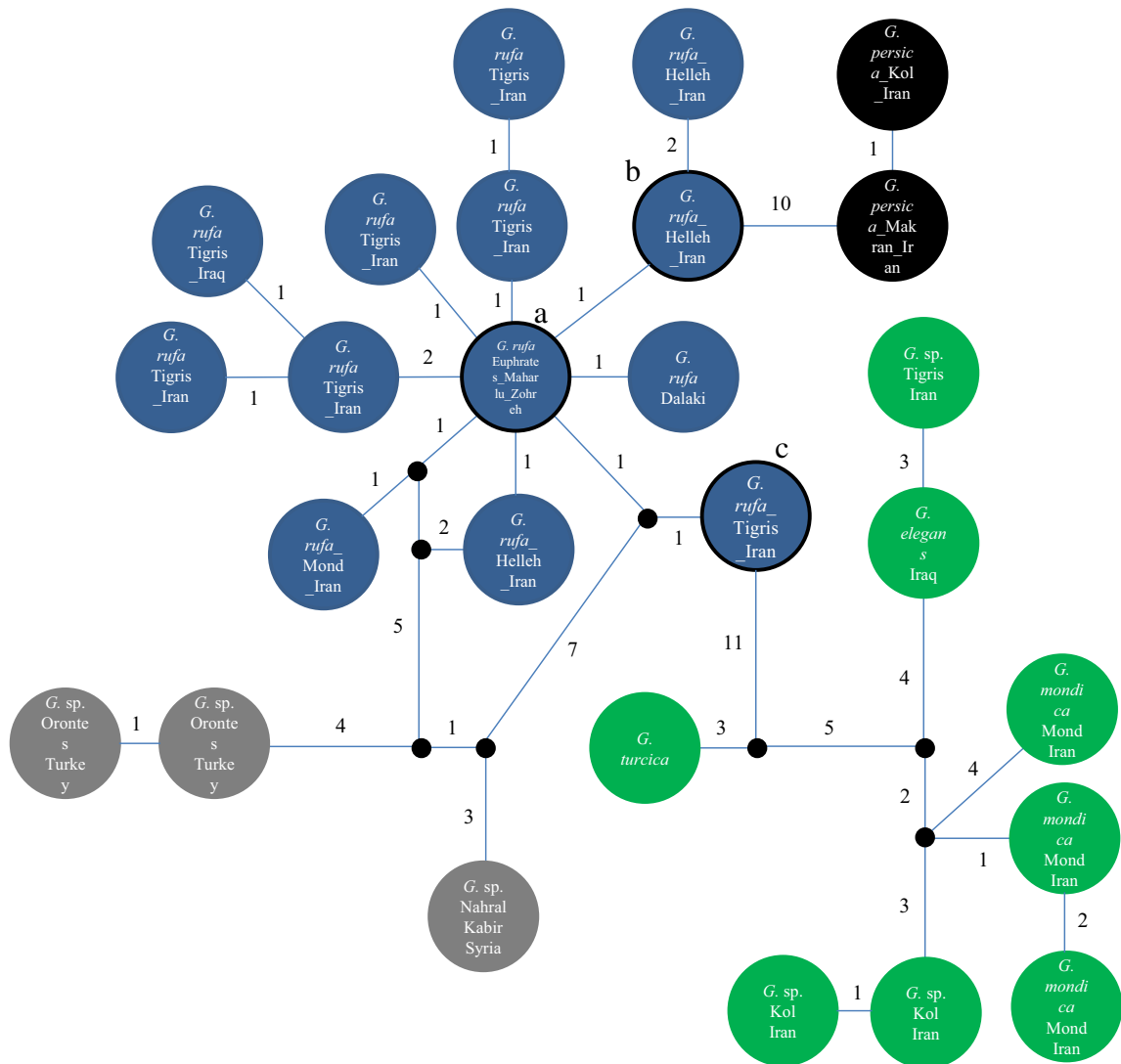


Fig. 2 Haplotype network showing mutational connections of different haplotypes in sub-clades D, E, F, and G. The *large circles* denote different haplotypes, the related species and the geographic locality. The size of the circles is not related to haplotype frequencies. The *colours of the circles* denote: *black* sub-clade D; *grey*: sub-clade E; *green*: sub-clade F; and *blue*: sub-clade G. The *blue circles with black outline* are the possible

ancestral haplotypes. The *blue lines* connecting the haplotypes denote the mutational links and the numbers beside each line indicate the number of point-mutational differences between each pair of neighbouring haplotypes. The *smaller black circles* show the hypothetical haplotypes that lead to observed haplotypes

haplotype *a* observed in the Euphrates (Turkey), Maharlu (Iran), and Zohreh (Iran) systems is apparently one of the ancestral haplotypes due to its wide geographic distribution and more connections to other haplotypes. Among the other haplotypes, haplotypes *b* and *c* also seem to be old haplotypes, since they are intermediate haplotypes located on the mutational path between haplotype *a* and the *G. persica* and *G. mondica* groups, respectively. Other haplotypes within sub-clade G are probably newer haplotypes with fewer connections and terminal positions in the network (Fig. 2).

Because our *G. rufa* samples are mostly from Iran with only a few specimens from other regions, samples of this species may not be sufficiently informative derive an inference regarding the centre of origin of sub-clade G. However, since an east–west direction of invasion is accepted (Tang et al., 2009) for labeonins, the direction of the *G. rufa* colonization also may be inferred from easterly drainages to the Tigris–Euphrates. This also may be confirmed by observation of intermediate haplotypes connecting the eastern *G. persica* (sub-clade D) and *G. mondica* (sub-clade F) to *G. rufa* (sub-clade G) (Fig. 2). Clearly, this inference cannot be confirmed until more specimens and additional molecular data are collected and analysed.

Disc shape

Menon (1964) synthesized his hypothesis regarding two waves of invasion of *Garra* from East Asia to the Middle East based on morphological attributes, including characters of the mental disc. He assumed

the reduced disc character as the ancestral state and the disc-bearing state as a derived state. We argue, however, that the mental disc may have been fully reduced independently in 6–7 lineages in the Middle East (Fig. 1). This reduction may have occurred once in clade I, in which *G. kemali* and *G. mendere-sensis* lack a mental disc, while *G. variabilis* and *G. rossica* have a small and partly reduced mental disc, respectively. Within clade II, 5–6 independent lineages apparently have reduced the mental disc, depending upon whether *G. festai* is or is not related to *Garra nana* (Heckel, 1843) (Fig. 3). Tree topologies presented by Behrens-Chapuis et al. (2015) place *G. culiciphaga*, *G. nana*, and *G. festai* in one group, while our data separate *G. festai* from the other two species. As disc evolution in labeonins had been shown to be bidirectional by Zheng et al. (2012), the question is whether the mental disc was reduced in convergence or developed in convergence. As the structure of the mental disc is identical or differs in very minor aspects in all disc-bearing species studied here, as well as *Garra* species from Africa (Stiassny & Getahun, 2007) and Asia (Menon, 1964), we see convergent reduction as the most parsimonious explanation of the phenomena described here.

Possible character displacement

Based on our own field data in which general habitat and microhabitats were noted, up to three *Garra* species with different disc forms can be found sympatrically (Table 2). This is especially true in

Fig. 3 Ventral views of heads showing the mental disc. From the left: *G. rufa*, FSJF 3368, 102 mm SL, Nalparez River, Iraq; *G. variabilis*, FSJF 2845, 95 mm SL, Tigris River, Turkey; *G. festai*, FSJF 3268, 65 mm SL, Ammiq Marshes, Lebanon. Heads are not shown in scale



Table 2 Middle Eastern *Garra* species with different mental disc states and their sympatric species

Drainage studied	Mental disc state		
	Fully developed	Absent	Reduced
Ceyhan	<i>G. rufa</i>	<i>G. culiciphaga</i>	–
Euphrates (epigean)	<i>G. rufa</i>	–	<i>G. variabilis</i>
Jordan	<i>G. sp.</i>	<i>G. nana</i>	–
Karun (subterranean)	<i>G. lorestanensi</i>	<i>G. typhlops</i>	–
Kol	<i>G. persica</i> & <i>G. sp.</i>		–
Mond	<i>G. rufa</i> & <i>G. mondica</i>	–	–
Orontes	<i>G. sp.</i>	<i>G. culiciphaga</i>	<i>G. variabilis</i>
Tigris	<i>G. rufa</i>	<i>G. elegans</i>	<i>G. variabilis</i>

springs, where open gravel fields with outflowing water occur closely adjacent to densely vegetated, stagnant microhabitats. Species with a fully developed mental disc are generally the only species in rapids and riffle habitats with fast-flowing water. Species with a reduced or absent mental disc are absent from fast-flowing waters and instead occur in slow-flowing streams, marshes and densely vegetated parts of springs. Different field collection notes reveal that sympatric *G. rufa* and *G. variabilis* show remarkable differences in microhabitat choice, with *G. rufa* being strongly connected to gravel and flowing water, and *G. variabilis* associated with submerged vegetation and standing water. Other sympatric species, for example, *G. typhlops* and *G. lorestanensis* collected at the opening of the subterranean water layer in Iran, seem to differ in their ecological niches. Based on several observations during different periods of the year, the disc-bearing species appear at the opening during the pluvial periods of the year when there is water flow, but the non-disc-bearing species (*G. typhlops*) can be observed at the locality all year-round. These observations may indicate niche isolation driven by disc form and water flow. This situation might be interpreted as indicating character displacement (Brown & Wilson, 1956; Gray et al., 2005; Pfennig & Pfennig, 2010, 2009; Robinson & Pfennig, 2013; Robinson & Wilson, 1994; Schluter, 2000), allowing the species to reduce competition in the subterranean habitat. Character displacement leading different species to avoid competition in sympatry may have allowed the ecological co-occurrence of other *Garra* species after their secondary contact. Admittedly, our data are not sufficient to rigorously support this hypothesis. Testing

of this hypothesis will require more data collected both in situ and used for more detailed phylogenetic assessment. Our observations indicating a possible relationship between mental disc shape and habitat preferences should be checked using a more quantitative approach recording habitat parameters and habitat selectivity for fish with different mental disc forms.

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