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Comparison of *Capoeta capoeta gracilis* (Cyprinidae, Teleostei) populations in the south Caspian Sea River basin, using morphometric ratios and genetic markers

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A b s t r a c t. The Siah Mahi, *Capoeta capoeta gracilis*, is a widely distributed taxon in the south Caspian Sea basin (north of Iran) that has not yet been surveyed at the intra-specific level. Besides its ecological significance, this species is important for inland water fishing, aquaculture, sport fishing and zoogeographical studies. Here, we describe patterns of morphological and molecular differentiation among six populations of *C. c. gracilis* located in the Aras, Sefidrud, Shirud, Tonekābon, Harāz and Gorgānrud river systems in Iran. Univariate analysis of variance revealed significant differences between means of the six samples for 56 out of 60 morphometric ratios. The first two ordination axes of the mean ratios of the six samples (59.11% of total variation) separated all of the samples from each other, although not necessarily with any clear geographic pattern. The overall assignment of individuals into their original groups was high (88.6%). The proportion of individuals correctly classified into their original groups was 92.5%, 78.9%, 96%, 89.7%, 93.3% and 83.3% for Aras, Gorgānrud, Sefidrud, Shirud, Tonekābon and Harāz, respectively. Molecular clustering also grouped individuals of each sample either in the same cluster or the same sub-cluster. There was a large-scale congruence between results of the morphological and molecular analyses.

Key words: Caspian Sea, Iranian ichthyofauna, RAPD, Siah Mahi

Introduction

Iran belongs to the Eurasian land mass (B ă n ă r e s c u & C o a d 1991), constituting a significant part of the Middle East both in terms of geographic area and zoogeography (C o a d 1987). The country has been divided into nineteen major drainage basins based on river systems (C o a d 1995; Fig 1). Iran's ichthyofauna contains both Ethiopian and Oriental elements, although it is principally part of the Palaearctic Realm (C o a d 1987).

The south Caspian Sea River basin encompasses 256,000 km^2, 15.5% of the whole country (Zakeri 1997). This basin is located in the European area of the Eurasia (B ă n ă r e s c u & C o a d 1991) and is one of the most diverse freshwater ecosystems in Iran (C o a d 1995).

The genus *Capoeta* Cuvier et Valenciennes, 1842 is found in eastern Europe and southwestern Asia (B ă n ă r e s c u & C o a d 1991) and contains about 10 species, of which 7 occur in Iran (C o a d 2005). It is one of the most taxonomically complex genera of the Cyprinidae family (C o a d 1995). Species and subspecies of *Capoeta* occur sympatrically (S a a d a t i 1977) and allopatrically in all basins of Iran. *Capoeta capoeta gracilis* (K e y s e r l i n g 1861), the focus of the current study, is found in the south Caspian Sea River basin.

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Scaphiodon gracilis was initially described by K e y s e r l i n g (1861) from "Wasserleitung bei Gaes, einige Meilen von Isphahan". The name has been generally attributed to *Capoeta capoeta* (Güldenstädt, 1773) in Iran. To date, four subspecies of *C. capoeta* have been described from Iran including: *Capoeta capoeta gracilis* (Keyserling, 1861), *Capoeta capoeta capoeta* (Güldenstädt, 1773), *Capoeta capoeta heratensis* (Keyserling, 1861) and *Capoeta capoeta sevangi* de Filippi, 1865.

Capoeta capoeta heratensis is a subspecies from the Tedzhen River basin (B e r g 1949). The subspecies can be distinguished by its two pairs of barbles in comparison with one pair in other subspecies. *Capoeta capoeta gracilis* (Fig. 2) is distinguishable from *Capoeta capoeta capoeta* (limited to Kura-Aras basin (B i a n c o & B ă n ă r e s c u 1982)) by having fewer dorsal fin rays on average, greater head length and depth, smaller eyes, longer snout and postorbital distance, greater body depth and caudal peduncle depth, a shorter postdorsal distance, a shorter dorsal fin base, lesser dorsal fin height, a longer anal fin base, a greater pectoral-pelvic fin distance and a shorter pelvic-anal fin distance (A b d u r a k h m a n o v 1962). *Capoeta capoeta sevangi* the subspecies found in the Araxes River basin (presumably including Iran), is distinguished from the type subspecies, *Capoeta capoeta capoeta* of the Kura River basin, by having a straight or slightly convex dorsal fin margin, as opposed to a slightly to moderately notched margin (B ă n ă r e s c u 1999).

C. c. gracilis is present in all rivers, lagoons, bays and water reservoirs in the south Caspian Sea River basin, from the coldest region in the west (Aras) to the most tropical region in the east (Gorgãnrud). Therefore, it is widely tolerant of extreme environmental conditions. There are a variety of local names for the taxon in the basin such as Tolkhoskoli, Gelkhorkoli, Siãhkoli, Tolkhos (at Tonekãbon city in Mãzandarãn province), Tilkhos (in Guilãn province) and Gelkhor.

Besides its ecological significance, *C. c. gracilis* is an important taxon for sport fishing (K i a b i et al. 1999), inland water fishing (in some rivers it comprises 33% of captured fishes), aquaculture (A b d o l i 2000), and zoogeography (A r m a n t r o u t 1980).

Although a few reports exist on the taxonomic status of *C. c. gracilis*, this subspecies has not yet been subjected to a comprehensive study at the inter-population level. Virtually nothing is known about the population structure of *C. c. gracilis* in river systems of the south Caspian Sea basin of northern Iran. It is vitally important to obtain detailed basic knowledge of the biology of this taxon, including information on population structure, as such information influences the development of management strategies for conserving biodiversity (T u r a n et al. 2005).

This study aims to investigate differentiation among six populations of *C.c. gracilis* throughout its range in the south Caspian Sea River basin on the basis of morphological characteristics and Random Amplified Polymorphic DNA (RAPD; W i l l i a m et al. 1990, W e l s h & M c C l e l l a n d 1990).

Materials and Methods

S a m p l e c o l l e c t i o n

For this study, 212 adult fish specimens ≥90 mm in length from snout to base of caudal fin gathered during 2005–2006. Specimens were collected from six isolated rivers over several ecological regions (Fig. 1) throughout the species' distribution range in the south Caspian Sea River basin: Aras River (A) in Azarbãyjãn province (40 specimens), Sefidrud (Se) in Guilãn

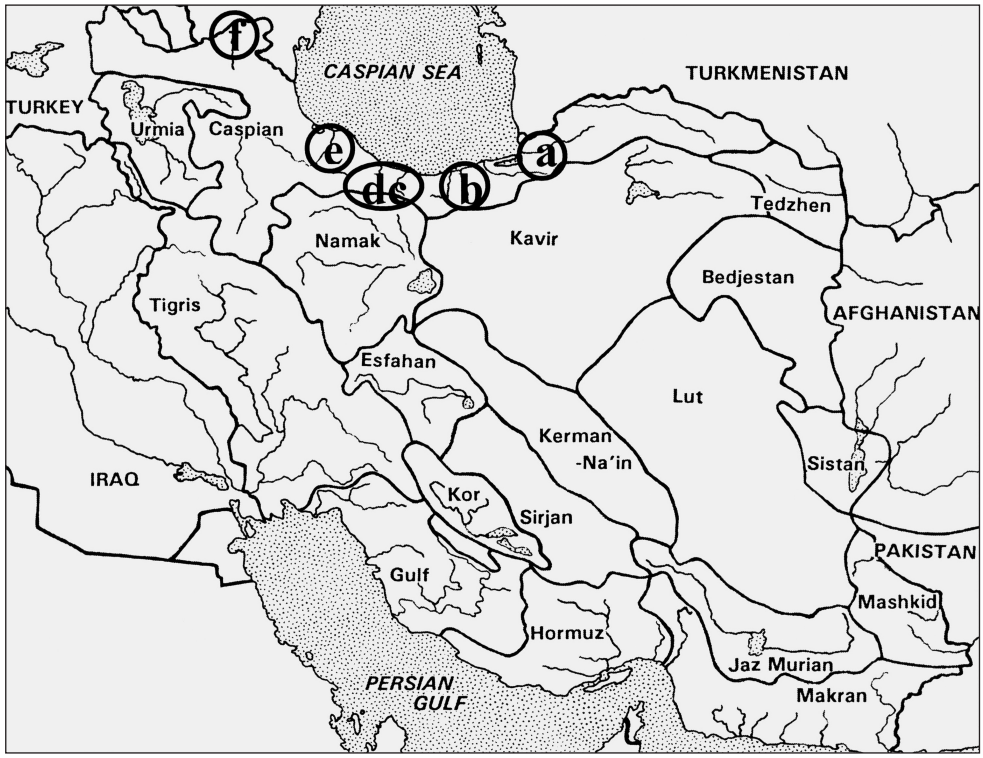

Fig. 1. Map of the 19 major drainage basins of Iran (the lake Mahãrlu basin lies between the Gulf and Kor basins) showing locations of sampling points: a, Gorgãnrud; b, Harãz; c, Tonekãbon; d, Shirud; e, Sefidrud; f, Aras (map from C o a d 1995).

province (25 specimens), Shirud (Sh), (49 specimens), Tonekãbon River (T), (30 specimens) and Harãz River (H) in Mãzandarãn province (30 specimens), and Gorgãnrud (G) in Golestãn province (38 specimens).

Specimens were deposited at the Ichthyology Laboratory, Department of Fisheries, University of Tehran, Iran for future study. Fish were caught by Electroshoker (P r i c e 1982) and Cast Net 1.5-centimeter mesh. The samples were sexed, photographed, tagged, and fixed in formalin solution (10 mL formalin, 0.9 g sodium chloride, and 100 mL water).

Three out of the six populations (Shirud, Sefidrud and Harãz) were subjected to molecular analysis. For the DNA study, 18 specimens were collected (six specimens from each population: three females and three males in order to account for sexual differences). This quantity is usually sufficient for studying inter-population differentiation. Live fish, were transported to the Ichthyology laboratory, Department of Fisheries and Environmental Sciences, University of Tehran, where blood samples were taken from the caudal vein using a syringe, mixed with EDTA (2mg/mL blood), and stored at 4 ºC until DNA extraction took place.

M o r p h o l o g i c a l c h a r a c t e r i s t i c s

In total, thirty morphometric characteristics were recorded from each fish specimen. Measurements were taken using electronic digital calipers precise to the nearest 0.01mm.

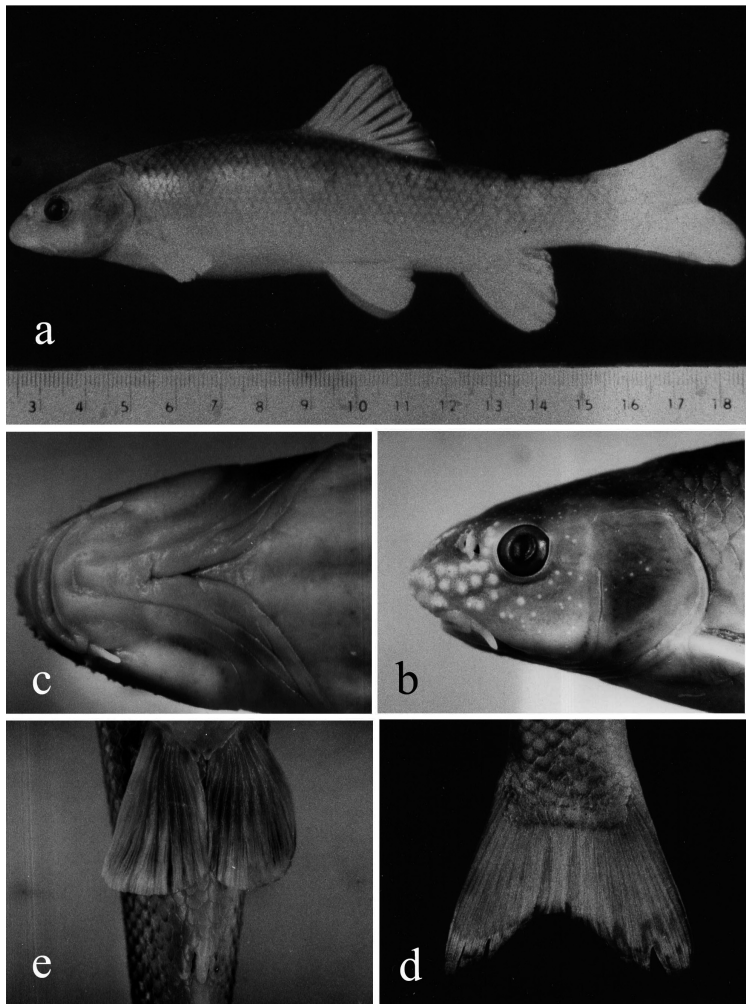

Fig. 2. A specimen of *C. c. gracilis* caught from the Gorgãnrud river (a) Lateral view of the entire specimen; (b) Lateral view of the head; (c) Ventral view of the head; (d) Caudal fin; and (e) Ventral view of the body between the pelvic and anal fins.

Measurements and their abbreviations followed H o l č í k et al. (1989), except where otherwise noted. Finally, 60 ratios were calculated from the morphometric data. Throughout this paper, these traits are referred to interchangeably as "traits", "characteristics", "data", "measurements" or "variables". The following abbreviations are used in the text and tables for morphometric characteristics and morphometric ratios:

Standard length, Sl [straight line distance from the anteriormost point of the snout (upper lip) to the base of the caudal fin (basis of central rays); (H o w e 2002)]; pectoral-pelvic length, P-V; ventral-anal length, V-A; anal-caudal length, A-C; pectoral-anal length, P-A; predorsal distance, pD; preanal distance, pA; postdorsal distance, poD; caudal peduncle length, lpc; caudal peduncle depth, hpc; caudal peduncle width, lapc; body depth, H; body width, laco; minimum body depth, h; head length, lc; head depth, hc; head width, lac; preorbital length (=snout length), prO; postorbital length, poO; inter-orbital distance, io;

inter-nasal distance, ina; eye diameter, Oh; height of dorsal fin, hD; length of dorsal fin base, lD, height of anal fin, hA; length of anal fin base, lA; pectoral fin length, lP; pectoral fin base, lPbs; ventral fin length, lV; ventral fin base lVbs; barbells length, lb; lc/Oh; lc/poO; lc/prO; Sl/lc; Sl/lpc; Sl/hD; Sl/H; lpc/h; P-A/hD; Sl/lD; Sl/lP; Sl/hc; Sl/lac; Sl/hpc; H/h; P-A/lA; P-V/lA; V-A/lA; A-C/lA; lpc/lA; Sl/Oh; lP/lV; hA/hD; lc/lb; hA/H; P-A/hA; P-V/hA; V-A/hA; A-C/hA; lpc/hA; lac/io; lc/hD; hD/lD; lP/lc; lc/hA; lc/lP; lc/lV; P-V/lP; lpc/hpc; Sl/pD; lc/ina; pD/poD; pD/lc; pD/hc; pD/lac; pD/lb; pD/lpc; pD/hpc; pD/h; pD/H; pD/lD; pD/hD; pD/hA; pD/lA; pA/pD; pD/Oh; pD/V-A; pD/A-C; pD/lP; pD/P-V.

DNA extraction

Total DNA was extracted from whole blood, following Maniatis (1989) and Kirby (1990) with minor modifications. Each 50 μl blood sample was mixed with 200 μl ice-cooled lysis buffer (0.32 M Sucrose, 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100) and centrifuged at 6000 rpm for 3 min. The aqueous phase was discarded and the remaining pellet was mixed with 500 μl of extraction buffer (10 mM Tris-HCl, 0.1 M ethylene diamine tetra acetic acid (EDTA) pH 8.0 and 0.1 M NaCl) and 10 μl proteinase K (10 mg/ml). Samples were incubated overnight at 37°C, or for 4 h at 65°C after which DNA was purified by successive extraction with phenol, phenol:chloroform:isoamylalcohol (25:24:1) and chloroform: isoamylalcohol (24:1). DNA precipitation was achieved by adding 1/10 volume sodium acetate and 2 volumes ice-cooled absolute ethanol and centrifuging at 10000 rpm for 15 min. Finally, DNA samples were air-dried for 30 min and then dissolved in 150 μl of modified TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). DNA stock was stored at -70°C until required.

DNA amplification protocol

PCR amplification was performed in a 25μl volumes, each containing 1XPCR buffer (500 mM KCl and 200 mM Tris-HCl, pH 8.4), 1.5 mM $MgCl_2$, 100 mM of dNTP, 1 Unit of Taq DNA polymerase (Gibco BRL), 5 pM of 10 base primer (Kit C, Operon Technologies Inc., Alameda, California), 25 ng genomic DNA, and overlaid with two drops of mineral oil as described in Williams et al. (1990) with minor modifications. Amplification was carried out by a thermocycler (Perkin-Elmer-Cetus, Norwolk, Connecticut) at 95ºC for 4 min for initial strand separation, followed by 40 cycles of 1 min at 94ºC, 1 min at 37ºC, 2 min at 72ºC, and a final 10 min extension at 72ºC. Negative (without DNA template) and positive (lambda DNA) controls were also included in all experiments. When any PCR product was detected in the negative control, the whole reaction set was discarded and repeated. Amplification products were separated by electrophoresis in 1.4% agarose gel in 0.5XTBE [10X TBE (0.9 M Tris, 25 mM EDTA, 0.9 M Boric acid)] based on Stellwagen (1998), stained with ethidium bromide, and photographed.

Statistical analysis

Morphometric ratios were log-transformed and standardized for numerical analysis. Morphological surveys in the present study were based on means of the six river samples for morphometric ratios. The coefficient of variation (CV) for each morphometric ratio was estimated.

Univariate analysis of variance (ANOVA) was carried out to test the significance of morphological differences among river samples and to test the significance of differences between species and sexes for all variables.

Table 1. Means of *C. c. gracilis* samples for 56 morphometric ratios and the coefficient of variation (C.V) for each variable. See Materials and Methods for abbreviations of morphometric ratios and samples.

Morphometric ratios		Means of the samples for 56 morphometric ratios						CV (%)	Morphometric ratios		Means of the samples for 56 morphometric ratios						CV (%)
		A	G	Se	Sh	T	H				A	G	Se	Sh	T	H	
1	lc/Oh	3.92	4.10	4.03	4.19	3.84	3.97	3	29	hA/H	0.65	0.71	0.74	0.78	0.69	0.77	7
2	pD/Oh	8.38	9.02	8.73	9.24	8.16	8.46	5	30	pD/H	1.92	2.15	2.15	2.14	2.09	2.16	4
3	pD/lc	2.14	2.20	2.16	2.20	2.11	2.13	2	31	Sl/pD	1.89	1.86	1.93	1.95	1.86	1.93	2
4	Sl/lc	4.06	4.10	4.21	4.30	3.97	4.13	3	32	P-A/pD	1.49	1.47	1.51	1.55	1.52	1.52	2
5	Sl/Oh	15.90	16.84	16.93	18.06	15.15	16.36	6	33	lpc/h	1.18	1.20	1.33	1.20	1.09	1.26	7
6	Sl/hD	4.78	5.21	5.16	5.40	4.93	4.74	5	34	lpc/hpc	1.04	1.08	1.17	1.05	0.94	1.09	7
7	P-A/hD	2.65	2.92	2.88	3.16	2.75	2.68	7	35	Sl/hpc	7.10	7.49	7.96	7.70	6.89	7.49	5
8	hA/hD	0.85	0.93	0.92	1.00	0.87	0.87	6	36	pD/hpc	3.73	4.01	4.10	3.95	3.69	3.94	4
9	SL/lP	5.25	5.48	5.50	5.57	5.21	5.10	4	37	pD/h	4.25	4.48	4.65	4.49	4.25	4.55	4
10	P-V/lP	1.75	1.80	1.79	1.90	1.72	1.57	6	38	Sl/hc	5.42	5.46	5.77	5.47	5.50	5.45	2
11	lc/prO	2.63	2.71	2.71	2.68	2.69	2.61	2	39	H/h	2.20	2.07	2.70	2.09	2.02	2.10	12
12	lc/lV	1.44	1.58	1.59	1.51	1.53	1.37	6	40	pD/hc	2.86	2.92	2.96	2.80	2.74	2.82	3
13	lc/lP	1.23	1.33	1.33	1.29	1.31	1.23	4	41	lc/poO	2.33	2.22	2.29	2.28	2.26	2.30	2
14	lP/lV	1.10	1.19	1.22	1.16	1.17	1.11	4	42	A-C/lA	2.86	2.74	2.71	2.67	2.61	2.91	4
15	lc/hD	1.17	1.27	1.22	1.25	1.24	1.14	4	43	lpc/lA	1.91	1.78	1.80	1.71	1.67	1.86	5
16	pD/hD	2.51	2.79	2.66	2.77	2.64	2.45	5	44	P-A/lA	7.20	6.92	6.78	7.34	6.81	7.32	4
17	pD/lP	2.76	2.93	2.83	2.85	2.79	2.64	3	45	V-A/lA	3.08	2.92	2.95	3.11	2.83	3.23	5
18	lc/ina	5.27	5.55	5.87	5.48	5.86	5.23	5	46	lP/lc	0.77	0.75	0.76	0.77	0.76	0.81	3
19	Sl/lD	6.87	6.92	6.68	7.16	6.48	6.49	4	47	hD/lD	1.44	1.32	1.29	1.32	1.38	1.37	4
20	lc/lb	6.00	6.26	5.70	6.82	6.20	5.23	9	48	P-A/pD	6.95	6.65	6.26	6.40	6.51	6.69	4
21	pD/lb	12.87	13.79	12.44	15.04	13.17	11.23	10	49	lc/hA	1.83	1.37	1.33	1.25	1.43	1.30	15
22	P-V/hA	1.88	1.85	1.79	1.83	1.87	1.67	4	50	A-C/hA	1.24	1.24	1.26	1.14	1.21	1.21	3
23	pD/lD	3.62	3.71	3.45	3.67	3.66	3.36	4	51	lpc/hA	0.83	0.81	0.83	0.73	0.76	0.77	5
24	P-V/lA	4.31	4.08	3.90	4.26	3.98	4.00	4	52	lac/io	1.75	1.82	1.78	1.30	1.78	1.73	12
25	Sl/lpc	6.83	6.95	6.73	7.34	7.27	7.01	3	53	pD/P-V	1.57	1.62	1.60	1.50	1.61	1.67	4
26	pD/lpc	3.60	3.72	3.50	3.76	3.90	3.63	4	54	pD/poD	1.52	1.59	1.50	1.48	1.55	1.54	3
27	pD/A-C	2.38	2.42	2.31	2.40	2.49	2.31	3	55	pD/hA	2.97	3.01	2.90	2.76	3.03	2.79	4
28	Sl/H	3.66	4.01	4.16	4.19	3.91	4.19	5	56	pD/V-A	2.20	2.27	2.13	2.06	2.30	2.07	5

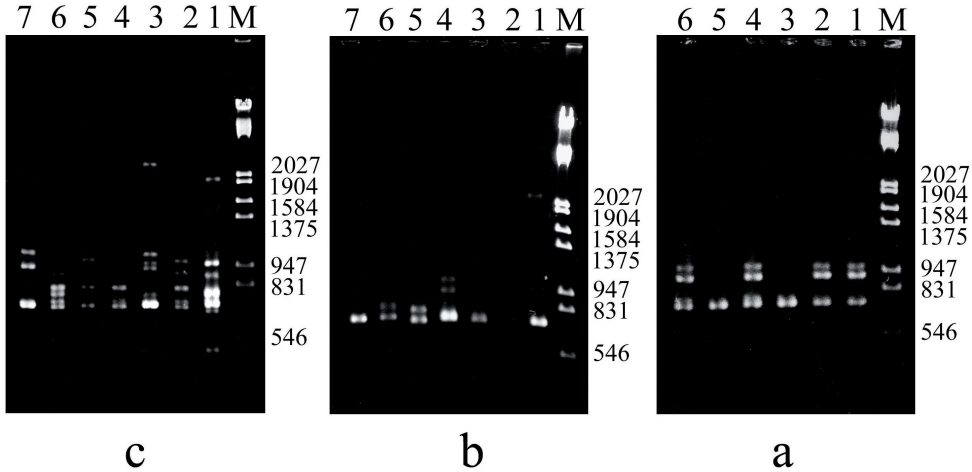

Fig. 3. Agarose gel electrophoresis of RAPD fragments obtained from river samples from (a) Harãz, (b) Sefidrud, and (c) Shirud. Lane M contains lambda DNA/Hind III, EcoR I digest. Other lanes show the separation of RAPD fragments amplified from genomic DNA samples using the decamer primer (OPC02). Lanes 1-3 and 4-7 contain DNA from male and female specimens, respectively.

A number of morphometric ratios that were significantly different, in the ANOVA, were submitted to a Pearson's product-moment correlation analysis (r) and then underwent to a data discarding process to reduce the dataset, eliminate redundancy among the variables, and to extract a number of independent characteristics.

In order to reveal multivariate morphological affinity of the *C. c. gracilis* river samples under study, the selected morphometric ratios obtained through the discarding process were subjected to a principal component analysis (PCA). Groups identified by PCA were validated by determining the number of individuals correctly vs. incorrectly assigned to each of group by discriminant function analysis (DFA).

In order to analyze molecular data, RAPD bands were scored manually in binary form using 1 to indicate presence and 0 to indicate absence of a band. To ensure consistent results, and to provide a conservative calculation of similarity coefficients, only repeatable and well-defined bands were included in statistical analysis (O k a m u r a 1993).

To evaluate the populations' genetic similarity, a cluster analysis (CA) was conducted on RAPD data. Clustering was based on J a c c a r d ' s (1908) similarity index, using an UPGMA (Unweighted pair group method with arithemetic average) algorithm.

Statistical analyses were performed using SPSS (SPSS Inc., Chicago, version 10.1), Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc version 2.02, R o h l f (1998)) and MINITAB (version 13.2, MINITAB Inc. 2000).

Results

M o r p h o l o g i c a l r e s u l t s

Interaction between morphometric ratios and sex were not significant, demonstrating a negligible effect of sex on observed variation. Therefore, data for both sexes were pooled for all subsequent analyses.

ANOVA revealed significant differences between means of the six river samples for 56 out of 60 morphometric ratios (only Sl/lac, P-A/hA, V-A/hA and pD/lac were not significantly different, $P>0.05$, and were therefore excluded from subsequent analyses).

Analysis of Pearson's product-moment correlation between 56 morphometric ratios revealed that there are significant functional associations among some of the variables (data not shown). In order to eliminate the nonorthogonality among the variables and select a number of independent morphological characteristics, a data discarding process was run on the 56 morphometric ratios. The data discarding process was carried out by conducting a cluster analysis on the 56 significant morphometric ratios. Cluster analysis organized the 56 morphometric ratios into 17 distinct clusters (Fig. 4). The single ratio with the highest CV (Table 1, columns 9 and 18), was selected from each of the 17 distinct clusters for subsequent analyses: P-A/hD, lc/lV, pD/lb, P-V/lA, pD/lpc, hA/H, Sl/pD, lpc/h, H/h, pD/hc, lc/poO, lpc/lA, V-A/lA, lc/hA, lac/io, pD/P-V and pD/V-A (see Fig. 4).

Examination of the estimates of the correlated Pearson's coefficients among the 17 selected ratios showed, only five significant correlations: pD/lb vs pD/P-V with ($r = -0.833$, $P < 0.05$), pD/lpc vs. lpc/h ($r = -0.877$, $P < 0.05$), hA/H vs. lc/hA ($r = -0.888$, $P < 0.05$), Sl/pD vs. pD/V-A ($r = -0.977$, $P < 0.01$) and V-A/lA vs. pD/V-A ($r = -0.823$, $P < 0.05$). Therefore, Pearson's analysis confirmed that the methodology utilized for discarding characteristics was appropriate.

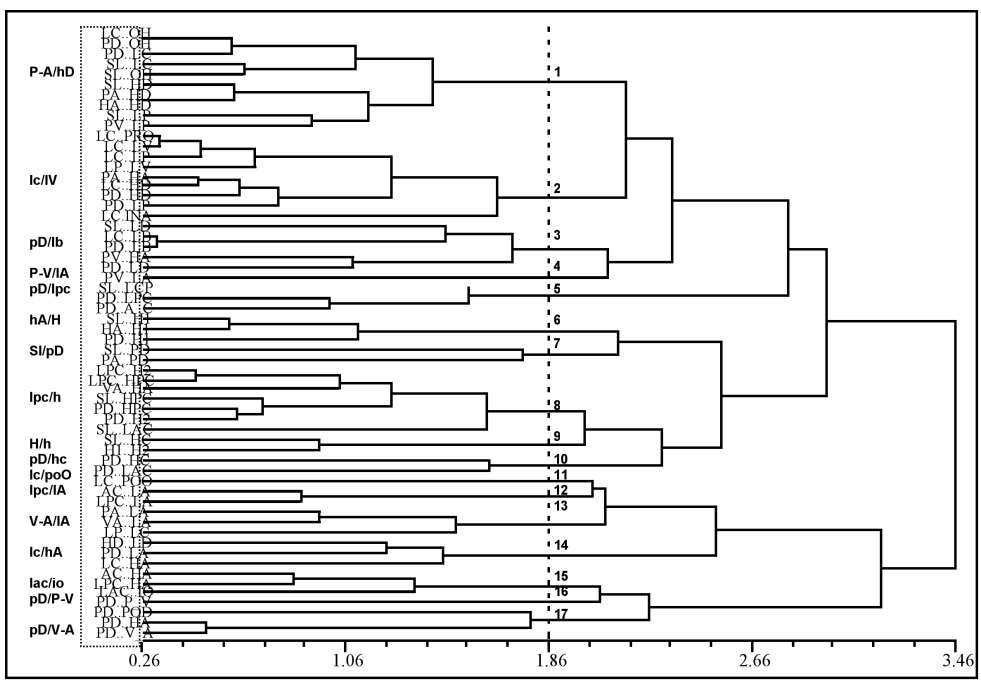

Fig. 4. Dendrogram showing the results of clustering 56 morphometric ratios. See Materials and Methods for abbreviations of morphometric ratios.

In order to determine which morphometric ratios most effectively differentiate populations, the contributions of variables to principal components (PC) were examined. PCA of 17 morphometric ratios showed that PC I accounts for 35.68% of the variation and

Table 2. Percentage of explained variance and weights of 17 morphometric ratios on the first three principal components for total samples of *C. c. gracilis*. Weights with the greatest significance on PC I and PC II appear in bold. See Materials and Methods for abbreviations of morphometric ratios.

Morphometric ratios	PC I	PC II	PC III
% of variance	31.869	28.522	20.868
P-A/hD	-0.121	**0.905**	0.308
lc/lV	-0.449	0.171	0.762
pD/lb	-0.519	0.708	-0.004
P-V/lA	-0.003	0.283	-0.756
pD/lpc	**0.855**	0.276	-0.245
hA/H	0.438	0.707	0.271
Sl/pD	**0.779**	0.596	-0.002
lpc/h	**0.848**	0.003	0.513
H/h	0.557	-0.139	0.602
pD/hc	0.430	-0.236	0.624
lc/poO	0.660	-0.178	-0.553
lpc/lA	0.680	-0.554	-0.292
V-A/lA	**0.760**	0.210	-0.515
lc/hA	-0.007	-0.617	-0.552
lac/io	-0.114	**-0.932**	0.328
pD/P-V	0.124	-0.710	0.255
pD/V-A	**-0.832**	-0.537	0.120

Table 3. Absolute number and percentage of samples correctly classified to their true population by Discriminant Analysis grouping procedure. See Materials and Methods for abbreviation of samples.

Samples	A	G	Se	Sh	T	H
A	37	0	0	0	0	0
G	0	30	1	3	0	0
Se	0	5	24	0	0	3
Sh	0	1	0	44	2	2
T	2	2	0	2	28	0
H	1	0	0	0	0	25
Total	40	38	25	49	30	30
Correct	37	30	24	44	28	25
Percentage	92.5	78.9	96	89.7	93.3	83.3

PC II for 23.43%, and that the most significant weightings on PC I were from pD/lpc, Sl/pD, lpc/h, V-A/lA, pD/V-A, P-A/hD, lac/io (Table 2). Visual examination of plotted PC I and PC II scores for each sample (Fig. 5a) revealed that all the samples were clearly distinct from each other. The overall assignment of individuals into their original group was 88.6%. The percentages of individuals correctly classified into their original group were 92.5%, 78.9%, 96%, 89.7%, 93.3% and 83.3% for Aras, Gorgãnrud, Sefidrud, Shirud, Tonekãbon and Harãz respectively (Table 3).

Molecular results

Twenty primers were used for a preliminary screening of two individuals from each sample, resulting in the identification of four informative primers for use in subsequent clustering

Table 4. Base sequences of selected primers and number of bands amplified by each in a RAPD analysis of individuals from three samples of *C. c. gracilis*. 1 = Sefidrud; 2 = Shirud; 3 = Harāz.

Primers	Sequences	Number of monomorphic bands			Number of polymorphic bands		
		1	2	3	1	2	3
OPC-02	GTGAGGCGTC	1	1	2	4	7	3
OPC-03	GGGGGTCTTT	0	1	0	3	5	6
OPC-04	CCGCATCTAC	0	0	1	5	9	6
OPC-05	GATGACCGCC	1	2	3	4	3	5
Combined		2	4	6	16	24	20

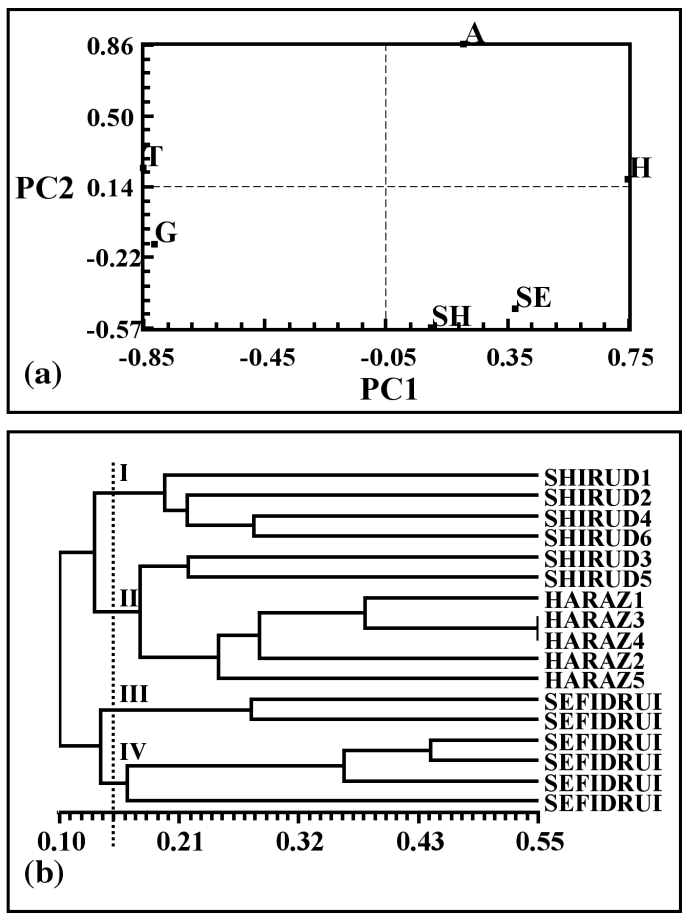

Fig. 5. (a) Plot of the first and second axes of a PCA based on means of the six samples of *C. c. gracilis* for 17 morphometric ratios; (b) UPGMA dendrogram representing phenetic relationships between specimens from three samples of *C. c. gracilis* constructed based on RAPD data. See Materials and Methods for abbreviation of samples.

analysis. These four primers (OPC02, OPC03, OPC04, and OPC05: Table 4) were selected on the basis of their ability to produce easily scorable and reproducible polymorphic bands (Wilkerson et al. 1993, Adamkewicz & Harasewych 1994) and their ability to produce polymorphic bands.

Amplification of individual samples with the initial set of 20 primers (Fig. 3) yielded a total of 90 bands, of which 72 (80%) were reproducible and thus used for the clustering analysis. Of the 72 loci generated, 60 were polymorphic (Table 4). This number of loci is expected to give a good sampling of the total genome and allow an accurate assessment of overall genetic diversity (B o u v e t 2004).

Results of cluster analysis are shown in Fig. 5b. There are four main clusters on the dendrogram, separated by a linkage distance of 0.15 centimorgans. Group I includes Shirud 1, 2, 3, 4 and 6. Group II is composed of two sub-clusters, the first comprising all individuals from Harãz, and the second comprising two individuals from Shirud (3 and 6). Group III contains only two individuals from Sefidrud (1 and 2). Group IV is comprised of Sefidrud 3, 4, 5 and 6.

Discussion

The morphological analysis of the six populations of *C. c. gracilis* in the north of Iran (the south Caspian Sea River basin) presented here (Fig. 1) revealed six distinct populations, differentiated to varying degrees, but not corresponding to any clear geographic pattern.

The Aras (in the west part of the basin) and Gorgãnrud (in the east part of the basin) river samples are from the coldest and most tropical regions of the south Caspian Sea River basin, respectively. It is well known that morphological characteristics can show high plasticity in response to differences in environmental conditions (A l l e n d o r f 1988, S w a i n et al. 1991, W i m b e r g e r 1992). Therefore, the distinctive environmental conditions of the Aras and Gorgãnrud relative to the other study areas may underlie the morphological differentiation between these two sites. Between-location differences in environmental conditions may also be the cause of differentiation between the Harãz and Sefidrud river samples and between these two samples and those from other sites.

Differentiation between samples from adjacent rivers, such as those from Tonekãbon and Shirud (with 5 km distance), may be due to the geographic isolation of rivers from each other allowing morphological differentiation to proceed independently in each river.

Examination of the loading of the morphometric ratios along PCA axes and the magnitude of their correlations revealed that the observed differences among river samples were mainly (90%) attributable to ratios of measurements involving the longitudinal body axis of the fish (pD/lpc, Sl/pD, lpc/h, V-A/lA, pD/V-A, P-A/hD, lac/io), indicating this region to be important in the description of population characteristics. Therefore, PCA indirectly revealed that the morphological differences are related to pD, lpc, Sl, h, V-A, lA, P-A, hD, lac and io. In addition, examination of ratios among samples revealed that pD/lpc was lowest in the Sefidrud sample and highest in the Tonekãbon. Sl/pD was lowest in the Gorgãnrud and Tonekãbon samples and highest in the Shirud sample. lpc/h was lowest in the Tonekãbon sample and highest in the Sefidrud. V-A/lA was lowest in the Tonekãbon sample and highest in the Harãz. pD/V-A was lowest in the Shirud sample and highest in the Tonekãbon. P-A/hD was lowest in the Aras sample and highest in the Shirud. lac/io was lowest in the Shirud sample and highest in the Gorgãnrud.

Overall, the present analysis suggests high morphological differentiation among *C. c. gracilis* populations. The differentiation detected between populations may result from different environmental and habitat conditions, such as temperature, turbidity, food availability, and water depth and flow. Consistent with this interpretation, previous studies have shown that the position of the eyes on the head is related to vertical habitat preference (A l e e v 1969), and that the location of the dorsal fin is related to the vertical position in the

water column (T u r a n 2005). Of course, in order to confirm these causal hypotheses, more detailed data on the environmental conditions from each sampled river would be required.

The morphological findings presented here were further explored using genetic techniques. Morphological analysis is a highly indirect method for assessing population genetic variation because phenotype can be strongly influenced by environmental factors; thus, morphology may not necessarily reflect population differentiation at the molecular level (Z w a r t j e s 2003). Because of these limitations, we supplemented our morphological results with Random Amplified Polymorphic DNA (RAPD) technique. The outcome of multivariate analysis of RAPD data was largely congruent with the morphological results. Since DNA-based markers such as RAPD are not influenced by environmental factors, the remarkable congruence between molecular and morphological results reported here establishes that the morphological differences between populations likely have a genetic basis and are not solely due to environmental influences.

Since the six populations of *C. c. gracilis* are geographically isolated, there is currently no opportunity for gene flow among them. Therefore, they may have diverged from each other through drift and/or differential selection. Furthermore, the large phenotypic and molecular differences between populations lead us to speculate that these may, in fact, have diverged sufficiently to constitute several distinct taxa (ecotypes, subspecies or even species); we hope to explore this possibility in future research.

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