

Phylogeny of *Oxynoemacheilus bergianus* (Derzhavin, 1934) (Nemacheilidae: Cypriniformes) in Iran using osteological characteristics

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Abstract

The phylogenetic relationships of *Oxynoemacheilus bergianus* from three Iranian inland waters, i.e. the Caspian Sea, Namak and Urmia Lake basins were investigated using osteological characters. 21 osteological characters were used and *Garra rufa* was the out-groups. A phylogenetic analysis based on the described characters resulted four most-parsimonious cladograms (36 steps, CI=0.69, RI=0.5). The strict consensus tree showed that the populations of *O. bergianus* are monophyletic group. It was confirmed that the populations of Qareh-chai, Qezel-ozan and Sefid rivers form a sister group to other examined populations of *O. bergianus*.

Keywords: Loach, Osteology, Phylogenetic relationships, Cladistic.

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Introduction

Oxynoemacheilus bergianus (Derzhavin, 1934), a nemacheilid species, was described from the Sefid River (southern Caspian Sea basin) and is also found in the Namak Lake, Urmia Lake and probably in Dasht-e Kavir basins of Iranian inland waters (Bănărescu and Nalbant 1966; Saadati 1977; Holčík and Oláh 1992; Abbasi et al. 1999; Esmaeili et al. 2017). Banarescu et al. (1978) considered this species as a subspecies of *O. angorae*. Nalbant and Bianco (Nalbant and Bianco 1998) then placed this species as a distinct species in the genus *Orthrias*, and finally Freyhof et al. (Freyhof et al. 2011) considered *O. bergianus* as a valid species (Fig. 1).

This species is characterized by a slightly emarginated caudal fin, the upper lip with a median gap and the lower lip interrupted with a bony projection underneath, small flank scaled and well-developed lateral line along the whole flank (Coad 2017). In addition, it is distinguished by a dorsal fin with 3-4 unbranched and 8-9 branched rays (usually 8), anal fin with 3 unbranched and 5 branched rays, pectoral fin with 8-11 branched rays, pelvic fin with 6-8 branched rays; total vertebrae 35-37 (Coad 2017).

There are remarkable differences in body shape of the Iranian populations of *O. bergianus* (Eagderi et al. 2016). Thus, they cannot be discriminated using morphometric and meristic characteristics (Eagderi et al. 2016). Osteological features may be able to detect their differences. Osteology is a useful tool to study the taxonomy and phylogenetic relationships of fishes (Eagderi and Adriaens 2010a; Eagderi and Adriaens 2010b; Jalili and Eagderi 2015a; Mafakheri et al. 2015a; Mafakheri et al. 2015b; Mafakheri et al. 2016) and, thus, the current phylogenetic relationships of nemacheilids is based on osteological features (Prokofiev 2009; Prokofiev 2010). Since there is no comprehensive phylogenetic hypothesis for *O. bergianus*, therefore, this study was conducted to resolve the phylogenetic relationships among the populations of *O. bergianus* in Iran using osteological characters.

Material and Methods

For osteological examination, the specimens of *O. bergianus* were collected from three basins, i.e. from the Sefid (49.4-64.0 mm SL, n=10) and Qezel-ozan rivers in southern Caspian Sea basin (47.4-55.5 mm SL; n=10); Qareh-chai (57.7-65.2 mm SL; n=10) and Kordan (64.4-68.3 mm SL; n=10) rivers in the Namak Lake basin, and Saqez-chai (51.3-60.4 mm SL; n=10) and Zarineh (47.0-49.8 mm SL; n=10) rivers in the Urmia Lake basin



Figure 1. Lateral view of *Oxynoemacheilus bergianus* from the Sefid River, Caspian Sea basin, Iran.

using electrofishing device. The specimens were anaesthetized by clove oil solution and then fixed using 10% buffered formalin.

The specimens were cleared and stained using alcian blue and alizarin red following the protocol of Taylor and Van dyke (1985). The skeletal structures were photographed using a digital camera (6 mega pixels) and a scanner (Epson v600) equipped with a glycerol bath. The skeletal elements were drawn based on the digital pictures using CorelDrawX7 software. The scale bare of all drawings were set to 1 mm.

Hypotheses of phylogenetic relationship were proposed by employing the cladistic method. A most parsimonious tree was computed based on 21 osteological characters using TNT Cladistics (Goloboff et al. 2000). No characters were treated as additive. Osteology of outgroup species i.e. *Garra rufa* (Heckel, 1843) was included as well. Characters were mapped on the most parsimonious trees. TNT Cladistics was also used for the calculation of the statistical consistency and retention indices. For an estimate of node support, we calculated Bremer support (decay index) and performed a standard bootstrap resampling (using 10,000 replicas). Nomenclature of the skeletal elements and some characters were based on Rojo (1991), Prokofiev (2010) and Jalili and Eagderi (2015b).

Results

Twenty-one osteological characters, i.e. character states, which show variability within the studied taxa, were identified and described as following:

1. (0) the anterior part of the vomer is concaved, (1) it is straight. Populations of the Kordan, Qezal-ozan and Zarineh rivers as well as *G. rufa* had character state (0); the others had the state 1 (Fig. 2).

2. (0) the notch on the lateral margin of the vomer is slight; (1) it is deep. The vomer bears a notch on its lateral margin; this notch was slight in *G. rufa* and specimens of the Sefid and Qareh-chai rivers. The apomorphic state was found in other populations of *O. bergianus*.

3. (0) there is a complete connection between the orbitosphenoid and ptersphenoid; (1) the connection between orbitosphenoid and ptersphenoid is incomplete. In all populations of *O. bergianus*, this connection was incomplete (Fig. 3b). The plesiomorphic state was only found in out-group (Fig. 3a).

4. (0) the lateral-ethmoid is connected to the vomer, orbitosphenoid and parasphenoid; (1) the lateral-ethmoid is connected to the vomer and orbitosphenoid; (2) lateral-ethmoid is only attached to the orbitosphenoid. In *G. rufa*, the ventral part of the lateral-ethmoid was broad and attached to the vomer, orbitosphenoid and the anterior part of the parasphenoid (Fig. 4a). In the Qareh-chai and Qezel-ozan populations, the lateral-ethmoid was positioned in the most posterior part of the neurocranium (Fig. 4d), whereas that of Zarineh River population was attached to the vomer and orbitosphenoid (Fig. 4b). An intermediate state can be found in the Sefid and Kordan rivers populations (Fig. 4c).

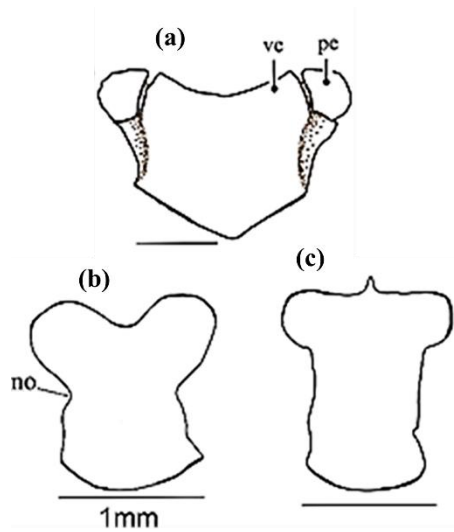


Figure 2. Ventral view of the vomer in (a) *Garra rufa*, (b) *Oxynoemacheilus bergianus* (Qezel-ozan River), and (c) *O. bergianus* (Qareh-chai) (no: notch of on the lateral margin of vomer; pc: preethmoid I; ve: vomer).

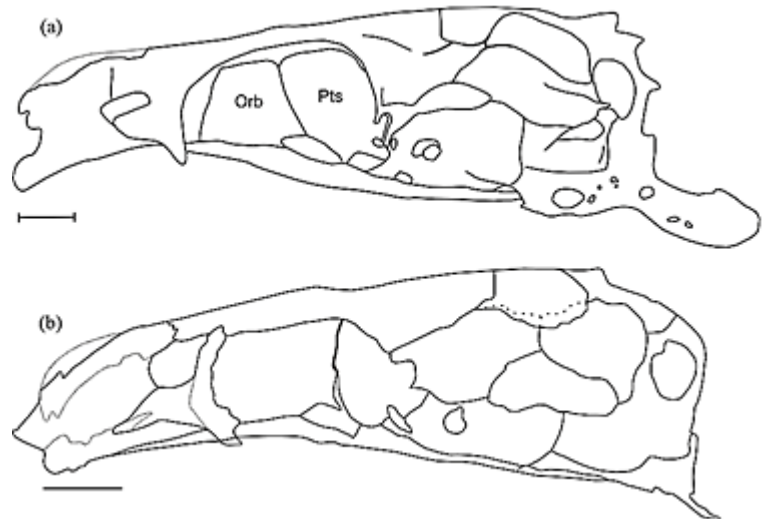


Figure 3. Lateral view of neurocranium of (a) *Garra rufa* and (b) *Oxynoemacheilus bergianus* (Sefid River) (Orb: orbitosphenoid; Pts: ptersphenoid).

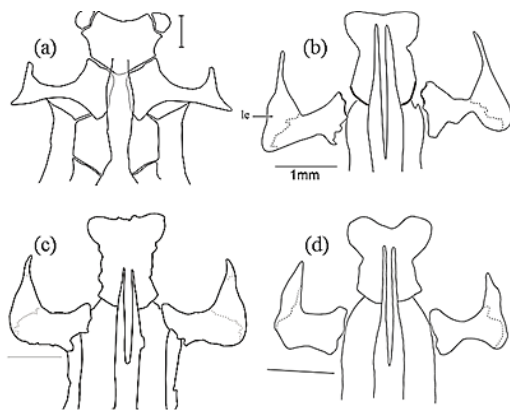


Figure 4. Ventral view of the anterior part of the neurocranium of (a) *Garra rufa*, (b) *Oxynoemacheilus bergianus* (Zarineh River), (c) *O. bergianus* (Sefid River) and (d) *O. bergianus* (Qezel-ozan River) (lc: lateral ethmoid).

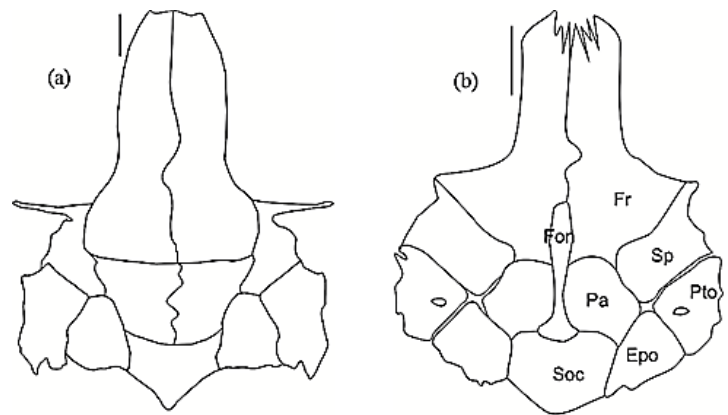


Figure 5. Dorsal view of the neurocranium of (a) *Garra rufa* and (b) *Oxynoemacheilus bergianus* (Qezel-ozan River) (Epo: epiotic; Fon: fontanel; Fr: frontal; Pa: parietal; Pto: pterotic; Soc: supraoccipital; Sp: sphenotic).

5. (0) the contact between the epiotic and sphenotic is present, and it divides the parietal and pterotic; (1) contact between the parietal and pterotic and between the sphenotic and epiotic are absent; (2) contact between the parietal and pterotic is present, and it divided the epiotic and sphenotic. In the populations of Qezel-ozan and Saqez-chai, the contacts between the parietal and pterotic, and sphenotic and epiotic were absent (Fig. 5b). In *G. rufa* and remaining populations of *O. bergianus*, the epiotic was connected to the sphenotic (Fig. 5a).

6. (0) the fontanel is absent; (1) it is present. In out-groups, the fontanel is absent (Fig. 5b).

7. (0) the sesamoid ossifications in the ethmoid region are absent; (1) they are present. The sesamoid ossification was absent in out-group taxa, as well as the Kordan, Sefid and Saqez-chai populations, but were observed in the Qezel-ozan, Qareh-chai and Zarineh loaches.

8. (0) a latero-external process of the ascending part of the premaxilla is absent; (1) it is present. The state (1) is found only in the Qareh-chai specimens (Fig. 6).

9. (0) the dorsal edge of the opercle is straight; (1) the dorsal edge of the opercle is concaved. The specimens of *O. bergianus* bear a straight dorsal edge (Fig. 7b) except those of the Sefid, Qareh-chai and Qezel-ozan

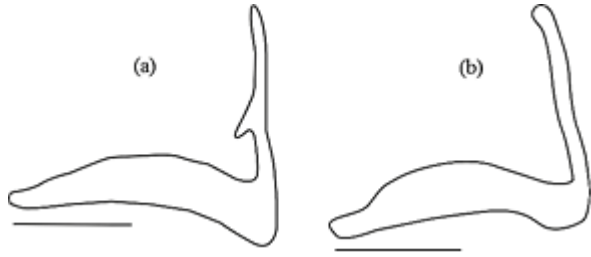


Figure 6. Premaxilla of (a) *Oxynoemacheilus bergianus* (Qareh-chai River) and (b) *O. bergianus* (Sefid River).

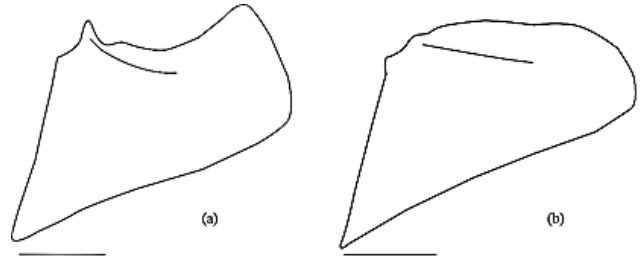


Figure 7. Opercle of (a) *Oxynoemacheilus bergianus* (Qareh-chai River) and (b) *O. bergianus* (Zarineh River).

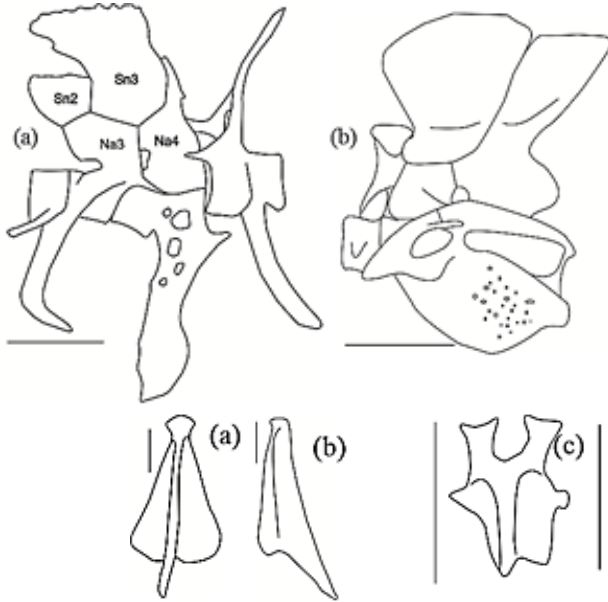


Figure 8. Lateral view of the swim bladder capsule in (a) *Garra rufa* and (b) *Oxynoemacheilus bergianus* (Sefid River) (Na: neural arch; Sn: supraneural).

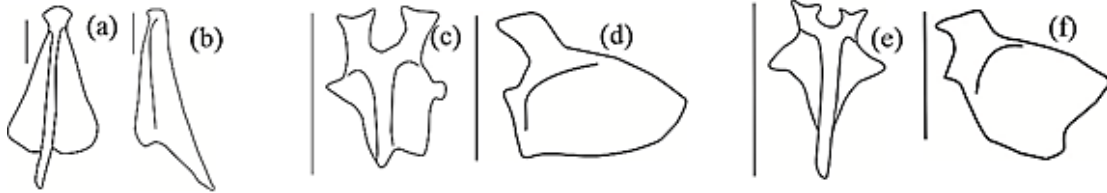


Figure 9. Dorsal (a, c and e) and lateral (b, d and f) view of the urohyal in (a, b) *Garra rufa*, (c, d) *Oxynoemacheilus bergianus* (Kordan River), and (e, f) *O. bergianus* (Qezel-ozan River).

populations.

10. (0) the swim bladder capsule is absent; (1) swim bladder capsule is present. In loaches, the swim bladder was present and formed from the fused second, third and fourth centra (Fig. 8b).

11. (0) the urohyal extra is absent; (1) a paired urohyal extra is present and are homologues; (2) a paired urohyal extra is present, but not homologues. In all studied taxa, a homologues pair of the urohyal extra was present (between hypohyals), with the exception of the Qareh-chai specimens that had unequal length and width.

12. (0) the posterior margin of the vertical plate of the urohyal is concaved; (1) it is truncate; (2) it bears a projection in its middle part. All the studied loaches but the Kordan one, possesses a projection in the posterior margin of the vertical plate of the urohyal (Fig. 9). The shape of this projection was pointed or rounded (Fig. 9b).

13. (0) the posterior margin of the horizontal part of the urohyal is blunt; (1) it bears V-shaped notch; (2) it is pointed. The plesiomorphic feature was found in *G. rufa* and loaches of the Kordan and Qareh-chai Rivers (Fig. 9). In all remaining loaches, it was pointed.

14. (0) the anterior part of the basihyal is straight; (1) the anterior part of basihyal is slightly concaved; (2) it is sharply concaved. It was straight in *G. rufa* and those of Zarineh River (Fig. 10).

15. (0) the middle part of the basibranchial-2 is narrow; (1) the posterior part of the basibranchial-2 is wider than its anterior part.

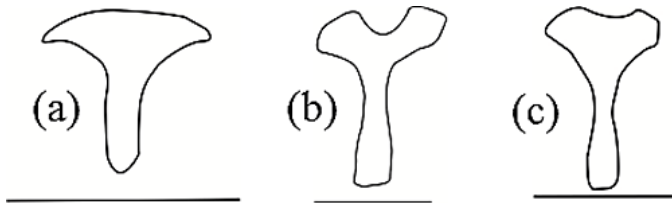


Figure 10. Dorsal view of basihyal of (a) *Garra rufa*, (b) *Oxynoemacheilus bergianus* (Sefid River), and (c) *O. bergianus* (Qezel-ozan River).

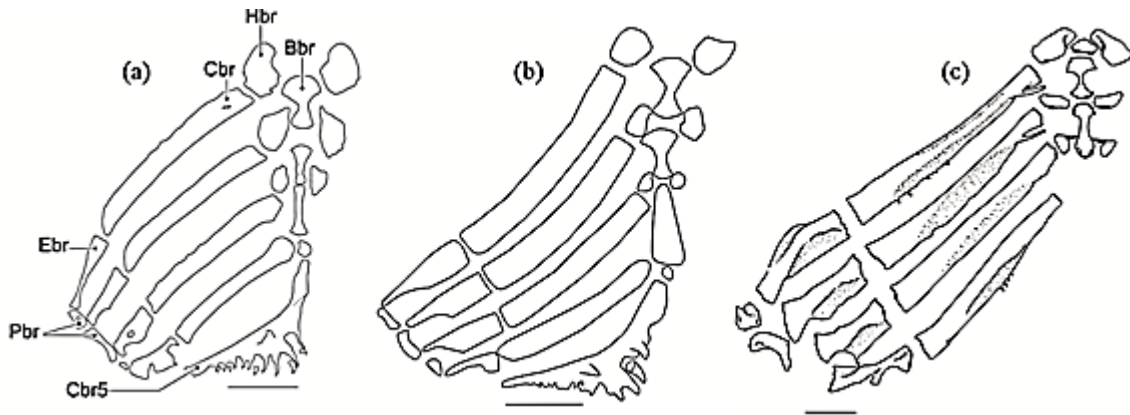


Figure 11. Branchial apparatus in (a) *Oxynoemacheilus bergianus* (Sefid River), (b) *O. bergianus* (Qareh-chai River) and (c) *Garra rufa* (Bbr: basibranchial; Cbr: ceratobranchial; Ebr: epibranchial; Hbr: hypobranchial; Pbr: inpharyngobranchial).

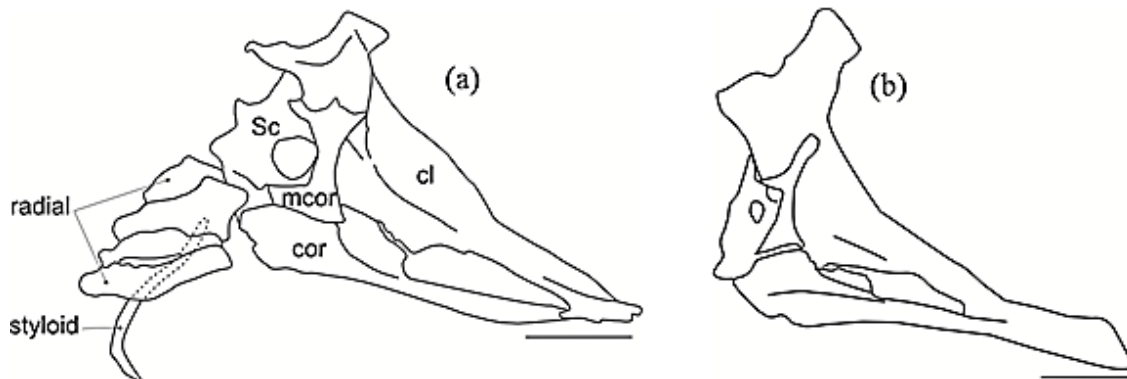


Figure 12. Lateral view of the pectoral girdle in (a) *Oxynoemacheilus bergianus* (Sefid River) and (b) *O. bergianus* (Saqez-chai River) (Cl: cleithrum; Cor: coracoid; Mcor: mesocoracoid; Sc: scapula).

16. (0) there are 3 rows of the pharyngeal teeth; (1) there is one row pharyngeal teeth. In all studied loaches, the skeleton of the branchial apparatus consists four basibranchials, three pairs of basibranchials and infrapharyngobranchials, four pairs of ceratobranchials and epibranchials, and the pharyngeal teeth were arranged in one row. In the out-group, there were two pairs of the infrapharyngobranchials and three basibranchials, and the pharyngeal teeth are arranged in three rows. In general, the middle part of the basibranchial-2 is narrow, but in two populations, including the Qareh-chai and Kordan rivers, the posterior part of the basibranchial-2 was wider (Fig. 11b).

17. In pectoral girdle, (0) the ventral part of the mesocorsoid is attached to coracoid and scapula; (1) it is only attached to coracoid. In the majority of studied specimens, the ventral part of the mesocorsoid was attached to the coracoid and scapula except the Zarineh River that it was only attached to the coracoid (Fig. 12b).

18. (0) the styloid bone of the pectoral fin is absent; (1) the pectoral fin bears a pair of the styloid bone. In the specimens from the Qare-chai, Sefid and Qezel-ozan rivers, a paired styloid bone was present (Fig. 13a). The styloid was thin and L-shaped bone, situated around the dorso-lateral part of the radials. This bone was absent in the other taxa.

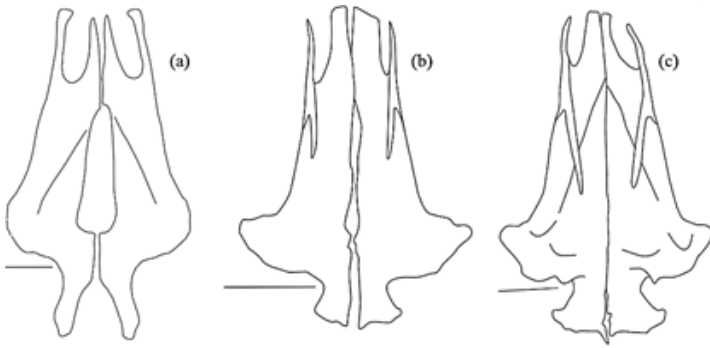


Figure 13. Ventral view of the pelvic bone in (a) *Garra rufa*, (b) *Oxynoemacheilus bergianus* (Saqez-chai River) and (c) *O. bergianus* (Sefid River).

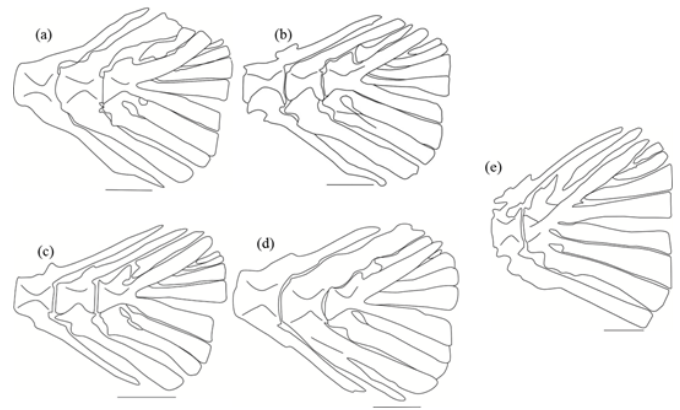


Figure 14. The Caudal fins of (a) *Oxynoemacheilus bergianus* (Qareh-chai River), (b) Sefid, (c) Zarineh and (d) Kordan rivers and (e) *Garra rufa*.

Table 1. Character state distribution. Data matrix for the out-group and six in-groups. 0=plesiomorphic state, 1 and 2 derived state, and ?=inapplicable state.

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
<i>Garra rufa</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sefid	1	0	1	2	0	1	0	0	1	1	1	2	2	1	0	1	0	1	2	1	0
Qezal-ozan	0	1	1	2	1	1	1	0	1	1	1	2	2	1	0	1	0	1	2	0	1
Qareh-chai	1	0	?	2	0	1	1	1	1	1	2	2	0	1	1	1	0	1	2	1	1
Kordan	0	1	1	2	0	1	0	0	0	1	1	1	0	1	1	1	0	0	2	0	1
Saqez-chai	1	?	1	2	1	1	0	0	0	1	1	2	2	1	0	1	0	0	1	0	0
Zarineh	1	1	1	1	0	1	1	0	0	1	1	2	2	0	0	1	1	0	1	1	0

19. (0) the posterior end of the pelvic bone is pointed; (1) it is broad and has a small lateral process; (2) the posterior end of the pelvic bone is broad and bears a developed lateral process. In all in-group taxa, the posterior part of the pelvic bone was generally broad with a lateral process, whereas it was well-developed in the loaches of the Caspian Sea and Namak Lake basins). In the loaches of Urmia Lake basin, the lateral process was small (Fig. 13b).

20. (0) the epural is symmetrical; (1) the epural is broad and asymmetrical. In specimens of the Sefid, Qareh-chai and Zarineh rivers, the epural was broad and asymmetrical (Fig. 14a, b, c). In other loaches and out-groups, the epural was narrow.

21. (0) the neural spine of the second centrum is narrow; (1) the neural spine of the second centrum is broad. The neural spine of the second centrum was broad in the loaches of Kordan, Qareh-chai and Qezel-ozan rivers (Fig. 14a, d). In other studied taxa, the neural spine of second centrum was narrow.

Analysis of 21 osteological characters with *G. rufa* assigned as out-groups (Table 1), was resulted six most parsimonious trees with a tree length of 36, a consistency index (CI) of 0.69 and retention index (RI) of 0.5. The strict consensus of the trees is shown in Figure 15.

Discussion

This is the first study that investigated the phylogenetic relationships of *O. bergianus* populations in Iran based on osteological characters scored from observations of the 7 taxa. The phylogenetic hypothesis based on the osteological characters showed that the populations of *O. bergianus* can be divided into four groups. The sister group of Sefid, Qareh-chai and Qezel-ozan was supported with 47% bootstrap value by 2 synapomorphic characters of a concaved posterior margin of opercle (9:1) and paired styloid bone in the pectoral girdle (18:1). Among this group, the specimens of the Qareh-chai River was diagnosed by 2 autapomorphic characters of a

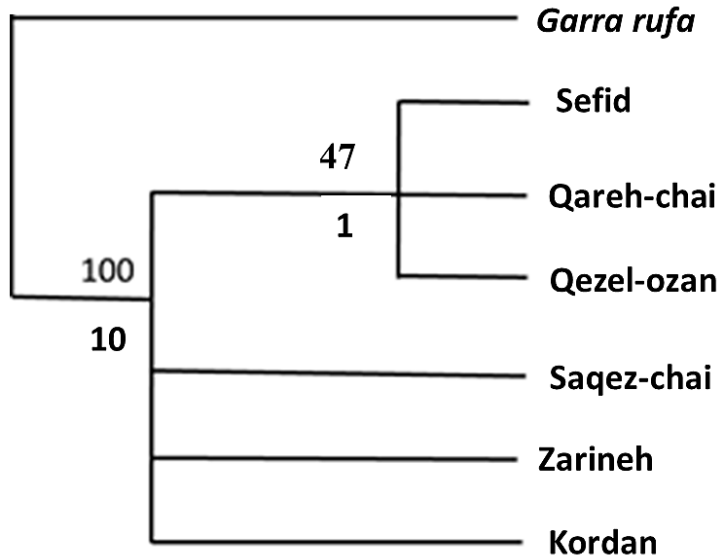


Figure 15. Strict consensus tree of the six *Oxynoemacheilus bergianus* populations and one outgroup species trees resulting from analysis of the data matrix shown in Table 1, with indication of standard bootstrap support values (top) and Bremer support values (bottom).

latero-external process of the ascending part of the premaxilla (8:1) and not homologues pair of the urohyal extra (11:2).

The population of the Kordan River is diagnosed by one autapomorphic characters of a truncate posterior margin of the vertical plate of the urohyal (12:1). The populations of the Urmia Lake basin were diagnosed by one characters, a broad posterior end of the pelvic girdle with a small lateral process (19:1). The population of the Zarineh River was diagnosed by 3 characters, including a contact between the lateral-ethmoid, vomer and orbitosphenoid (4:1), a straight anterior margin of the basihyal (14:0) and a contact between the ventral end of the mesocorsoid and coracoid (17:1).

Origin of the family Nemacheilidae goes back to the Oligocene era from the south of China (Sawada 1982). The family Nemacheilidae was distributed from the Southeast Asia via two directions *i.e.* Siberia and southern Asia toward west. In the southern direction, connection between the Ganges and Indus river basins to the Persian Gulf basin was occurred during the late Miocene and early Pleistocene. This geological event allows the family Nemacheilidae to move from the Yunnan (China) to Anatolia (Turkey) along with a speciation wave from the genera *Nemacheilus* and *Triplophysa* in the east and the genus *Paraschistura* and *Oxynoemacheilus* in the west. After entrance of the Oriental fishes to the Persian Gulf basin, sea level rising and drying of the coastal river led to isolation of these new members of the family Nemacheilidae (Sawada 1982).

The presence of the genus *Oxynoemacheilus* from Albania to central region of Iran, may indicate its origin in the Persian Gulf basin. Along with distribution of this genus, the ancestor of *O. bergianus* probably moved toward the Aras-Kura river drainage via the Taurus Mountains in the east of Turkey where the Tigris and Aras-Kura Rivers drainage originates from its southern and northern slopes, respectively, due to stream capture events. The presence of *O. bergianus* populations in the Caspian Sea basin (the Aras River) and Urmia Lake basins may show their connection in the past due to geological events as further migration path of this species toward the Sefid River in the Caspian Sea basin. Possible corridors between these basins could be inter-drainage connections in the northern part of the Urmia Lake basin in Khoy (Ghara-Tappeh) via the Aras River, which drains into the Caspian Sea, and stream capture events occurring in the southeast of this basin, as the headwaters of some rivers draining to the Caspian Sea (e.g., Qezel-ozan River) are located in proximity to the south-eastern Urmia Lake basin (Zarrineh River) (Khaefi et al. 2017). This conclusion is supported by geological reports

indicating the origin of the modern Urmia Lake *c.* 10,000-30,000 years ago (Darvishzadeh 2007). Additionally, the Lake Urmia was formed during the late Pliocene-Pleistocene and may have had a Pleistocene connection to the Caspian Sea (Coad 2017). A number of the Lake Namak River drainages in its northern part are located in proximity to the southern Caspian Sea basin. The close phylogenetic relationship of *O. bergianus* in the Namak Lake and the Caspian Sea basin may be of recent Pliocene origin of this endorheic basin, which has already been proposed by Berg (1940).

Acknowledgments

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