Analysis of mitochondrial DNA sequences of *Turcinoemacheilus* genus (Nemacheilidae; Cypriniformes) in Iran

Jamshidi S.[1*], Abdoli A.[2], Sherafatian M.[3], Golzarianpour K.[4]

Received: February 2012 Accepted: January 2013

Abstract

Members of Nemacheilidae Family, *Turcinoemacheilus* genus were subjected to molecular phylogenetic analysis in this study. This genus was reported in 2009 to inhabit in Karoon River drainage, in contrary to previous assumption that it was the endemic species in the Basin of Tigris River. It was sampled from three stations placed in different tributaries in Karoon drainage and evaluated to understand the molecular phylogenetic relationships of *Turcinoemacheilus* in Iran. The mitochondrial cytochrome *b* gene (cyt *b*) and control region were used to infer phylogenetic relationships. PCR amplification of control region was not carried out successfully, possibly due to the high divergence of this sequence in the studied genus. The amplified fragments of cyt *b* were sequenced then analyzed by the use of phylogenetic software. Only one divergent position was seen in all three samples stations located in amino acid position 365. GTR and *p*-distances of cytochrome *b* gene for *T. kosswigi* computed from different stations of running water in Karoon drainage showed these samples belong to different populations and fall in intraspecific differences. In this study, examination of the molecular phylogeny using Bayesian analysis, maximum parsimony or neighbor-joining define the phylogentics of *Turcinoemacheilus* genus as a monophyletic clade which is sister-clade of *Nemacheilus* and *Schistura* genera. This report is the first report of *Turcinoemacheilus* molecular data and could describe molecular phylogeny of this genus in loaches.

Keywords: Molecular phylogenetics, Karoon drainage, Bayesian analysis, Maximum parsimony, Neighbor-joining

1-Department of Fisheries Science, Faculty of Marine Sciences, Tarbiat Modares University, P.O.BOX:46414-356, Noor, Mazandaran, Iran

2- Department of Biodiversity and Ecosystem Management, Environmental Sciences Research Institute, Shahid Beheshti University, Tehran, Iran

3- School of Biology, College of Science, University of Tehran, P.O.Box:14155-6455, Tehran, Iran

4- Department of Biology, Faculty of Sciences, Gonbad Kavous University, Gonbad, Iran

*Corresponding author's email: shjamshidi@modares.ac.ir

Introduction

The Nemacheilidae Family is the largest group in the Cobitoidea, including numerous morphologically similar species. The nemacheilid loaches of Western Asia are poorly known and many taxonomic problems remain at the species level (Tang et al., 2006), due to their small size and limited commercial value (Kottelat, 1990). In order to resolve these phylogenetic relationships, mitochondrial DNA (mtDNA) sequences, especially the cytochrome *b* (cyt *b*) gene and the control region (CR) are frequently utilized for the study of population genetics and phylogenetics of fishes (Liu and Chen, 2003; Peng et al., 2004).

Nalbant and Bianco(1998) reported at least 3 genera and 14 species of nemacheilid loaches from Iran. *Turcinoemacheilus kosswigi* is the only member of the *Turchinoemacheilus* genus and morphologically distinct from all other members of the Nemacheilidae Family and it is only recently recorded from Iran and characters of the genus are the same as the species *T. kosswigi*. This species was described for the first time by Bănărescu and Nalbant (1964) from the upper part of the Tigris River in Anatolia. Nalbant and Binaco (1998) added two more localities in Tigris River for this species. Breil and Bohlen (2001) reported *T. kosswigi* in Karasu River at Mercan in Euphrates drainage system and described habitat and behavior of the fish in an aquarium. Since then, one additional genus and species, *Ilamnemacheilus longipinnis*, has been described by Coad and Nalbant (2005). Nalbant and Binaco (1998) mentioned the possibility of the presence of *T. kosswigi* and genus *Triplophysa* in Iran. Golzarianpour et al. (2009) reported this genus from Karoon drainage in Dez River in Iran and elucidated special differences of *Turcinoemacheilus* with the other loaches. In their study, Euphrates-Tigris and Iran samples were equal in some parameters, which distinguish this slender loach from the other nemachelid loaches. Their research showed some *T. kosswigi* characters differ from different tributaries. This reason and non existence of any data on molecular phylogenetic of *T. kosswigi* were purpose of starting our research.

In order to understand the phylogeny history of these fishes, and detecting possibility of existence different population in this species; First, samples were collected from different stations of Dez River located in Southern and South-west part of Iran, in Karoon drainage in Bazoft, Marboreh and Khoram tributaries. Molecular genetics and phylogenetic studies were then followed for assessing phylogenetic inferences including evaluating intraspecies differences, or possibly introducing new species by finding significant molecular differences among samples taken from different stations.

Materials and methods

Sample collection

At least 10 specimens fin tissues in each station were pooled. This species originally was collected from drainage of Karoon River. Fin tissues of samples were

preserved in absolute ethanol before DNA extraction. The sequences of cytochrome *b* gene in cypriniformes order (from GenBank database) were used as references and comparing with our samples (Table 1). The cytochrome *b* sequences of *Pseudobagrus tokiensis* from Siluriformes order (Fig.1) and *Misgurnus bipartitus* from Cobitinae subfamily (Fig. 2) were used as outgroups.

Table 1: GenBank accession numbers of samples used for molecular phylogenetic analysis in this study.

Taxonomy	Accession No.(cyt *b*)	Classification
Turcinoemacheilus kosswigi (Bazoft)	GQ338826	Nemacheilinae
Turcinoemacheilus kosswigi (Khoram)	GQ338827	Nemacheilinae
Turcinoemacheilus kosswigi (Marboreh)	GQ338828	Nemacheilinae
Lefua costata	DQ105196.1	Nemacheilinae
Oreonectes platycephalus	DQ105197.1	Nemacheilinae
Micronemacheilus pulcher	DQ105199.1	Nemacheilinae
Sectoria heterognathos	DQ105200.1	Nemacheilinae
Schistura fasciolata	DQ105201.1	Nemacheilinae
Schistura thai	DQ105202.1	Nemacheilinae
Paracobitis potanini	DQ105203.1	Nemacheilinae
Paracobitis variegates	AY625697.1	Nemacheilinae
Nemacheilus subfuscus	DQ105224.1	Nemacheilinae
Nemacheilus putaoensis	DQ105226.1	Nemacheilinae
Nemacheilus polytaenia	DQ105227.1	Nemacheilinae
Schistura kloetzliae	DQ105228.1	Nemacheilinae
Triplophysa stenura	DQ105246.1	Nemacheilinae
Triplophysa stewarti	DQ105248.1	Nemacheilinae
Triplophysa stoliczkae	DQ105249.1	Nemacheilinae
Triplophysa orientalis	DQ105251.1	Nemacheilinae
Barbatula nuda	DQ105252.1	Nemacheilinae
Barbatula barbatula	DQ105254.1	Nemacheilinae
Acanthocobitis botia	GQ478433.1	Nemacheilinae
Schistura corica	GQ478440.1	Nemacheilinae
Schistura beavani	GQ478443.1	Nemacheilinae
Schistura spilota	EF508596.1	Nemacheilinae
Nemacheilus masyae	EF508597.1	Nemacheilinae
Barbatula toni	EU670771.1	Nemacheilinae
Oxynoemacheilus merga	EF562774.1	Nemacheilinae
Triplophysa bleekeri	FJ406578.1	Nemacheilinae

Barbatula sturyani	EF562766.1	Nemacheilinae
Barbatula zetensis	EF562770.1	Nemacheilinae
Oxynoemacheilus pindus	EF562773.1	Nemacheilinae
Oxynoemacheilus bureschi	GQ199449.1	Nemacheilinae
Barbatula vardarensis	DQ465714.1	Nemacheilinae
Orthrias barbatulus	AF263098.1	Nemacheilinae
Paramisgurnus dabryanus	AY625701.1	Cobitinae
Misgurnus bipartitus	DQ105237.1	Cobitinae
Lepidocephalus octocirrhus	DQ105245.1	Cobitinae
Cobitis macrostigma	DQ105229.1	Cobtinae
Niwaella cf. laterimaculata	DQ105236.1	Cobitinae
Vanmanenia pingchowensis	AY625727.1	Balitorinae
Crossostoma stigmata	DQ105220.1	Balitorinae
Beaufortia szechuanensis	AY625726.1	Balitorinae
Pseudogastromyzon tungpeiensis	DQ105221.1	Balitorinae
Jinshaia abbreviate	DQ105211.1	Balitorinae
Sinogastromyzon szechuanensis	DQ105213.1	Balitorinae
Lepturichthys fimbriata	AY625695.1	Balitorinae
Balitora elongate	DQ105217.1	Balitorinae
Metahomaloptera omeiensis	DQ111990.1	Balitorinae
Leptobotia pellegrini	DQ105204.1	Botiinae
Parabotia fasciata	AY625709.1	Botiinae
Botia superciliaris	AY625704.1	Botinae
Myxocyprinus asiaticus	AF036176.1	Catostomidae
Ictiobus bubalus	FJ226364.1	Catostomidae
Cyprinus carpio	NC_001606	Cyprinidae
Gyrinocheilus aymonieri	DQ105256.1	Gyrinocheilidae
Psilorhynchus homaloptera	NC_011210	Psilorhynchidae
Pseudobagrus tokiensis	AB054127.1	Siluriformes

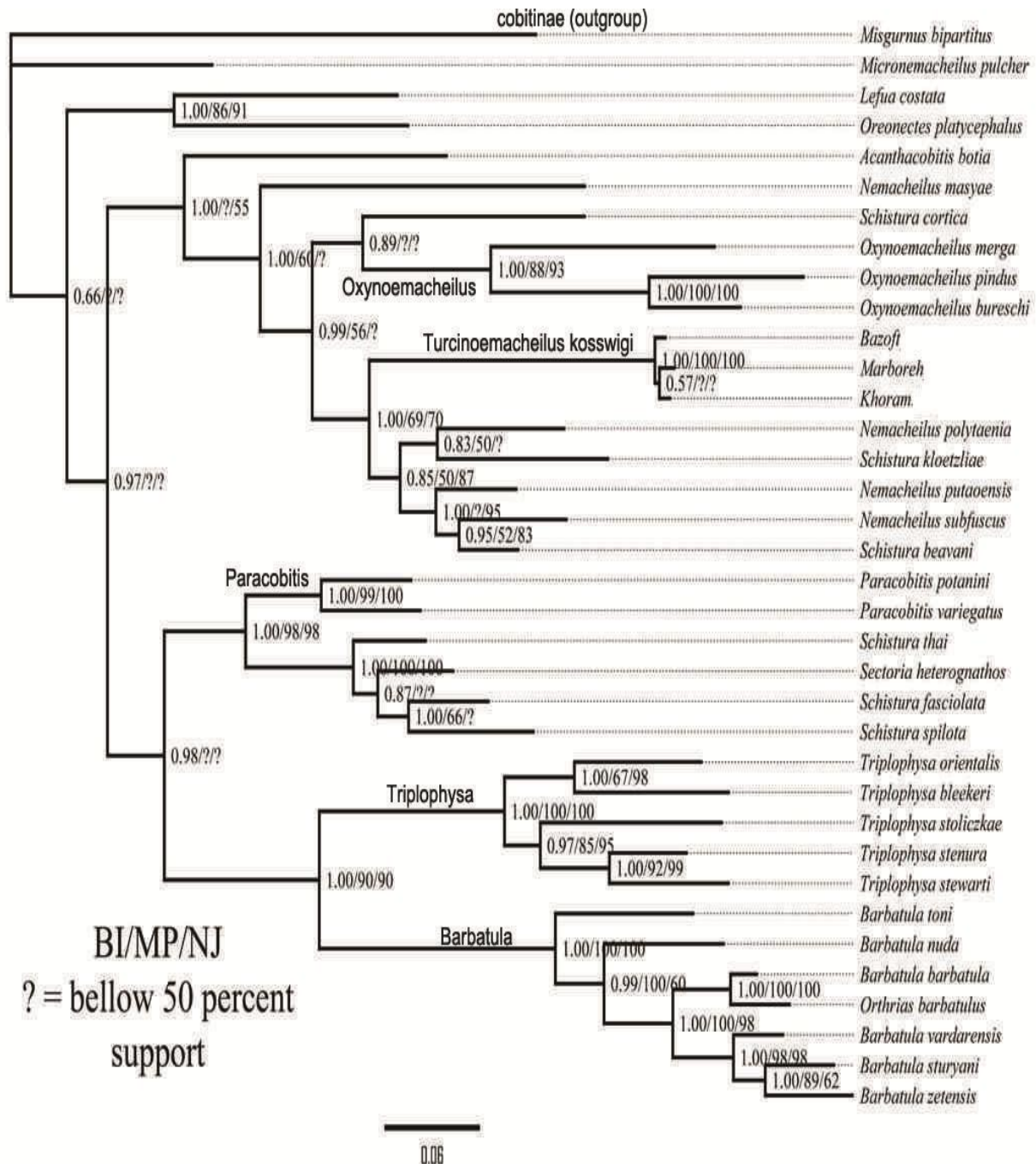

Figure 1: Phylogeny of *Turcinoemacheilus kosswigi* in Cypriformes order based on 50% major rule consensus tree obtained from Bayesian analysis of cytochrome *b* sequences. Numbers above the nodes means posterior probabilities of BI/Bootstrap values of MP/Bootstrap value of neighbor-joining (NJ); *Pseudobagrus tokiensis* from Siluriformes order were used as outgroups.

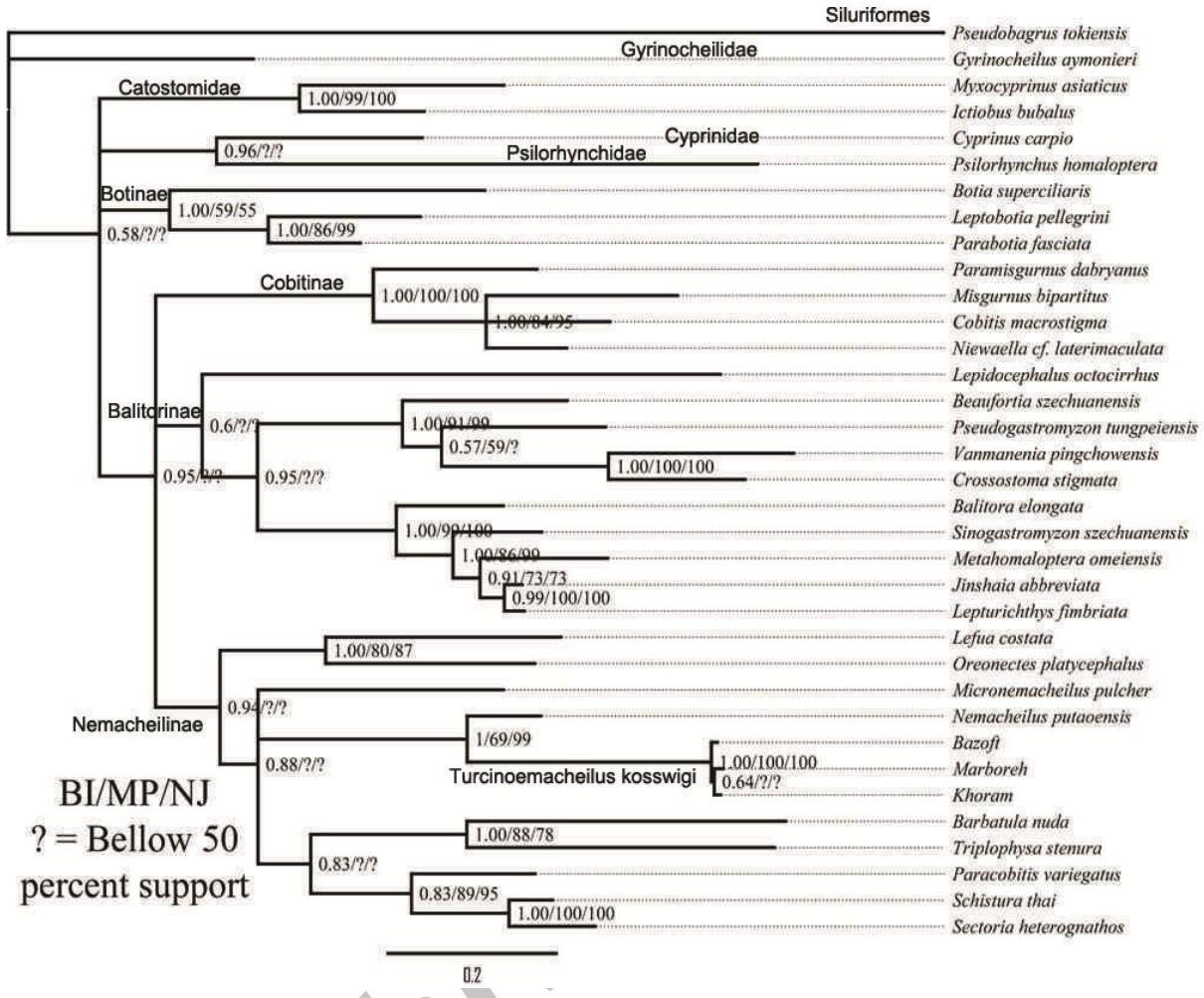

Figure 2:Phylogeny of *Turcinoemacheilus kosswigi* in Nemacheilinae subfamily based on 50% major rule consensus tree obtained from Bayesian analysis of cytochrome *b* sequences. Numbers above the nodes means posterior probabilities of BI/Bootstrap values of MP/Bootstrap value of neighbor-joining (NJ); *Misgurnus bipartitus* from *Cobitinae* subfamily were used as outgroups.

DNA extraction

Total DNA was extracted from 50 mg of fin tissue using Roche DNA extraction kit (Roche, Germany). Each tissue sample was homogenized with liquid nitrogen and then tissue lysis buffer of Roche kit and Proteinase K were added followed by incubation at 55ºC for 1 h. After that, DNA was extracted according to the instruction manual.

PCR amplification of cytochrome b and control region genes

Primers L14724 (5'-GAC TTG AAA AAC CAC CGT TG-3') and H15915 (5'-CTC CGA TCT CCG GAT TAC AAG AC-3') were used for cytochrome *b* gene amplification (Xiao *et al*, 1997) and DL1 (ACC CCT GGC TCC CAA AGC) and DH2 (ATC TTA GCA TCT TCA GTG) (Xiao et al., 2001; Tang et al., 2006) were utilized for control region gene

amplification. PCR amplifications were performed on the Eppendorff thermalcycler in 25 µl reaction volumes containing: 2.5 µl of 10X PCR buffer, dNTPs (10mM) 0.5µl, 0.5 µl of each primers (20pmol/µl), *Taq* DNA polymerase (5u/µl) 0.125µl, $MgCl_2$ (50Mm) 0.75 µl, 1 µl of extracted total DNA and ddH_2o. Approximately 1200bp fragments of cytochrome *b* and about 930bp fragments of control regions were expected to be amplified by using the following PCR condition; initial denaturing (94ºC) for 5 min followed by 35 cycles; 94ºC, denaturing for 30s, 52-58 ºC, annealing for 45 s, 72 ºC, extension for 1 min and one cycle of final extension at 72 ºC for 8 min. PCR products were then visualized by running 3µl of each on 0.9% TBE agarose gel and staining with ethidium bromide. Remaining amounts of products were run on 1.5% agarose gel and cut from the gel and purified with High Pure PCR Purification Kit (Roche, Germany) for cytochrome *b* gene sequencing. The purified fragments were sequenced by ABI sequence analyzer I England (Macro Gene Company).

Sequence analysis of cytochrome b

Alignment of sequences was performed using SEAVIEW alignment editor (Gouy et al., 2010), which drives CLUSTAL W2 (Larkin et al., 2007) program. Sequence variations and the pairwise distances were analyzed using PAUP* version 4.0b10 (Swofford, 2003). Nucleotide composition was measured and also base compositional bias was calculated with a chi-square (χ^2) test of base heterogeneity as implemented in PAUP*. Number of transitions (T_i) and transversions (T_v) versus divergence were analyzed and plotted by DAMBE version 5.2.13(Xia and Xie, 2001) for assessing the degree of nucleotide saturation. Statistical selection of best-fit models of nucleotide substitution was carried out using jModelTest (Posada, 2003). General Time Reversible + I + G model were used for phylogenetic analysis in Cypriniformes and Nemacheilinae. Data were analyzed by maximum parsimony (MP) and neighbor-joining (NJ) using PAUP*. Bootstrap analysis was used with 1000 replications to approximate support for the resulting topologies. Bayesian analyses were carried out with MrBayes version 3.0b (Huelsenbeck and Ronquist, 2001). In conducting Bayesian analysis, starting trees were random. Four simultaneous Markov chains were run for 5,000,000 generations. The first 25% fraction of the trees was discarded as before burn-in and the rest were used to calculate posterior probability and to reconstruct phylogenetic relationships. Trees are drawn by Figtree version 1.3.1(Morariu et al., 2008). All sequences of cytochrome *b* gene in *Turcinoemacheilus* genus (this study) are available in GenBank under Accession numbers GQ338826 to GQ338828.

Results

Sequence analysis of T. kosswigi samples obtained from three different stations

No bands were obtained for control region in our three different stations samples with DL1 and DH2 primers even with different temperature optimizations. This situation

could be due to variation in tRNA-Pro and tRNA-Phe regions in this genus which causes no amplification by these primers (Xiao, 1997; Xiao et al., 2001). However, bands with the size of about 1200 bp were obtained for cytochrome *b* gene from all samples. The average nucleotide composition in the 1118bp of cytochrome *b* region is: T: 33.4%, C: 23%, A: 29.4% and G: 14.2%. Base frequencies were heterogeneous across all taxa (for data set in Cypriniformes order, $\chi 2$ =179.118, df =102, p=0.000 and for data set in Nemacheilinae subfamily, $\chi 2$ = 154.509, df = 105, P = 0.001). Nucleotide diversity (π) from Tajima's Neutrality Test in three different station samples was 0.012224. 1098bp in all three 1118bp sequences were identical and only one divergent site was seen in all three different stations samples, which was in position 365. Unique differences in Bazoft, Marboreh and Khoram tributary samples were 6, 7 and 6bp, respectively. The transition/transversion rate ratios are k1 = 13.845 (purines) and k2 = 2.566 (pyrimidines). Number of transitions and transversions versus divergence is plotted in Figure 3. This plot demonstrates the level of substitution saturation of the sequences of cytochrome *b* among species belonging to the order of Cypriniformes (a) and the Nemacheilinae subfamily (b). As calculated in 3 codon positions, the number of transition is higher than the number of transversions, which notifies that these sequences are not saturated and can be exercised to determine divergences (Tang et al., 2006).

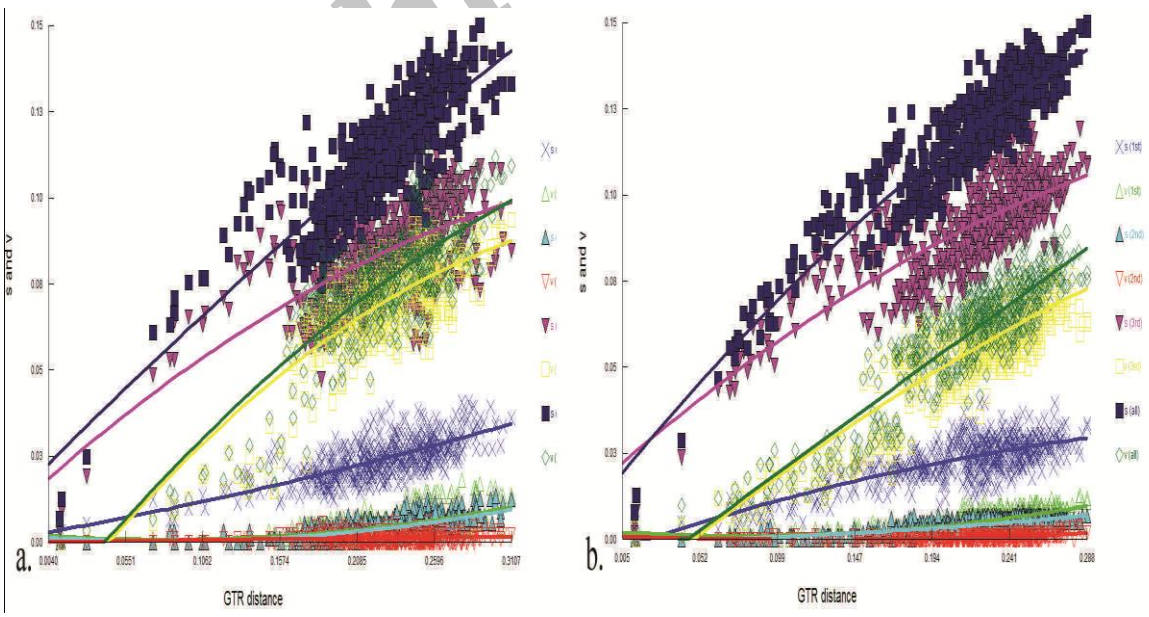

Figure 3: Plots of the number of transitions and transversions versus divergence to demonstrate the level of substitution saturation of the sequences of cytochrome *b*.

Phylogenetic analysis of Turcinoemacheilus genus

Pair-wise distances among all taxa in Nemacheilinae subfamily were calculated by General time-reversible model. Rates of nucleotide substitution assumed to follow gamma distribution with shape parameter of 0.963. This model was selected as the best-fit model for this set of data. Part of the distance matrix calculated by PAUP* is shown in Table 2. GTR distances between *Turcinoemacheilus* samples were about 0.012. The mean intra-taxa distance for sequences of Nemacheilinae data set was 0.269 with standard deviation of 0.061. Descriptive statistics of these distances described in Table 3. The *z*-score calculated for distances between *Turcinoemacheilus* samples taken from different locations were around - 4.2. Considering that this data set contains available cyt*b* sequences of species belonging to Nemacheilinae subfamily and that *Turcinoemacheilus* distances are extreme values in this data set, it is reasonable to deduce that differences between these samples are intraspecific differences.

Table 2: Part of GTR distance matrix.

N	Minimum	Maximum	Mean	Std. Deviation	Skewness		Kurtosis	
Statistic	Statistic	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Std. Error
675	0.01204	0.37859	0.26946	0.0615843	-1.215	0.094	1.931	0.188

Table 3: Descriptive statistics of GTR distances of species in Nemacheilinae subfamily.

1	*Misgurnus bipartitus*									
2	*Bazoft*	0.3379								
3	*Marboreh*	0.3403	0.01295							
4	*KhoramII*	0.33008	0.01204	0.01292						
5	*Micronemacheilus pulcher*	0.27213	0.26761	0.26811	0.2615					
6	*Nemacheilus putaoensis*	0.28373	0.17894	0.17838	0.18154	0.21744				
7	*Nemacheilus polytaenia*	0.2915	0.20807	0.20728	0.20544	0.21288	0.12572			
8	*Schistura kloetzliae*	0.30013	0.20913	0.21312	0.20678	0.2344	0.13238	0.14705		
9	*Triplophysa stenura*	0.31911	0.29618	0.29861	0.29964	0.26786	0.26128	0.28323	0.28375	
10	*Oxynoemacheilus merga*	0.35022	0.25679	0.26123	0.25277	0.31256	0.22989	0.23169	0.27428	0.33544

Discussion

Non-coding control region in vertebrates evolves rapidly. Control region, especially the tRNA-pro end, has been suggested to have one of the highest substitution rates of all the mitochondrial genes (Brown, 1985; Meyer, 1993). Mutation rate of the CR can be 2-5 times higher than that of mitochondrial protein-coding genes (Meyer, 1993); this reason caused DL1 and DH2 primers even with different temperature optimizations could not amplify control region. Slower rate of substitution in control region was found in salmonid fishes (Shedlock et al., 1992). In this study, total content of T+A is much higher than C+G which fell within the range of GC content typical for vertebrates (Nei and Kumar, 2000). In Thang et al. (2005) study, Botiidae family has content T+A much higher than C+G. Sequences with high substitution rates often experience substantial substitution saturation, especially in the third codon position of protein-coding genes. This saturation can misrepresent the phylogenetic information contained in the sequences, leading to incorrect phylogenetic inference. Our plots show that both transitions and transversions fall on a straight line with transitions consistently outnumbering transversions, then the sequences have not experienced substitution saturation and are good for phylogenetic analysis.

In parallel studies, *p*-distances index were measured and compared to intraspecific distances to obtain phylogenetic inferences. Xiao (1997) suggested *p*-distance of 0.0325 between Fuijan (*D. compressus*) and Sichuan (*D. tumirostris*) of genus *Distoechodon* and mentioned that they belong to one species. Also the distance between two different species of genus *Xenocypris* (*X. fangi* and *X. microlepis*) ranges were 0.0438 to 0.0467. Liu (2002) notified intraspecific distances from cyt *b* about 0.005 for *compressus* and 0.007 for *tumirostris* and mentioned the two *tumirostris* samples are from different water systems. Also they concluded that sequence divergence of cyt *b* gene supports the hypothesis that those are two different species. Tang et al. (2005) reported *p*-distance intraspecific of Asian Botiinae range from 0.002 to 0.028. Aiki et al. (2009) introduced average genetic distance using *p*-distance of *Lefua echigonia* populations between 0.005 ± 0.002 to 0.065 ± 0.008. *P*-distance between *Turcinoemacheilus* samples were 0.012 for Bazoft and Khoram samples and 0.013 in the other two samples. Among 372 studied amino acids, 12 sites are variable in these three stations samples. Composition distances for samples were 0.017 for Bazoft and Marboreh, 0.023 for Marboreh and Khoram, and 0.020 for Bazoft and Khoram. According to the mentioned studies, *p*-distances of cyt*b* gene for *T. kosswigi* obtained from different stations of running water in Karoon drainage belong to different populations and fall in intra-specific differences. The position of *Turcinoemacheilus* genus showed in Figure 1 with names derived from the sampled stations (Bazoft, Marboreh and Khoram tributaries) within Cypriniformes order in a phylogenetic tree. Nemacheilinae and Cobitinae are sister clades. Cobitidae in contrary to the conclusion of Tang et al. (2005) forms a polyphyletic group with *Lepidocephalus octocirrhus* being jointed to monophyletic clade of Balitorinae.

In agreement with the phylogenetic trees drawn by Mayden et al. (2008) and Tang et al. (2005), Botiinae forms a monophyletic clade with good support from all three methods. Balitoridae and Nemacheilidae are recovered as monophyletic groups although in our study only Bayesian posterior probabilities support this topology. So-called enigmatic genus *Psilorhynchus* is jointed to *Cyprinus carpio* and confirms the results given by Mayden et al. (2008) using complete mitochondrial genome in their phylogenetic analysis. Also, Figure 2 shows the phylogenetic relationship of species within Nemacheilinae which rooted by an out-group selected from cobitinae subfamily. Based on available sequences of cyt *b* on GeneBank database and our three mentioned methods of phylogenetics, only three genera, *Triplophysa*, *paracobitis* and *Oxynoemacheilus* form their own monophyletic clades. Our species (*Turcinoemacheilus kosswigi*) forms a monophyletic clade, which is sister to a clade consisting of *Nemacheilus* and *Schistura* genera. In conclusion, As it has been verified, Bayesian method is the most efficient character-based method for accurately reconstructing a phylogeny (Simmons and Miya 2004). In our analysis the most successful trees in revealing deep phylogenetic relationships were those inferred by this method. Samples of *Turcinoemacheilus kosswigi* obtained from different stations of running water in Karoon drainage belong to different populations and fall in intra-specific differences. Moreover, location of *Turcinoemacheilus* in Nemacheilinae subfamily compared with other subfamilies of Cypriniformes order; Cobitinae, Balitorinae and Botinae. This study is the first report to demonstrate *T.kosswigi* ´s molecular genetic data. For a better decision and resolution on haplotypes diversity on each population or introducing new species in this genus, it is recommended to collect more samples from more different Karoon drainage tributaries in Iran.

Acknowledgments

We would like to thank Department of Animal Science in National Institute of Genetic Engineering and Biotechnology (NIGEB) for their assistance and providing facilities. We are also grateful to the Faculty of Environmental Science of Shahid Beheshti University for sampling.

References

Aiki, H., Takayama, K., Tamaru, T., Mano, T., Shimada, M., Komaki, H. and Hirose, H., 2009. Phylogeography of the Japanese eight-barbel loach *Lefu echigonia* from the Yamagata area of the Tohoku district, Japan, *Fisheries Science* 75: 903–908.

Bănărescu, P. M. and Nalbant, T. T., 1995. A generical classification of Nemacheilinae with description of two new genera (Teleostei: Cypriniformes: Cobitidae).Trav. Mus. Hist. Nat. “Grigore Antipa” 35, 429–496.

Breil, M. and Bohlen, J., 2001. First record of the loach fish *Turcinoemacheilus kosswigi* in the Basin of Euphrates River, with first observations on habitat and

behavior. Zoology in Middle East 23: 71-76.

Brown, W., 1985. The mitochondrial genome of animals, In: Macinttyre, R. (Ed.), Molecular Evolutionary Genetics. Plenum Press, New York, pp. 95–130.

Coad, B.W. and Nalbant, T.T., 2005. A new genus and new species of a remarkable nemacheilid fish from Iran (Pisces,Ostariophysi:Nemacheilidae).- Travaux du Musém National d´Histoire Naturelle 10: 303-308.

Golzarianpour, K., Abdoli, A., Kiabi, B. and Frayhof, J., 2009. First record of fresh water loach *Turcinoemacheilus kosswigi* (Banarescu and Nalbant, 1964) from Iran, Karoun drainage, Zoology in Middle East 47: 57-62.

Gouy, M., Guindon, S. and Gascuel, O., 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular Biology and Evolution* 27(2):221-224.

Huelsenbeck, J.P. and Ronquist, F., 2001. MrBayes: Bayesian inference of phylogeny. *Bioinformatics* 17, 754–755.

Kottelat, M., 1990. Indochinese nemacheilines: a revision of nemacheilinae loaches (Pisces,Cypriniformes) of Thiland, Burma, Laos,Cambodia and southern Vietnam.-Munich.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D. Gibson, T.J. and Higgins, D.G., 2007. Clustal W and Clustal X version 2.0, Bioinformatics,23(21) 2947-2948.

Liu, H., 2002. Mitochondrial DNA sequence variations and systematics of the genus *Distoechodon*(Teleostei:Cyprinidae), *Journal of Applied Ichthyology*18(3): 129-218.

Liu, H. and Chen Y., 2003. Phylogeny of the East Asian cyprinids inferred from sequences of the mitochondrial DNA control region, *Canadian Journal of Zoology* 81: 1938–1946.

Mayden, R., Tang, K.L., Wood, R.M., Chen, W-J., Agnew, M.K., Conway, K.W., Yang, L., Simon, A.M., Bart, H.L., Harris, P.M., Li, J., Wang, X., Saitoh, K., He, S., Liu, H., Chen, Y., Nishida, M. and Miya, M., 2008. Inferring the Tree of Life of the order Cypriniformes, the earth's most diverse clade of freshwater fishes: Implications of varied taxon and character sampling Journal of Systematics and Evolution 46(3):424–438.

Meyer, A., 1993. Evolution of mitochondrial DNA in fishes, in: Hochachka, P.W., Mommsen, T.P. (Eds.), Biochemistry and Molecular Biology of Fishes, Vol. 2. Elsevier, Amsterdam, pp. 1–38.

Morariu, V. I., Srinivasan, B. V., Raykar, V. C., Duraiswami, R. and Davis, L. S., 2008. Automatic online tuning for fast Gaussian summation, Advances in Neural Information Processing Systems (NIPS)

Nalbant, T.T. and Bianco, PG., 1998. The loaches of Iran and adjacent region with description of six new species (Cobitoidea), *Italian Journal of Zoology* 65: 109–123.

Nei, M. and Kumar, S., 2000. Molecular Evolution and Phylogenetics, Oxford University Press, New York.

Peng, Z., He, S. and Zhang, Y., 2004. Phylogenetic relationships of glyptosternoid Fishes (Siluriformes: Sisoridae) inferred from mitochondrial cytochrome *b* gene sequence. *Molecular Phylogenetics and Evolution*, 31: 979–987.

Posada, D., 2008. jModelTest: Phylogenetic Model Averaging. Journal of Molecular Biology and Evolution, 25(7) 1253-1256.

Shedlock, A. M., Parker, J. D., Crispin, D. A., Pietsch, T. W. and Burmer, G. C., 1992. Evolution of the salmonid mitochondrial control region, *Molecular Phylogenetics and Evolution*, 1:179–192.

Simmons, M. P. and Miya, M., 2007. Efficiently resolving the basal clades of a phylogenetic tree using Bayesian and parsimony approaches: a case study using mitogenomic data from 100 higher teleost fishes. Mol Phylogenet Evol 2004, 31(1):351-362.independence of Far Eastern species in subfamily Leuciscinae (Pisces: Cyprinidae) inferred from mitochondrial DNA analyses. Genes Genet Syst, 82(4):329-340.

Swofford, D. L., 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tang, Q., Xiong, B., Yang, X. and Liu, H., 2005. Phylogeny of the East Asian botiine loaches (Cypriniformes, Botiidae) inferred from mitochondrial cytochrome *b* gene sequences, *Hydrobiologia*, 544: 249-258.

Tang, Q., Liu, H., Mayden, R. and Xiong, B., 2006. Comparison of evolutionary rates in the mitochondrial DNA cytochrome *b* gene and control region and their implications for phylogeny of the Cobitoidea (Teleostei: Cypriniformes)**,** *Molecular Phylogenetics and Evolution*, 39: 347-357.

Xiao, W., 1997. Molecular systematics of the subfamily Xenocyprinae(Teleostei,Cyprinidae) and some other related groups with consideration of the coevolution between Xenocyprinae and Hexaitidae(Fllagellate,Diplomonadida), Unpubl.PhD Diss. Institute of Hydrobiology, Chinese Academy of Science, Wuhan, China.

Xiao, W., Zhang, Y. and Liu, H., 2001. Molecular systematics of Xenocyprinae (Teleostei: Cyprinidae): taxonomy, biogeography, and coevolution of a special group restricted in east Asia, Molecular Phylogenetic and Evolution, 18: 163–173.

Xia, X. and Xie, Z., 2001. DAMBE: Data analysis in molecular biology and evolution. *Journal of Heredity*, 92:371-373.