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Genetic diversity for three populations of Rainbow Trout (*Oncorhynchus mykiss*) based on sequencing of mtDNA genes

Taqi Abdullah[1], Mohammadreza Nassiri[2], Omid Safari[3], Ali Javadmanesh[4*]

1 Assistant Lecturer of Genetic Science, Faculty of Agriculture, Basrah University, Iraq.

2 Professor of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Iran.

3 Associate Professor of Fisheries, Faculty of Natural Resources and Environment, Ferdowsi University of Mashhad, Iran.

4 Assistant Professor of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Iran.

* Corresponding author's email: javadmanesh@um.ac.ir

A B S T R A C T

Loss of genetic diversity decreases the population or species will or may affect the ability of a population being in a new environment affect or cause disease. Estimation of genetic diversity in fish farms will play an essential role in managing it properly. Therefore, this study aimed to estimate the genetic variation between the three population of rainbow trout including Iranian, French, and Danish using mitochondrial DNA COX1 and Cytb genes were performed. Ninety samples from three populations salmon farms in the area of Kalat Naderi city of Mashhad collected. DNA was extracted and COX1 and Cytb gene were amplified by PCR and then sequenced. The results showed a low level of genetic variation within studied populations. Phylogenic and haplotype analysis revealed that the genetic distance between Iranian trout population and French was much lower than Danish. This finding might indicate that the Iranian population might have the similar gene pool with and originated from the French population.

Keyword: Rainbow trout, polymerase chain reaction, cytochrome oxidase B, phylogenetic tree.

Introduction

The origin of a rainbow trout (*Oncorhynchus Mykiss*) is McCloud River of California (Carlander, 1969). The rainbow trout of California migrated to other area and inoculated by other subspecies, therefore the high genetic diversity have been produced in rainbow trout (Pavlov *et al*., 2004).

Currently, more than 75 subspecies of rainbow trout have been observed in the world, among these, there are commercial rainbow trout from the Denmark, France, Norway, Italy and England subspecies in Iran (Mahmodi *et al*., 2014). The unconventional inoculation of rainbow trout species in Iran have caused severe effect on the genetic reserve of Iranian rainbow trout and increased inbreeding coefficient. The overcrossing of productive fish in rainbow trout subspecies leads to reduced growth, phenotypic abnormalities, and increased casualties (McCusker *et al*., 2000). In order to the efficient exploitation and programmer for the hatchery, subspecies identification and breeding are very important for different populations (Lin *et al*., 2002). The study on the genetic diversity of hatchery rainbow trout subspecies is the first steps of the breeding program. Genetic diversity is necessary for the long-term survival of a species (Bataillon *et al.*, 1996). There are some traditional methods for assessing genetic diversity such as electrophoresis (Jaayid and Aziz, 2009). In the recent year, genetic diversity estimation and identification for various fish populations were done by PCR-based methods such as RAPD, RFLP, microsatellite, and the sequencing of mitochondrial DNA nucleotide. Among these, direct sequencing can be used to provide the highest yield of genetic information as well as the highest possible accuracy and this method was known as a standard method for estimating genetic diversity (Maqsood and Ahmad, 2017). The using of the mitochondrial genome in genetic population is common because mtDNA has unique characteristics such as high copy, simple separation from the genome, the small size of the genome and a large number of mutation (Billington and Hebert, 1998). The COX1 and the cytochrome oxidase B (Cytb) are the main genes of the mitochondrial genome that used for genetic diversity of rainbow trout (Shed'ko, 2002). Since there is no information on the geographical distribution of rainbow trout in Khorasan Razavi, therefore, there is a deep divorce in the information needed to design protective strategies in this area. The genetic diversity of rainbow trout species is affected by its geographical distribution; thus, the collection of genetic information in this area makes it possible to implement the appropriate conservation strategy. Therefore, this study aimed to determine the genetic diversity of three species of Iranian, French, and Danish rainbow trout populations in the Kalat Nader city using COX1 and Cytb genes.

Materials and Methods

The 90 rainbow trout species from three populations (30 samples from each Iranian, French, and Danish populations) were collected from a hatchery in Kalat Nader, Mashhad, Iran (Fig 1). All fish were taken manually and then anesthetized with clove solution (10 g per liter of distilled water). Then 1-2 cm from the caudal fin was collected and transferred to a 5 ml tube containing 96% ethanol. The samples were transferred to the

laboratory on ice and then stored at -20 ° C.

Sample preparation and DNA extraction
Approximately 25 mg of caudal fin samples were homogenized in 300 µl digestion buffer (100 mM NaCl, 15 mM EDTA pH = 8, 10 mM Tris pH = 8 and 0.5% SDS Which was dissolved in 85 ml distilled water). Then, 20 µl of Proteinase K enzyme (20 mg/ml) was added to the solution. The sample was shaken well and then incubated for 1 hour at 37 ° C. The sample was centrifuged at 10,000 rpm for 10 seconds. The DNA extraction was performed from 200 µl of the supernatant using the standard protocol of DNA extraction kit (Isogene Diatom DNA Prep.100-Russia). The quantity and quality of DNA extraction were assessed by electrophoresis on a 1% agarose gel and spectrophotometry methods.

Fig 1. The map of sampling area (red circle).

Polymerase chain reaction

The specific primers were designed by Primer Premier 5 (PRIMER Biosoft-USA) that can be used in order to amplify a 624 bp fragment from mitochondrial COX1 gene, PCR reactions were conducted using a pair of primers as follows:

Forward	5' GGCATTCCCTCGAATAAATAACATA 3'
Reverse	5' TCCACGTCTATCCCTACAGTGAACA 3'

Moreover, a fragment of 735 bp from Cytb gene of Rainbow trout. The Forward and reverse primers were:

Forward	5' AAGAACCTGGAATATCGGAGTTGTA 3'
Reverse	5' GAGGCGACTTGTCCGATAATAGA 3'

The PCR reaction was performed using Amplicon Red Mastermix 2X (Amplicon-Denmark). Each reaction contained 8 µl distilled water, 1 µl of forward and reverse primers, 1 µl DNA sample and 10 µl Master Mixer PCR. PCRs were performed using a T-Personal thermo-cycler (Biometra-Germany). PCR program consisted of 35 cycles of 94°C for 30 sec (denaturation), 64°C for 30 sec (annealing) and 72°C for 45 sec (extension). An initial denaturation was performed at 95° C for 5 min and after completion of 35 cycles; a final extension was carried out at 72°C for 10 min.

PCR Product Purification and Sequencing Purification of PCR products was carried out using ethanol precipitation method (5). Subsequently, the quality of the samples was investigated on the agarose gel, 40 µl of each sample were sent for sequencing to the Macrogen Company (South Korea). The samples were sequenced using the ABI 3130 machine by the Sanger automatic method.

Analysis of sequencing results

In order to the estimation of sequence homology and identifying, BLAST tool and the blast method at NCBI were used. The phylogenetic analysis and genetic interval matrix were investigated by Mega v.7 software and Tumora et al., 2004 algorithm (13). A haplotype network **(**5.0.0, 1) was inferred by the Cytb sequences of rainbow trout populations (www.fluxus-rngineering.com).

Results

DNA extraction from all samples was successfully performed (Fig 2). The nanodrop curve showed that DNA samples had optimal quality. The PCR product electrophoresis on 1% agarose gel had a specific band on 624 bp for COX1 and 735 bp for Cyt b genes (Fig 3). Therefore, this result confirmed that primers were succeeded for amplifying of both genes.

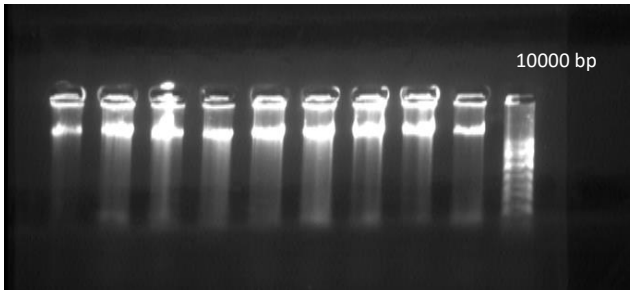

Fig 2. Electrophoresis of extracted DNA samples on %1 agarose gel

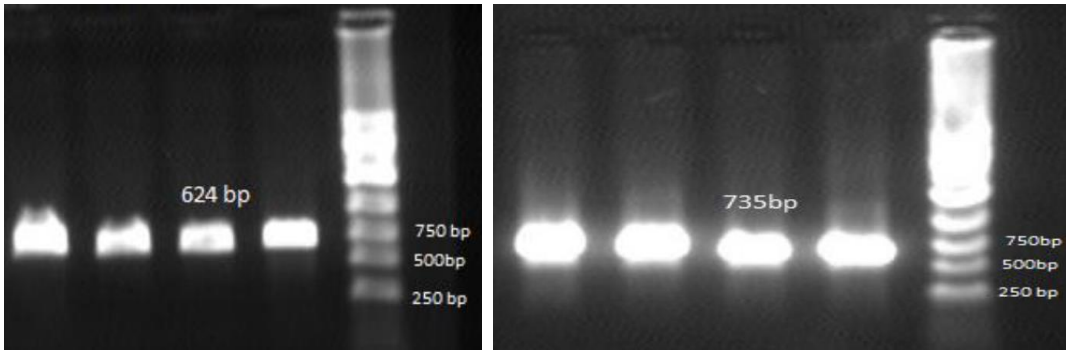

Fig 3. Electrophoresis of PCR samples on %1 agarose gel for COX1 (left) and Cytb (right) genes.

In order to estimate the sequence homology and identity, the BLAST tool at NCBI was used. The phylogenetic analysis and genetic interval matrix were investigated by Mega v.7 software and Tumora *et al*., 2004 algorithm (Tamura *et al*., 2004).

By comparing the sequences studied for COX1 gene with the sequences in the NCBI database, it was found that there were a low overlap and high homology between the sequences in this base with our studied sequences. The study of the phylogenetic tree plotted in order to determine the genetic diversity between the three species studied in this study and three species registered at the World Bank Gene showed that the three species of rainbow trout in Iran, France and Denmark have the lowest genetic distance and they are in a sister's custody (Fig 4)

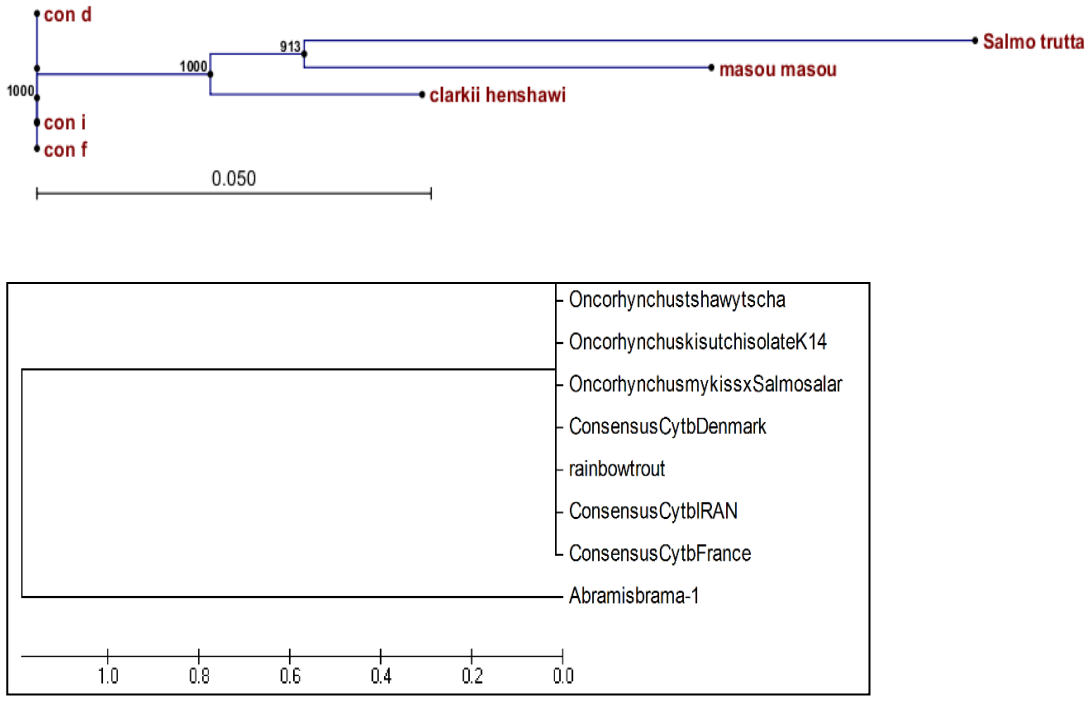

Fig 4. Top: The phylogenetic tree for studied rainbow trout populations (con f: French), (con i: Iranian), and (con d: Danish) based on COX1 sequencing. Bottom: The phylogenetic tree based on Cyt b gene sequence of Iranian, French, and Danish rainbow trout.

The various species of rainbow trout have been networked based on the differences in haplotype. As shown in (Fig 5). The three isolated of Iranian rainbow trout (Red color circle), France rainbow trout (Green color circle) and Danish rainbow trout (Blue color circle) were genetically identical or close together and they are located in a network. The other rainbow trout (yellow circle) subspecies were present in this network, but there are genetics distance between these. The network indicated that two groups of rainbow trout populations coexist in studied samples. The isolates of Iranian rainbow trout (red color) were very close to French rainbow trout (green color) (Fig 5). A neighbor-joining tree using the same data set for the network analysis were confirmed this, which is shown in Fig 4.

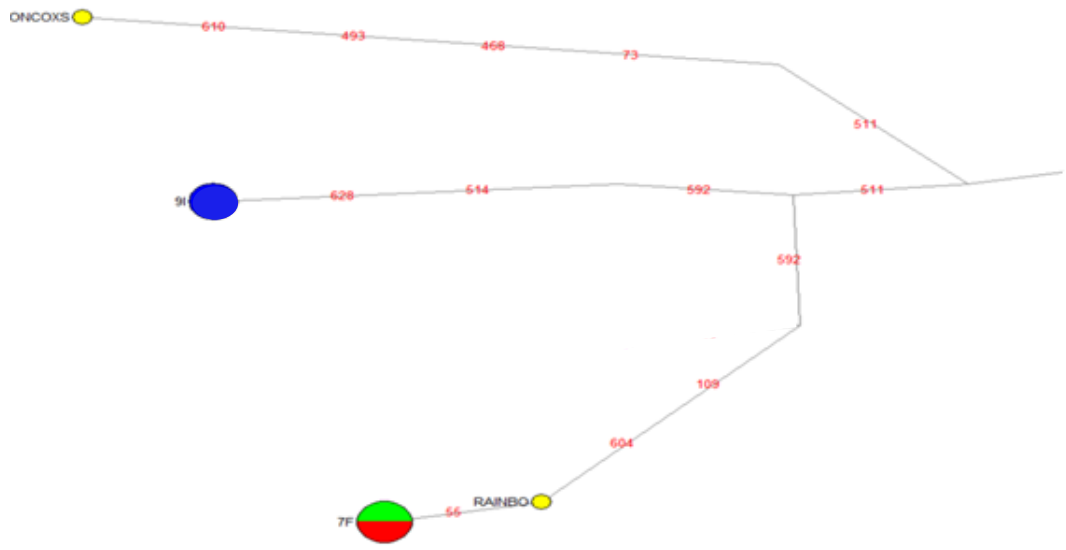

Fig 5. Network of haplotypes obtained based on sequence data for mtDNA Cyt b gene of rainbow trout. Circle sizes are approximately proportional to haplotype frequency: smallest circle represents a single individual; largest circle represents the population (Iranian in red color, French in green color, Danish in blue color, other *Oncorhynchus* species in yellow color). The numbers indicate to the position of mutation between the fish populations.

The study on genetic diversity of three species of Persian, French and Danish rainbow trout in the with using Cyt b gene that Iranian and French Rainbow trout had 99% identifying. In addition, the Danish rainbow trout had a 0.009 genetic distance with Iranian and French Rainbow trout. The results of genetic diversity among three populations that study in this research and other rainbow trout populations in the NCBI GenBank presented in Fig 4. The Iranian rainbow trout is very similar to the species registered at the NCBI. The genetic diversity Analysis confirmed that Iranian, French and Danish had lowest genetic distance with subspecies such as *Oncorhynchus mykiss* x Salmo salar, rainbow trout, *Oncorhynchus nerka*, *Oncorhynchus tshawytscha* and *Oncorhynchus kisutch* isolate K14 and highest genetic distance with subspecies such as *Oncorhynchus clarkii stomias* voucher, Oncorhynchus clarkii isolate *O. clarkii* and *Oncorhynchus clarkii henshawi*. The highest genetic distance of the studied species was observed with *Abramis brama* fish, which is a fish of the Cyprinidae family and has been investigated to verify the accuracy of the phylogenetic tree.

Discussion

To date, many researchers had been focused on the genetic diversity of the Cytb gene in rainbow trout populations (Billington and Hebert, 1998, Pavlov *et al*., 2004, Shed'ko, 2002). The Russian researcher's studies on the genetic diversity of rainbow trout showed that the gene sequence of these fish had a similarity of 99.6 to 100%. Hence, they reported that genetic diversity of rainbow trout population in the east and west of the Kamchatka Peninsula Sea was close to zero (Pavlov *et al*., 2004). The cytochrome b region is a coding region with low mutations and variations, but the presence of genetic diversity in this gene is known as a breeding characteristic of animal species. Iranian researchers estimate the genetic diversity between Persian and French rainbow trout species using the microsatellite marker. Their results demonstrated that there is significant genetic diversity between the Iranian rainbow trout and France rainbow trout (Mahmodi *et al*., 2014). Also, they reported that there was no significant genetic difference between the two Iranian and French subspecies when the genetic marker was the Cytb gene. However, they suggested that the Cytb gene was a suitable marker for genetic diversity among Iranian and Danish rainbow trout. Iraji *et al*. (2014) focused on the growth hormone gene (GH) to study genetic diversity among French and Iranian rainbow trout. Their results confirmed that two subspecies had different restriction enzyme digestion pattern on the agarose gel and genetic diversity (Iraji *et al*., 2014). Researchers concerns about the protection of native species in the industry. On the other hand, the pattern of genetic diversity in fish breeding programs is very useful and hatchery can be better management of production. Generally, the effect of reducing genetic diversity in breeding populations is the loss of adaptability to the environment. Therefore, one of the challenges in hatchery management is the estimation of genetic diversity. In this study, the 735 bp fragment of the cytb gene for propose estimation of genetic diversity in Iranian rainbow trout, French rainbow trout, and Danish rainbow trout were amplified and sequenced. The results showed that there is genetics distance among 3 subspecies, but genetic distance among Iranian and French rainbow trout was very negligible. Therefore, this study reported that the ancestral origin of Iranian and French rainbow trout was from an area.

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التنوع الوراثي لثلاث مجموعات من تراوت (*Oncorhynchus mykiss*) على أساس تسلسل جينات mtDNA

تقي عبد الله [1]، محمد رضا نصيرى[2]، اميد صفرى[3] و علي جوادمنش [2*]

1 كلية الزراعة، جامعة البصرة، العراق.

2 كلية الزراعة، جامعة الفردوسي في مشهد، إيران.

3 كلية الموارد الطبيعية والبيئة، جامعة الفردوسي في مشهد، إيران.

* javadmanesh@um.ac.ir

المستخلص

يؤدي فقدان التنوع الجيني إلى تقليل عدد الجماعات أو الأنواع التي ستؤثر أو قد تؤثر على قدرة الجماعة في بيئة جديدة تؤثر أو تسبب المرض. يلعب تقدير التنوع الوراثي في مزارع الأسماك دورًا أساسيًا في إدارته بشكل صحيح. لذلك ، تهدف هذه الدراسة إلى تقدير التباين الوراثي بين الجماعات الثلاثة من التراوت بما في ذلك الإيرانية والفرنسية والدنماركية باستخدام الميتوكوندريا DNA COX1 و .Cytb جمعت تسعين عينة من ثلاث مجموعات من مزارع سمك السلمون في منطقة مدينة كلات نادر في مشهد. تم استخراج الحمض النووي وتم تضخيم الجين COX1 و Cytb بواسطة PCR ثم تسلسلها. أظهرت النتائج انخفاض مستوى التباين الوراثي داخل المجموعات المدروسة. كشف التحليل الوراثي والنمط الفرداني أن المسافة الوراثية بين مجموعة التراوت الإيرانية والفرنسية كانت أقل بكثير من الدنماركية. قد يشير هذا الاكتشاف إلى أن الجماعات الإيرانية قد يكون لديهم تجمع جيني مماثل مع الجماعات الفرنسية.

الكلمة الأساسية: تراوت Rainbow ، تفاعل البلمرة المتسلسل ، السيتوكروم أوكسيديز ب ، شجرة النشوء والتطور.