Maternal ancestry of red tilapias based on D-loop sequences analysis of seven tilapia populations

Bingjie Jiang[1], Jianjun Fu[2], Zaijie Dong[12], Min Fang[1], Wenbin Zhu[2], Lanmei Wang[2]

[1] Wuxi Fisheries College, Nanjing Agricultural University, Wuxi, Jiangsu, China

[2] Key Laboratory ofFreshwater Fisheries and Germplasm Resources Utilization,Ministry of Agriculture, Freshwater Fisheries Research Center of Chinese Academy of Fishery Sciences, Wuxi, Jiangsu, China

Corresponding Author:

Zaijie Dong[12]

East Shanshui Road 9,Wuxi,Jiangsu,214081, China

Tel: 86-510-85558831;Email address: dongzj@ffrc.cn

Commented [e1]: space

Commented [e2]: space

Abstract

Background. Many tilapia species originally from Africa have been widely introduced and have become economically important as food fish in China. The genetic background information for those populations is seriously deficient, which requires more research, especially for red tilapia strains.

Methods. In the present study, the mtDNA D-loop sequences were used to evaluate the genetic relationship and diversity of seven tilapia populations widely cultured in China, especially for speculating the maternal ancestry of red tilapia strains. Three red tilapia varieties (*Oreochromissp*) [Chinese Taiwan (TW), Israel (IL), and Malaysia (MY)] and four wild-type or breeding populations [*O. aureus* (AR), *O. niloticus* (NL), *O. mossambicus* (MS), and GIFT stain of *O. niloticus*] were collected and analyzed in this study.

Results.A total of 146 polymorphic sites and 32 haplotypes of D-loop sequences were detected among 332 fishes, and four major haplotypes were shared among populations. TW and NL populations had more number of haplotypes (20, 8 respectively). The haplotype diversity (Hd) and nucleotide diversity (π) of each population ranged from 0.234 to 0.826, and 0 to 0.060, respectively. The significant positive Tajima's D values of neutral test were detected in NL, IL and MY populations ($P < 0.05$), which indicated these populations might have not experienced historical expansion. According to the pairwise *F*-statistics, highly significant genetic differentiation was detected among populations ($P < 0.01$), excepted between IL and MY populations ($P > 0.05$). The nearest K2P genetic distance (D = 0.014) was detected between MS and TW populations, whereas, the farthest (D = 0.101) was found between GIFT and AR populations. The result of molecular variance analysis (AMOVA) showed that there was extremely significant genetic variation detected among seven populations ($P < 0.01$), which contained 63.57% of total variation. In view of the genetic relationship of red tilapia strains with other populations, TW and IL were detected with more similar genetic structures related to MS, and MY showed with closely genetic distance to GIFT (or NL), which could provide more genetic evidences for red tilapia maternal ancestry.

Key words: tilapia;D-loop; population genetics; maternal ancestry; genetic diversity;

Introduction

Tilapia as a common name has been applied to various cichlids from three distinct genera, which includes *Oreochromis*, *Sarotherodon* and *Tilapia* (Trewavas 1983). The farmed tilapia production worldwide is over 6.4 million tons annually in 2015 and China is the largest tilapia producer in the world(FAO 2016).Red tilapia is instead a name used for several different manmade tilapia variants that sport and attractive red coloration. These variants are the result of

Commented [e3]: space

Commented [e4]: space

Commented [e5]: this should be in full spellings at the first usage

Commented [e6]: put colon after varieties

Commented [e7]: separate

Commented [e8]: stain or strain?

Commented [e9]: space

Commented [e10]: among the populations

Commented [e11]: space

Commented [e12]: Trewavas, 1983

Commented [e13]: was

Commented [e14]: FAO, 2016

Commented [e15]: space

continuous selective breeding (Wohlfarth et al. 1990). Many farmers prefer to cultivate red tilapia since it is much sought after in certain markets. Because of their high protein content, large size, high feed conversion rate (FCR), rapid growth, and palatability, red tilapias are the focus of major farming efforts in China (Romana-eguia 1999).

In recent years, due to the increasing demand of market in China, many red tilapia populations have been cultured in China farms. However, there are genetic introgressiveand hybridization in the genetic resourcebecause of them inter-specific hybridization breeding.The problems of slow growth rate and color separation often occur in the practice, which greatly affect the promotion and marketing of red tilapia. In China, the genetic diversity studies of tilapia populations based on molecular markers were carried out in tilapia populations, such asresearches using TRAP (Ma et al. 2012), microsatellites (Zhang et al.2010) and ISSR (Zhong etal. 2012).The research on tilapia mainly focuses on growth and development (Lith et al. 2005), culture (Muendo et al. 2006), and breeding (Fujimura et al. 2010) in other countries. There was rareinformation on genetic diversity and genetic ancestries of red tilapia. The origin of the red tilapia was generally thought to be attributed to cross-breeding of mutant reddish-orange *O. mossambicus* and other populations including *O. aureus*, *O. niloticus* and *O.hornorum*(Wohlfarth et al. 1990; Sandeep et al. 2012), but the specific source of the three strains of red tilapia are ambiguous.

The D-loop sequence is a non-coding region of mitochondrial DNA (mtDNA), with high rate of evolution and no restructuring, which becomes one of the most commonly used mtDNA sequences for addressing the evolutionary relationship of close relatives and/or subspecies (Murgra et al. 2002). At present, the D-loop sequence had widely used in aquaculture species, concerned in genetic structure (Ryota et al. 2002), genetic differentiation (Brown 2002), species validities (Tang2007), phylogeny and molecular differentiation (Ekerette 2018).In this study, D-loop sequence was used to evaluate the genetic diversity of seven tilapia populations and also used to estimate the maternal ancestry of three strains of red tilapia.

Materials & Methods

Sample collection

Seven tilapia populations were collected, including three red tilapiastrains[Chinese Taiwan (TW), Israel (IL), and Malaysia (MY)] and four wild-type or breeding populations of tilapia [GIFT strain of *O. niloticus*, *O.aureus*(AR), *O. niloticus*(NL), *O. mossambicus* (MS)]. TW and IL populations were transferred from Fujian Province, China in 2014, and MY population was introduced from Malaysia 2009 (Yang et al. 2015). All tilapia populations were domesticated and bred in an experimental aquaculture farm in Wuxi (Jiangsu Province, China).48 fin clips were sampled from each population, and then soaked in absolute ethanol, until DNA extraction.

DNA extraction and amplification

Genomic DNA was extracted using phenol-chloroform method (Sambrook 2001). The integrity was detected by 1% agarose gel electrophoresis, and purity and concentration of DNA

Commented [e16]: et al.,

Commented [e17]: ,

Commented [e18]: Introgression and hybridization

Commented [e19]: space

Commented [e20]: their

Commented [e21]: space

Commented [e22]: there should be a comer after et al. Do note this for subsequent in-text citations.

Commented [e23]: is

Commented [e24]: space

Commented [e25]: had wide uses

Commented [e26]: there should be a comma after a surname. Do take note for subsequent in-text citations.

Commented [e27]: Ekerette et al., 2018. This was not a one author publication so acknowledge all authors by adding et al.

Commented [e28]: Tilapia fish were sampled from seven populations

Commented [e29]: space

Commented [e30]: space

Commented [e31]: space

were detected by NanoDrop spectrophotometer. The DNA concentration of each sample was adjusted to about 20 ng / L, kept under -20℃ until use.

Primers of D-loop was designed according to the complete sequence of tilapia mtDNA (Accession NO: NC_014060) from the National Center for Biotechnical Information (NCBI). The D-loop sequence of 867bp was amplified using the primer pair (sense primer: 5'-CTACTTCTTCCTCTTCCTTGT-3', anti-sense primer: 5'-TCCGTCTTAACATCTTCAGT-3'), which were synthesized bySangon Biotech (Shanghai) Co.Ltd. The PCR amplification was performed on an Eppendorf Mastercycler Pro 384 PCR thermocycler (Eppendorf, Germany). Amplifications were performed in a volume of 50 μl, containing 5 μL 10× PCR Buffer, 3 μL $MgCl_2$(0.25 mM), 4 μL dNTPs (2.5 mM), 1 μL Taq polymerase (2.5 U/μL), 1 μL of each primer (10 μM), 2µl genomic DNA (20 ng/µl), and 33 µl DNase/RNase-free deionized water. PCR amplification was performed under the following conditions: pre-denaturing 2 min at 94 ℃; 35 cycles of denaturing 40s at 94 ℃, annealing 55s at 55 ℃, prolonging 1 min at 72 ℃; final prolonging10 min at 72 ℃; and then held at 12 ℃. Subsequently, the reaction product was detected by 1% agarose gel electrophoresis, and the bidirectional sequencing was carried out by the ABI3730XL sequencing instrument of Shanghai Majorbio Company.

Sequence arrangement and data analysis

The sequences were edited using BioEdit version 7.0.9 software (Hall 1998). To ensure accuracy, all DNA fragments were sequenced in two directions, and the sequencing results of the two directions should be manually checked to prevent the ambiguity of the base or sequencing error. After the completion of the splicing, all sequences were used for homologous alignment and length determination by BioEdit version 7.0.9.

Genetic variation parameters were calculated by DNAsp5.1 (Librado 2009), including polymorphic (segregating) sites (S), number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π), average number of nucleotide differences (k) and Tajima's D. For phylogenetic analysis, Mega 5.05 software (Kimura 1980; Tamura et al. 2011) could be utilized to calculate the genetic distance of Kimura 2-parameter (K2P) distance model among populations, and to constructNeighbour joining (NJ) and Network map for haplotypes. Evaluation of Neighbour joining (NJ) used 1000 replications for bootstrapping. Arlequin 3.5 software (Excoffier 2010) was used to analyze nucleotide composition, *F*-statistics (F_{st}) and analysis of molecular variance(AMOVA) among seven tilapia populations.

Results

Variation and haplotype distribution of D-loop sequences in tilapias

After comparing and sorting the D-loop sequence of tilapia in seven tilapia populations, the fuzzy sequences were deleted and 332 homologous sequences were obtained. The nucleotide frequencies of the seven tilapia populations were consistent, and clearly, the rate of A +T

Commented [e32]: space

Commented [e33]: could be or was?

Commented [e34]: space

Commented [e35]: space

Commented [e36]: in the seven tilapia

Commented [e37]: This should be part of Materials and Methods. Take it to line 125.

(average value is 64.3%) was higher than of C + G (average value is 35.7%). Nucleotide composition showed that the contents of A, T, C, G were 31.8%, 32.5%, 21.6% and 14.1% respectively.

A total of 32 haplotypes were found in the D-loop sequences, deposited in the GenBank database under the accession numbers MH515150-MH515185 (except MH515152, MH515172, MH515175, MH515182). In detail, the different numbers of haplotype (from 1 to 20) were detected among populations (Table 1). 4 of these haplotypes were shared haplotypes (Hap_2, Hap_22, Hap_23, Hap_24), of which 2 haplotypes were composed of NL and GIFT populations (Hap_22, Hap_23), while the others were unique to each population. A total of 4 dominant haplotypes (Hap_2, Hap_22, Hap_24, Hap_26) accounting for them were 34.90%, 12.30%, 14.50% and 14.50%, respectively.

Genetic diversity and genetic distance among seven tilapia populations

The genetic diversity parameters of tilapia populations based on D-loop sequence are shown in Table 2. A total of 146 polymorphism sites were found, the overall haplotype diversity (Hd) of the tilapia populations was 0.817, and each population was ranged from 0 to 0.834. The average number of nucleotide differences (K = 0-47.32) and nucleotide diversity (π = 0-0.060) were in the similar result. Among them, AR population had the lowest genetic diversity (Hd = 0, π = 0), TW and NL populations had a higher haplotype diversity (Hd = 0.834, 0.826 respectively), and the highest nucleotide diversity (π) was in NL population (π = 0.060). Tajima's text indicated that the Tajima's D value of TW, GIFT and MS populations were negative, and other populations were positive. Among them, NL, MS have reached a significant level (P < 0.05), GIFT, IL and MY populations have reached extremely significant levels (P < 0.01).

The pairwise genetic distance was calculated by using Kimura 2-parameter (K2P) model among seven tilapiapopulations (Table3, below diagonal). The inter-specific distance between seven populations was from 0.014 to 0.101. The lowest inter-specific distance (0.014) was between MS and TW populations, while the highest inter-specific distance (0.101) was between GIFT and AR populations. In this study, the NJ tree based on K2P model genetic distance was shown in Figure 1. The NJ tree revealed the same phylogenetic relationship among populations. According to the phylogenetic tree, haploidtype was obviously divided into two branches: AR populations and other six populations. Among this six populations, two small branches could be divided, MS and TW populations were clustered and then clustered with IL population; GIFT and MY populations were clustered and then clustered with NL population.

Genetic differentiation between tilapia populations

The results of analysis of molecular variance (AMOVA) were shown in Table 4. Based on the results of genetic differentiation analysis, the variance of genetic variation among the seven tilapia populations was 63.57%, and the inter-population genetic differentiation index was 0.636, reaching a very significant level (P< 0.01). Seven populations were divided into 2 groups that were wild-type or breeding populations and red tilapia. The genetic differentiation index among populations within groups accounted for 0.633, reaching a very significant level (P< 0.01). From the above results, it can be concluded that the genetic variation of tilapia was derived from

Commented [e38]: 14.1%, respectively

Commented [e39]: of the tilapia

Commented [e40]: were detected

Commented [e41]: delete

Commented [e42]: space

Commented [e43]: inter-population

Commented [e44]: inter-population

Commented [e45]: Inter-population

Commented [e46]: The same? What are you comparing it with? I think this statement needs explanation or you expunge it completely.

Commented [e47]: Haplotype?

inter-populations, and the main significant genetic differentiation originated within groups among populations. The pairwise *F*-statistics values (Fst) of seven tilapia populations (Table 3, upper right corner) showed that the genetic differentiation among the other populations was very significant ($P < 0.01$), except that the genetic differentiation of MY and IL populations ($P > 0.05$).

Network map of haplotype of tilapia populations

The MJ network diagram of tilapia constructed by haplotype was described in Figure 2. MJ network presented a star-like profile, which was linked to a lot of haplotypes from different regions, and the shared haplotypes and dominant haplotypes were found clearly. Obviously, 3 shared haplotypes were composed of two populations (GIFT and NL, MY and IL), one shared haplotype was composed of six populations (except AR) and a dominant haplotype was composed of a population (AR).

Maternal ancestry of red tilapias

According to the genetic distanceresearch between red tilapias and wild-type or breeding populations(Table3, below diagonal), it was calculated that TW, IL red tilapias and MS populations had the closest genetic distance, which were 0.014 and 0.032 respectively.The genetic distance between MY red tilapia and GIFT population was the closest (0.034).The NJ tree clearly divided the tested samples of red tilapias into twoindependent branches: TW and IL clustered into one branch, MY clustered into other branch, and the former branchwas clustered with MS, while the latter branch was clustered with GIFT and NL.

Discussion

Genetic diversity and population dynamics

In the present study, the content of A+T (64.3%) in tilapia D-loop sequence was higher than the content of G+C (35.7%), which was in line with the distribution characteristics of base content in the D-loop (controlregion)of many fishes (Broughton et al. 2001).

In the D-loop sequences (867bp) of seven tilapia populations, 146 polymorphic (segregating) sites (S) and 32 haplotypes were detected, suggested that D-loop sequence could be an effective marker for detecting genetic diversity of tilapia populations.Overall, the tilapia populations had high haplotype diversity and nucleotide diversity, indicated that the populations containingabundant genetic resource for further use in breeding or practice. In detail, NL had the higher genetic diversity ($Hd > 0.5$, $\pi > 0.005$), which was consistent with microsatellite DNA markers (Romana-eguia et al. 2004; Yang et al. 2011), isozyme (Zhao et al. 1997). It was speculated that the original NL population introduced was larger and it had a potential for further selection breeding.The genetic diversity of red tilapia populations (TW, IL, MY) was higher thanthe other tilapia populations in China, whichwas consistent with itsgenetic background belongs to cross-breeding of tilapia. However, genetic diversity of AR population was the lowest ($Hd = 0$, $\pi = 0$), which was found to be similar to the results of the previous reports assessed by RAPD (Xia et al. 1999), two human microsatellite probes (Wang et al. 2000), and mtDNA restriction enzyme analysis (Cao 1997). The main reason may be due to the small population size had introduced in China, and generation of mass breeding carried out,which might resulted in a

Commented [e48]: This should be part of discussion as the implications of results

Commented [e49]: delete

Commented [e50]: space

Commented [e51]: ,

Commented [e52]: space

Commented [e53]: space

Commented [e54]: space

Commented [e55]: Your explanation of the phylogenetic tree is ambiguous. See how you can articulate it better

Commented [e56]: space

Commented [e57]: indicating

Commented [e58]: contain abundant

Commented [e59]: space

Commented [e60]: This is vague. Do you mean that TW, IL MY are cross breeding populations? If so, articulate this sentence clearly.

Commented [e61]: delete

Commented [e62]: space

decline in population genetic polymorphism. The genetic purity of AR population was adverse to the preservation of pure breed, it is necessary to introduce AR population again in order to improve its genetic diversity and avoid the decline of its excellent quality by inbreeding; however, the purity of this population also could be used inhybridization with other populations (or strains). The GIFT and MS populations also were detected with low haplotype diversity (Hd< 0.5) and low nucleotide diversity ($\pi < 0.005$), indicating that its population may have recently experienced bottleneck effect or founder effect produced by minority populations(Grant et al. 1998). The high purity of AR, GIFT and MS populations had great significance that the excellent economic traits obtained through long-term multi-generation breeding, could be stably inherited to the next generation and stabilized in the genetic process in the breeding population. Therefore, AR, GIFTand MS were often used as parents to breed red tilapia and stabilized excellent traits in red tilapia populations. The Tajima's D value of some red tilapia (TW) andwild-type or breeding populations (MS,GIFT) were negative,it may due to the larger scale breed after family selection of small number of breeder was carried in hatchery.

Genetic relationships of tilapia populations

According to the previous researches (Wohlfarth et al. 1990; Sandeep et al. 2012), red tilapias were originated from *O. mossambicus*, *O. aureus*or, *O.niloticus*.Four relative wild-type or breeding populationswere used forexploring the maternal ancestors of three strains of red tilapia. The MJ network of D-loop sequence haplotypes in tilapia individuals which weredistributed into three different majorregions and red tilapia populations existed in two regions. Four dominant haplotypes were shared by seven populations, where Hap_24 was a shared haplotype of MY and IL populations, Hap_22 and Hap_23 were shared haplotypes of GIFT and NL populations, and Hap_2 was the shared haplotype of six populations except AR population. It was suggested that six populations (except AR) may originate from the similar maternal ancestors dominated by dominant haplotypes.

The analysis of molecular variance (AMOVA) by grouping (wild-type or breeding populations and red tilapia) and non-grouping showed that the main genetic variation was derived from inter-population, which is similar to the results of Habib et al. (2010), the low variance within population and high variance between populations was reported among Channa fishes. The genetic fixed index (*F*st) was commonly used to examine the genetic variation of populations and the contribution of this variation to genetic differentiation (Holsinger 2009). Significant *F*st values (*F*st> 0.25, $P < 0.01$) were found in this study, which demonstrated that higher level of genetic differentiation among the seven tilapia populations except red tilapias. The results indicated that red tilapia populations may evolve independently after separating from the common ancestor, but those three strains of red tilapia were closed.

Analyzedof maternal ancestry of red tilapias

While the maternal ancestors of the existing 3 strains of red tilapias are not well documented, their derivation is generally attributed to crossbreeding of mutant reddish-orange *Oreochromismossambicus* with other species including *O. aureus*, *O. niloticus* and *O. hornorum*(Wohlfarth et al. 1990; Sandeep et al. 2012). mtDNAhas the characteristics of maternal

Commented [e63]: space

Commented [e64]: space

Commented [e65]: space

Commented [e66]: space

Commented [e67]: space

Commented [e68]: space

Commented [e69]: populations

Commented [e70]: space

Commented [e71]: space

Commented [e72]: the seven

Commented [e73]: fixation index

Commented [e74]: differentiation exist among

Commented [e75]: Space

Commented [e76]: space

inheritance, simple structure, rapidly evolution. It can more intuitively preserve the characteristics of population mutations and has been widely applied in genetic diversity of populations. Therefore, the phylogenetic tree constructed by mtDNA can directly reflect the origin of the maternalancestry (Cann 1994).

Based on the K2P genetic distances among seven tilapia populations, two branches were constructed in the NJ dendrogram, and it was speculated that these populations might be derived from two different primary maternal ancestors, which was consistent with the results of MJ network. In details, the three strains of red tilapia might derive from different maternal origin, MS and GIFT (or NL) populations, respectively. TW and IL populations were closely related to each other and were clustered with MS, which was confirmed that two strains of red tilapia were produced from local crossbreeding rare mutant-colored (reddish-orange) female *O.mossambicus* (Wohlfarth et al. 1990). GIFT strain was selected from four *O. niloticus* strains imported directly from the African and four strains widely cultivated in Asia (Eknath et al. 1993). The K2P genetic distance between MY red tilapia and GIFT population was relatively small (D = 0.034), speculated that MY population was bred by GIFT population or that MY and GIFT populations may come from similarly artificially selected from NL population.In addition, the genetic differentiation degree between red tilapia (IL, TW, MY) and its breeding source population (MS, GIFT or NL) was relatively small, demonstrating that close genetic relationship was maintained between the breeding varieties and breeding source population; this highly homology was related to the characteristics of maternal inheritance and non-recombination of mtDNA (Mabuchi, 2010).

Conclusions

In this study, we used the D-loop sequences to estimate genetic structure of seven tilapia populations mainly cultured in China, furthermore, we analyzed the maternal ancestry of three strains of red tilapia, which would provide more basic data for reasonable protection and further utilization of tilapia populations in future. In brief, the IL and TW red tilapia strains were derived from *O. mossambicus* population, whereas MY red tilapia was derived from GIFT or *O.niloticus*.

Animal Ethics

This study was approved by the Bioethical Committee of Freshwater Fisheries Research Center (FFRC), Chinese Academy of Fishery Sciences (CAFS) (BC 2013863, 9/2013). The methods of all experiments were carried out in accordance with the Guide for the Care and Use of Experimental Animals of China.

Commented [e77]: space

Commented [e78]: space

Commented [e79]: space

Acknowledgements

This work was supported by the Central Public-interest Scientific Institution BasalResearch Fund from Chinese Academy of Fishery Sciences (2017HY-XKQ0203) and Jiangsu NaturalScience Foundation for Young Scholar (BK20160203).

References

An LP (2006) Study on phylogeny of part horse populations of eastern Asia based on mitochondrial cytochrome b gene sequence, Thesis for M.S., Gansu Agricultural University, Supervisor: Luo YZ, pp.1-61.

Broughton RE, Milam JE, Roe BA (2001) The complete sequence of the zebrafish (*Danio rerio*) mitochondrialgenome and evolutionary patterns in vertebrate mitochondrial DNA [J]. Genome Research, 11(11):158-1967.

Brown KH, Thorgaard GH (2002) Mitochondrial and nuclear inheritance in an androgenetic line of rainbow trout, *Oncorhynchus mykiss* [J]. Aquaculture, 204 (3): 323-335.

Cann R L, Stoneking M, Wilson A C (1994) Mitochondrial DNA and human evolution[J]. Journal of Bioenergetics &Biomembranes,26(3):251.

Cao Y, Xia DQ (1997) Studies on the genetic variation in mitochondrial DNA of *Oreochromisniloticus* and *O. aureus* [J]. Fish China, 21:360-5.

Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics, 144:2001-2014.

Ekerette, EE, Ikpeme EV, Udensi OU, Ozoje MO, Etukudo OM, Umoyen AJ, Durosaro SO, and Wheto M (2018) Phylogeneticsand molecular divergence of tilapia fish (*Oreochromis*species) using mitochondrial D-loop and cytochrome b Regions. American Journal of Molecular Biology, 8: 39-57.

Excoffier L, Lischer HE (2010) Arlequin suite ver.3.5: a new series of programs to perform population genetics analyses under Linux and Windows [J]. Molecular Ecology Resources, 10 (3): 564-567.

FAO (2016) Food and Agriculture Organization of the United Nations (FAO), Fisheries and aquaculture department, Global Aquaculture Production, 1950-2016.

Fujimura K, Okada N (2010) Development of the embryo, larva and early juvenile of Nile tilapia *Oreochromisniloticus*(*Pisces:Cichlidae*) development staging system [J]. Development, Growth &Differentiation, 49:301-324.

Grant WS, Bowen BW (1998) Shallow population histories in deep evolutionary lineages of marine fishes: Insights from sardines and anchovies and lessons for conservation [J]. Journal of Heredity, 89 (5):415-426.

Habib M, Lakra WS, Vindhya M, Praveen K, Barman AS, Akanksha S, Kuldeep KL, Peyush P and Asif AK (2010) Evaluation of cytochrome b mtDNA sequences in genetic diversity studies of *Channamarulius* (*Channidae: Perciformes*). Molecular Biology Reports, 6:41-49.

Hall TA, (1998) BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT [J]. Nucleic Acids Symposium Series, 41: 95-98.
Holsinger KE, Bruce SW (2009) Genetics in geographically structured populations: defining, estimating and interpreting *F*ST [J]. Nature Reviews Genetics, 10: 639-650.
Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences [J]. Mol. Evol.16: 111–120.
Librado P, RozasJ (2009) DnaSP v5: asoftware for comprehensive analysis of DNA polymorphism data [J]. Bioinformatics, 25(11):1451–1452.
Lith D, Cherop L, Munguti J, et al. (2005) Growth and economic performance of Nile tilapia (*Oreochromisniloticus L.*) fed on two formulated diets and two locally available feeds in fertilized Ponds [J]. Aquaculture Research, 36:746-752.
Ma QN (2012) Screening of TRAP markers and analysis of genetic diversity in red tilapia[D].Thesis for M.S., Nanjing Agricultural University.
Mabuchi K, Senou H, Nishida M (2010) Mitochondrial DNA analysis reveals cryptic large-scale invasion of non-native genotypes of common *Carp* (*cyprinuscarpio*) in Japan. Molecular Ecology, 17(3): 796-809.
Muendo PN, Milstein A, Dam A et al. (2006) Exploring the trophic structure in organically fertilized and feed-driven tilapia culture enviroments using multivariate analyses [J]. Aquaculture Research, 37: 151-163.
Murgra R, Tola G, Archer SN, Vallerga S, Hirano J (2002) Genetic identification of grey mullet species (*Mugilidae*) by analysis of mitochondrial DNA sequence: application to identify the origin of processed ovary products (*Bottarga*). Marine Biotechnology, 4 (2):119-26.
Romana-eguia MRR, Eguia RV (1999) Growth of five Asian red tilapia strains in saline environments. Aquaculture, 173:161–170.
Romana-eguia MRR, Ikeda M, Basiao ZU et al. (2004) Genetic diversity in farmed Asian Nile and red hybrid tilapia stocks evaluated from microsatellite and mitochondrial DNA analysis [J]. Aquaculture, 236(1-4):131-150.
Ryota Y, Akira G (2002) Phylogeography of a freshwater *Sculpin*, *Cottusnozawae* from the northeastern part of Honshu Island, Japan[J]. Ichthological Research, 49 (2): 147-155.
SambrookJ, Russell DW (2001) Molecular Cloning: Alaboratory manual, third ed. Cold Spring Harbor Laboratory Press, New York.
Sandeep M, Sun F, Liu F, Li J, David PB, Yue GH (2012) Novel polymorphic microsatellites from Florida red tilapia and cross-species amplification in Mozambique and Nile tilapia [J].Genet, 91:e97-e99.
Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol., 28: 2731-2739.
Tang WQ, Hu XL, Yang JQ (2007) Species validities of *Coiliabrachygnathus* and *C.nasus*taihuens is based on sequence variations of complete mtDNA control region[J] Biodiversity Science, 15(3):224-231.

Trewavas E (1984) Tilapiine fishes of the genera *Sarotherodon*, *Oreochromis* and *Danakilia*. British Museum (Natural History), London, England.Groot S J D.[J]. Aquaculture, 42(1):95-96.
Wang JK, Xia DQ and Wu TT (2000) Studies on genetic polymorphism of *O.aureus* of founder in China with DNA finger printing [J]. Journal of Nanjing Agricultural University, 23(3):61-63.
Wohlfarth GW, Rothbard S, Hulata G, Szweigman D (1990) Inheritance of red body coloration in Taiwanese tilapias and in *Oreochromismossambicus*. Aquaculture, 84:219-234.
Xia DQ, Cao Y, Ting WU, Wang T (1999) A study on genetic variation of tilapias fish with RAPD analysis and its application to heterosis [J]. Journal of Fisheries of China, 23(1):27-32.
Yang H, Li DY, Cao X, Zou ZY, Xiao W, Zhu JL (2011) Genetic potential analysis of six tilapia populations by microsatellite DNA markers. Hereditas (Beijing), 33:768-75 [in Chinese].
Yang H, Zhu WB, Dong ZJ, Li F, Gong CP, Liu N, Yuan XH (2015) Morphological variation analysis of three populations of red tilapia [J]. Shanghai Ocean University, 24(5):678-684.
Zhang YD, Gan X, Tang ZS, Chen Z, Su XH, Tang ZY, Li LP, Lin Y (2010) Analysis of genetic diversity in six tilapia populations [J]. Journal of Northwest A & F University, 38(10):58-66.
Zhao JL, Li SF, Li CH, Li JL (1997)Study of biochemical genetic marker of different strains of Nile tilapia *Oreochromisniloticus*[J]. Journal of Shanghai Fisheries University, 6:166-70.
Zhong JX, Li JQ, Zhong R, et al. (2012) ISSR analysis on genetic relationships of five Nile tilapia *O. niloticus* populations [J]. Journal of Fujian Fisheries,34(5):349-353.

Commented [e80]: Authors should ensure that references are in line with the journal's format

Table 1 Distribution of theD-loop haplotypes in tilapia populations

haplotype	Accession	Wild-type or breeding populations				Red tilapias			Sum
		NL	AR	MS	GIFT	TW	MY	IL	
Hap_1	MH515150			1					1
Hap_2	MH515151	5		46	1	18	18	28	116
Hap_3	MH515153					1			1
Hap_4	MH515154					1			1
Hap_5	MH515155					1			1
Hap_6	MH515156					7			7
Hap_7	MH515157					1			1
Hap_8	MH515158					1			1
Hap_9	MH515159					3			3
Hap_10	MH515160					1			1
Hap_11	MH515161					1			1
Hap_12	MH515162					1			1
Hap_13	MH515163					1			1
Hap_14	MH515164					1			1
Hap_15	MH515165					1			1
Hap_16	MH515166					1			1
Hap_17	MH515167					1			1
Hap_18	MH515168					2			2
Hap_19	MH515169					1			1
Hap_20	MH515170					2			2

Commented [e1]: Table 1:

Commented [e2]: space

Hap_21	MH515171				1			1
Hap_22	MH515173	9		32				41
Hap_23	MH515174	3		14				17
Hap_24	MH515176					29	19	48
Hap_25	MH515177						1	1
Hap_26	MH515178		48					48
Hap_27	MH515179					1		1
Hap_28	MH515180	15						15
Hap_29	MH515181	3						3
Hap_30	MH515183	8						8
Hap_31	MH515184	3						3
Hap_32	MH515185	1						1

Commented [e3]: Are the accession numbers officially published by NCBI for public access?

Table 2 Genetic diversity parameters of mtDNA D-loop sequence of seven tilapia populations

	NL	AR	MS	GIFT	TW	IL	MY	Sum
S	116	0	6	70	101	74	71	146
h	8	1	2	3	20	3	3	32
Hd	0.826	0	0.043	0.457	0.834	0.513	0.504	0.817
π	0.060	0	0.0003	0.004	0.024	0.040	0.039	0.054
k	47.32	0	0.255	3.380	20.50	34.38	33.55	45.36
Tajima's D	2.374*	0	-2.094*	-2.784**	-0.464	3.754**	3.874**	2.523

[1]S Polymorphic sites, h Haplotypes, Hd Haplotype diversity, π Nucleotide diversity, k Average number of nucleotide differences

[2]Note: * significant difference (P<0.05); ** very significant difference (P<0.01);

Commented [e1]: :

Commented [e2]: Be consistent in the arrangement of the populations. You can model the arrangement in table 1 for all the tables (2 and 3).

Commented [e3]: Their p-values should be presented along with the Tajima's D values on the table.

Table 3 Pairwise K2P genetic distances (below diagonal) and fixation indexes (*F*ST, above diagonal) among seven tilapia populations using D-loop

	MS	AR	NL	GIFT	IL	TW	MY
MS		0.999**	0.639 **	0.971**	0.379**	0.128**	0.612**
AR	0.093		0.612**	0.978**	0.794**	0.876**	0.800**
NL	0.076	0.070		0.333**	0.263**	0.463**	0.181**
GIFT	0.078	0.101	0.045		0.571**	0.798**	0.395**
IL	0.032	0.095	0.064	0.050		0.133**	0.079
TW	0.014	0.095	0.073	0.070	0.036		0.369**
MY	0.050	0.097	0.057	0.034	0.042	0.049	

[1]Note: **. very significant difference (P<0.01);

Commented [e1]: :

Commented [e2]: Be consistent in the arrangement of the populations. You can model the arrangement in table 1 for all the tables (2 and 3).

Table 4 Analysis of molecular variance (AMOVA) of 7 populations of tilapia mtDNA D-loop

Commented [e1]: :

	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation Index
No group	Among populations	6	5848.14	20.31	63.57	0.636**
	Within populations	325	3781.13	11.63	36.43	
	Total	331	9629.27	31.94		
Two groups (Wild-type or breeding populations and red tilapia)	Among groups	1	900.47	-0.5567	-1.760	0.639**
	Among populations within groups	5	4947.67	20.62	65.06	0.633**
	Within populations	325	3781.13	11.63	36.70	-0.018
	Total	331	9629.27	31.70		

Commented [e2]: What is the implication of this rows

[1] Note: d.f. degrees of freedom; **. very significant difference (P<0.01);

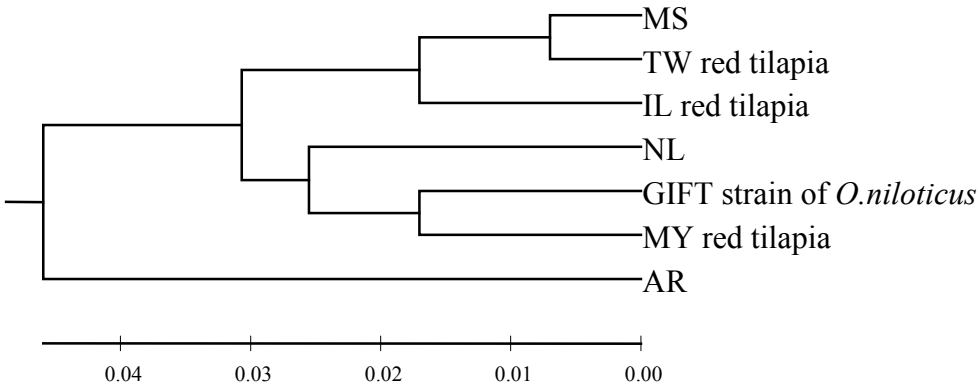

Fig. 1 NJ tree based on D-loop sequences of seven tilapia populations

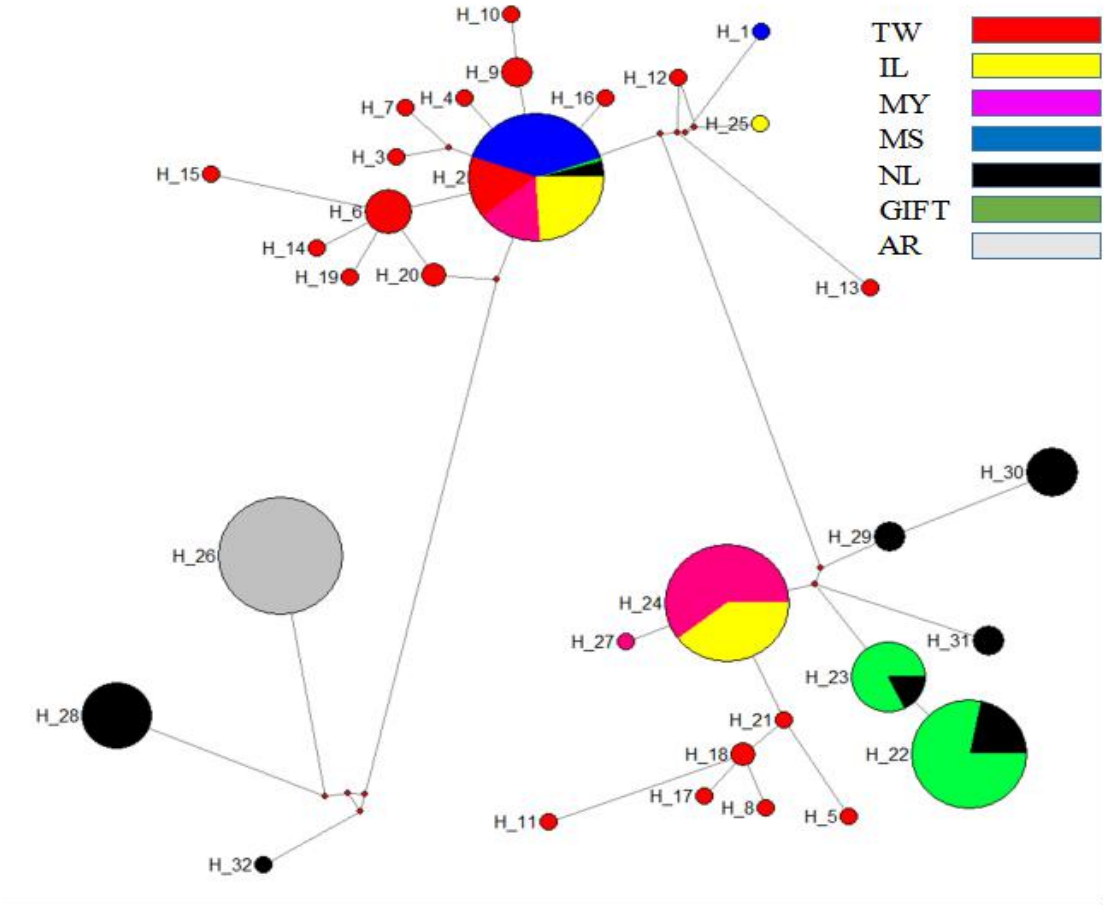

Fig.2 Haplotypes network of the mtDNA D-loop sequences for seven tilapia populations