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Use of sequence GNRH III for philogenetic analysis of red hard lipped barb carp, green hard lipped barb carp, mangot hard lipped barb carp and beureum panon

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Abstract

The aim of this study was to analyze the relationship between hard lipped barb carp strains based on encoding sequences of GnRH III. This research method uses an explorative method with descriptive analysis. Primers GnRH III-F (5'-GGACCTAAGAGCATGGAGTGG AAAGGAAG-3 ') and GnRH III-R (5'-GGGCTCGAGCACTCTTCCTCGTCTG TTGG-3') used in the GnRH III coding sequence analysis. Based on the coding sequence analysis of GnRH (forward and reverse directions) the genetic distance of red hard lipped barb carp and green hard lipped barb carp is 0.0033 and 0.0049, red hard lipped barb carp, green hard lipped barb carp, with mangot hard lipped barb carp of 0.0054 and 0.0093 and the three hard lipped barb carp with beureum panon of 0.0603 and 0.0558. The coding sequence of GnRH III red, green, mangot hard lipped barb carp has similar amino acid sequences with sGnRH *Osteochilus hasselti* (no. accession AFH41001.1) of 98% and beureum panon of 96%. The results showed that the coding sequence of GnRH III could be used in determining the kinship relationship of hard lipped barb carp strains.

Key words : Phylogenetic, GnRH III, hard lipped barb carp strain

1. Introduction

Hard lipped barb an indegenous tropical fish is a synchronous batch spawner fish capable of spawning several time simultaneously between males and females during the peak spawning period. Spawning period for hard lipped barb occurred three months prescribed by the gonadal development and sexual maturation are influenced by the gonadotropin releasing hormone (GnRH) (Lethimonier et al., 2004; Prayogo et al., 2011). In the fish's pituitary gland, the main function of GnRH III is to stimulate the synthesis and release of gonadotropins which further stimulates gonadal development (Dubois et al., 2002). In fish, there is a high diversity of GnRH amino acid sequences in 14 species, but from these variants the asam amino residues 1, 4, 9 and 10 are always conserv (Lethimonier et al., 2004). GnRH gene can be used as a phylogenetic analysis. GnRH gene encoding sequences selected as a determinant of diversity because it can detect the changes in the evolution of GnRH in every type of fish (Guilgur et al., 2007).

In Cyprinidae including hard lipped barb carp there are two types – of GnRH, namely GnRH II and GnRH III, the same as salmon _ have two types of GnRH (GnRH II or cGnRH-II and GnRH III or sGnRH) (Vickers *et al.*, 2004). GnRH II in hard lipped barb carp acts as LH (*Luteinizing Hormone*) while GnRH-III as FSH (*Follicle Stimulating Hormone*) (Prayogo *et al.*, 2011).

GnRH III (sGnRH) is related to ovarian and testicular maturation (Nabissi *et al.*, 2000; Fernald & White, 1999). GnRH III expression was detected in gonadal goldfish tissue (Lin & Peter, 1996; Pati & Habibi, 1998), rainbow trout (Uzbekova *et al.*, 2001),

pejerrey (Guilgur *et al.*, 2007) and seabream (Nabissi *et al.*, 2000), shows the role of GnRH III in reproductive control. GnRH III gene expression in rainbow trout gonad tissue showed variations in sexual cycle, indicating that GnRH III in fish varied. This encoding sequence of GnRH can be used in phylogenetic analysis of hard lipped barb carp strains because it can detect nucleotide changes in the GnRH gene sequence which is indicated by the percentage of similarity of sequences between hard lipped barb carp strains.

2. Materials And Methods

The materials used in this study include: isolation of DNA using wizard® genomic DNA purification kit (Promega), ethanol 70%, isopropanol. Amplification of DNA using gotaq® green master mix, primer GnRH III-F, and GnRH III-R (Table 1) for encoding sequence GnRH III. The sample in this study is the brain tissue of red, green, mangot and javaen hard lipped barb.

Table 1. Primer u	sed with So	equence Bases
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Primer	Sequence Bases
GnRH III-F	GGACCTAAGAGCATGGAGTGGAAAGGAAG
GnRH III-R	GGGCTCGAGCACTCTTCCTCGTCTGTTGG
Prayogo et al. (2	2011)

The method used in this study is exploratory method without using experimental design. Data obtained from this study will be analyzed using Genetic programs (R) version 7.0 (UPGMA/

Unweighted Pair Group Method with Arithmetic Mean) method sequences coding for GnRH III to get fenogram of hard lipped barb carp strains. The nucleotide sequences from GnRH III amplicon sequencing of the four strains of hard lipped barb carp (red, beurem panon, green and mangot) were analyzed *on-line* using the BLASTN / P program, especially the sequence of GnRH protein coding for hard lipped barb carp with the Osteochillus GnRH gene sequence found in genbank.

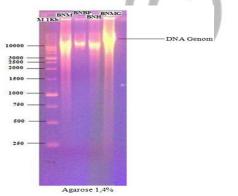
Fish brain genomic DNA of red hard lipped barb, green hard lipped barb, mangot hard lipped barb and Javaen hard lipped barb was isolated by Wizard® Genomic DNA purification Kit (Promega).

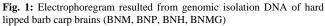
Composition of reaction amplification using PCR method consists of: 12.5 μ l go taq® green master mix, 1.5 μ l of GnRH III-F and 1.5 μ l GnRH III-R primers, 2.0 μ l DNA template and 7.5 μ l nuclease free water with a total volume of 25 μ l. The PCR program used in the amplification was 35 cycles consists of 95°C for 2 min (predenaturation), 95°C for 30 sec (denaturation), 55°C for 30 sec (annealing) and 72°C for 1 min (extension). After 35 cycles end with a final extension at 72°C for 5 minutes (Prayogo *et al.*, 2011).

3. Result And Discussion

Genomic DNA Isolation

Electrophoregram of genomic DNA resulting from hard lipped barb carp brain isolation is presented in Figure 1. Based on 1 kb DNA markers, genomic DNA size is above 10000 bp (10 kbp) because DNA consists of exons and introns so that the size of hard lipped barb carp brain genome DNA fragments is longer than with 1 kb DNA marker.





M = 1 kb DNA ladder marker (Promega), BNMG = brain of mangot hard lipped barb carp, BNM = brain of red hard lipped barb carp, BNBP = brain of bereum panon hard lipped barb carp, BNH = brain of green hard lipped barb carp

Amplification of GnRH III

Electrophoresis of PCR results on red hard lipped barb carp, panon, green and mangot samples produced a DNA fragment, and a relatively thick fragment of 500 bp for red, green and mangot nilem samples, while 600 bp of hard lipped barb carp beureum panon (Figure 2). The fragment is thought to be a sequence of encoding GnRH hard lipped barb carp, in accordance with the results of the study (Prayogo *et al.*, 2011). The target fragment is 500 bp fragment specific.

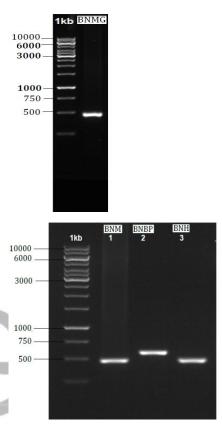


Fig. 2. Results of sequencing of PCR products coded BNMG, BNM, BNBP, BNH

M=1~kb~DNA ladder marker (Promega), BNMG = brain of mangot hard lipped barb carp, BNM~ = brain of red hard lipped barb carp, BNBP = brain of bereum panon hard lipped barb carp, BNH~ = brain of green hard lipped barb carp

The size of the GnRH DNA fragments in mangot (BNMG), red (BNM), and green (BNH) strains nilem were the same as 500 bp, and the bereum panon (BNBP) strain was 600 bp (Figure 6). The difference in size of the GnRH DNA fragment indicates the difference in the constituent nucleotide sequence of the GnRH III hormone among the strains of BNBP with BNMG, BNM and BNH. GnRH III nucleotide similarity analysis of four nilem strains using Genetyx R software version 7.0 shows the difference between the nilem strain, both forward and reverse directions (Figures 3 and 4).

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BNBP_GnRH III_arah F.txt		-GGCTGATCAGTTGGTATGCTAGTGTGTGTGTGGAGGTTAGTCTTTGCCAGCACTGGTC	59
BNH_GnRH III_arah F.txt BNMG GnRH III arah F.txt		GGTCTGSTCAGTTSTTATGCTGSTGTGTGTGTGGAGGTTAGTCTTTGCCAGCACTGGTC GTGCTGGTCAGTTSTTATGCTGGTGTGTGTGTGGAGGTTAGTCTTTGCCAGCACTGGTC	60
			60
BNM_GnRH III_arah F.txt	1	gggctgbrcagc-brtatgctbbrgtgtgtgtgtgggttagtctttgccagcactggtc	59
BNBP GnRH III arah F.txt	60	ATACSGTTGGCTTCCTGGTGGAAASAGAAGTGTTGGFSAAGTGGAGGCAACATTFAGGGT	119
BNH GnRH III arah F.txt	61	ATATSGTTGGCTTCCTGGTGGAAAAAGAAGTGTTGGTGAAGTGGAGGCAACATTCAGGGT	
BNMG GnRH III arah F.txt	61	ATAFGGTTGGCTTCCTGGTGGAAAAAGAAGTGTTGGAGAAGTGGAGGCAACATTCAGGGT	
BNM GnRH III arah F.txt	60	ATAT SGTTGGCTTCCTGGTGGAAAAAGAAGTGTTGGTGAAGTGGAGGCAACATTCAGGGT	119
BNBP_GnRH III_arah F.txt	120	BAGETTATCCTCTTGTTCTCATTTGGTCTGTTABATTTTAAGTSTTTGTCCCATTS	
BNH_GnRH III_arah F.txt	121	засыттатсстсттетералаа-тетталарынатыластастратетеление	179
BNMG_GnRH III_arah F.txt	121	васыптатсстсттетестстваааа-геттараанаптараетаствателета	
BNM_GnRH III_arah F.txt	120	видартитостоттарартсералла-готтарарартараластроградотранара	178
BNBP GnRH III arah F.txt	178	EATGTCTAGTTTAGTATCCGGGAAGTGTTGTAACATTGGGTGGCATGAAAAACTGAAAAT	237
BNH GnRH III arah F.txt	180	CTAAC	184
BNMG GnRH III arah F.txt	180	CTAAC	184
BNM GnRH III arah F.txt	179	CTAAC	183
BNBP_GnRH III_arah F.txt	238	ACTCATAGTACTGAGATGTGAGTATGTGTGTGTACTAACATCGAGTAAAAATCTGTCTCCAT	297
BNH_GnRH III_arah F.txt	185	AAAATTCTGTCTCCGT	200
BNMG_GnRH III_arah F.txt	185	aaaarrctgtctccgr	200
BNM_GnRH III_arah F.txt	184	hanartctgtctccgr	199
BNBP GnRH III arah F.txt	298	CAGATGATGGATGCTGGTGATGCAGTACTGTCCATTCCTGCAGACTCTCCAATAGAGCGG	357
BNH GnRH III arah F.txt	201	CAGATGATGGATGCTGGTGATGCAGTACTGTCTATTCCTGCAGACTCTCCAATGGAGCGG	260
BNMG GnRH III arah F.txt	201	CAGATGATGGATGCTGGTGATGCAGTACTGTCTATTCCTGCAGACTCTCCAATGGAGCGG	260
BNM GnRH III arah F.txt	200	CAGATGATGGATGCTGGTGATGCAGTACTGTCTATTCCTGCAGACTCTCCAATGGAGCGG	259
BNBP_GnRH III_arah F.txt	358	CTTTCACCGATACACATAGTAAGAGCAFGACTGTTFCTCTTTCTTAFFGGAAAACATCTG	417
BNH_GnRH III_arah F.txt	261	CTTTCACCGATACACATAGTAAGAGCACAACTGTTCCTTTCTTACTGGAAAACATCTG	
BNMG_GnRH III_arah F.txt	261	CTTTCACCGATACACATAGTAAGAGCACACTGTTCTTCTTACTGGAAAACATCTG	
BNM_GnRH III_arah F.txt	260	ctttcaccgatacacatagtaagagcacaaactgtctttctt	319
BNBP GnRH III arah F.txt	418	CAGCTTENTTEDCAGESTECNACTGATCABENATSECCTECTSETATAATENCAGGTGA	477
BNH GnRH III arah F.txt	321	CAGCTTCATTTTCCAGAGTGABACTGATCACTGATTTCCTCCTTTTTATAATCACAGGTGA	
BNMG GnRH III arah F.txt	321	CAGCTTCATTTTCCAGAGTGABACTGATCACTGATTTCCTCCTTTTTATAATCACAGGTGA	
BNM_GnRH III_arah F.txt	320	CAGCTTCATTTTCCAGAGTGAAACTGATCACTGATTTCCTCCTCTTTTATAATCACAGGTGA	
BNBP_GnRH III_arah F.txt	478	ATGAGGTGGATGCTGAAGGTTTGCCTCTAAAAGAACAGAGATTTCCCAACAGACGAGGAA	
BNH_GnRH III_arah F.txt	381	ATGATGTGGATGCTGAAGGTTTGCCTCTGAAAGAACAGAGATTTCCCAACAGACGAGGAA	
BNMG_GnRH III_arah F.txt	381	ATGATGTGGATGCTGAAGGTTTGCCTCTGAAAGAACAGAGATTTCCCAACAGACGAGGAA	
BNM_GnRH III_arah F.txt	380	atga <mark>r stggatg</mark> ctgaaggtttgcctcts <mark>baagaacagagatttcccaacagacgagaa</mark>	439
BNBF GnRH III arah F.txt	538	SAGTGCTCGAGCCCAAAAAAN	559
BNH GnRH III arah F.txt	441	GAGTGCTCGAGCCCAAATT	459
BNMG GnRH III arah F.txt	441	GAGTGCTCGAGCCCAAANA	459
BNM GnRH III arah F.txt	440	GAGTGCTCGAGCCCAAATT	458
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Fig. 3. Alignment of the nucleotide bases of the sequences encoding GnRH III-forward

Alignment of the nucelotide base sequence of the coding sequence GnRH III forward direction in each hard lipped barb carp strain has many similarities, especially in hard lipped barb carp mangot, green and red. The position of nucleotides to 179 to 282 in beureum panon showed different nucleotide variations from the three hard lipped barb carp, this indicates the distant genetic relationship with mangot, green and red strains (Figure 3). Alignment of the nucleotide bases of the sequences encoding the GnRH III gene reverse direction is presented in Figure 4.

BNBP GnRH III arah R.txt	1	GACTCTGTTCTTAGAGGCAACCTTCAGCATCCACCTCATCCACCTGTATTATAACA	58
BNH GnRH III arah R.txt		GGATCTCTCTTTAGAGGCAACCTTCAGCATCCACATCACCTGTGATTATAAAA	
BNMG_GnRH III_arah R.txt	1	NNNNNFCTNFTNFTA-GAGGCAACCTTCAGCATCCACAFCATTCACCTGFATTATAAAA	59
BNM_GnRH III_arah R.txt	1	васистритрираввоаасстисавсатосасфисатисасствираттатаафф	58
BNBP_GnRH_III_arah R.txt	59	<u>Бародаратрарталсаеттрарсартеераатралостосаелеттттссартале</u>	118
BNH_GnRH III_arah R.txt	61	GGRGGARATCRGTGATCAGTTCCACTCTGGRRAATGRAGCTGCAGATGTTTTCCAGTAAG	120
BNMG_GnRH III_arah R.txt	60	GERGEARATCRETEGATCAGTTECRCECTGGRRAATGRAGCTGCAGATGTTTTCCAGTAAG	119
BNM_GnRH III_arah R.txt	59	<u>Брресарут; реголтслоттроротовруговратотосоргатоттосоргало</u>	118
BNBP GnRH III arah R.txt	119	AAAGAGAAACAGTCATGCTCTTACTATGTGTATCGGTGAAAGCCGCTCTATTGGAGAGTC	178
BNH GnRH III arah R.txt	121	AAAGAGGAACAGTEGEGCTCTTACTATGTGTATCGGTGAAAGCCGCTCCATTGGAGAGTC	180
BNMG GnRH III arah R.txt	120	AAAGAGGAACAGTEGEGCTCTTACTATGTGTATCGGTGAAAGCCGCTCCATTGGAGAGTC	179
BNM_GnRH III_arah R.txt	119	AAAGAGGAACAGT GTGCTCTTACTATGTGTATCGGTGAAAGCCGCTCCATTGGAGAGTC	178
BNBP GnRH III arah R.txt	179	TGCAGGAATBBACAGTACTGCATCACCAGCATCCATCTGABBGAGACAGABTTTTAC	238
BNH GnRH III arah R.txt	181	TGCAGGAATAGACAGTACTGCATCACCAGCATCCATCTGACGGAGACAGAATTTTGT	240
BNMG GnRH III arah R.txt	180	IGCAGGAATAGACAGTACIGCATCACCAGCATCCATCATCIGACGGAGACAGAATTITGT	239
BNM_GnRH III_arah R.txt	179	IGCAGGAATABACAGTACTGCATCACCAGCATCCATCTGACBGAGACAGAATTTTBT	
BNBP GnRH III arah R.txt	239	EDEATGEERSEACACACACACCACCACCACCACCACCACCACCACCACCA	298
BNH GnRH III arah R.txt	241	TREFACECA-FACAFCAGE	
BNMG GnRH III arah R.txt	240	таргастра-тасатсарг	257
BNM_GnRH III_arah R.txt	239	TAGTACTCA-TACATCAGT	
BNBP GnRH III arah R.txt	299	CACCCARTGTTACAACACTTCCCCGGATACTAAACTAGACATGCAATGGGACAAACACTTA	358
BNH GnRH III arah R.txt	259	ACTT	263
BNMG GnRH III arah R.txt	258	ACTT	262
BNM_GnRH III_arah R.txt	257	<u>ACTT</u> F	261
BNBP GnRH III arah R.txt	359	АААААГриалавассартсарадсаададдааррисассотратотососсас	418
BNH GnRH III arah R.txt	264	AAFAFFFFFAACAFTTTCR6A-RCCRCAAGAGGATAAFCTCACCCTGRATGTTGCCTCCAC	322
BNMG GnRH III arah R.txt	263	AAFAFFFFFAACAFTTTCAGA-ACCACAAGAGGATAAFCTCACCCTGAATGTTGCCTCCAC	321
BNM_GnRH III_arah R.txt	263	AAFAFFFFAACAFTTTCAGA-ACCACAAGAGGATAAFCTCACCCTGAATGTTGCCTCCAC	320
BNBP GnRH III arah R.txt	419	TTCACCAACACTTCTC TTTCCACCAGGAAGCCAACC FATGACCAGTGCTGGCAAAGACT	478
BNH GnRH III arah R.txt	323	TCACCAACACTTCTTTTTCCACCAGGAAGCCAACCATATGACCAGTGCTGGCAAAGACT	382
BNMG GnRH III arah R.txt	322	TTCACCAACACTTCTTTTCCACCAGGAAGCCAACCATATGACCAGTGCTGGCAAAGACT	381
BNM_GnRH III_arah R.txt	321	TTCACCAACACTTCTTTTTCCACCAGGAAGCCAACCATATGACCAGTGCTGGCAAAGACT	
BNBP GnRH III arah R.txt	479	AACCTCCAACACACACACTACTACCAACTGGACCAGCAACCTTCCTT	538
BNH GnRH III arah R.txt	383	RACCICCAACACACACACCAGCATTARCAACTGGACCAGCAACCITCCITICCACTCCAT	
BNMG GnRH III arah R.txt	382	AACCTCCAACACACACCAGCATTAACAACTGGACCAGCAACCTTCCTT	
BNM_GnRH III_arah R.txt	381	RACCICCAACACACACACACACACCACCATTAACAACTGGACCAGCAACCITCCTTTCCACTCCAT	
BNBP GnRH III arah R.txt	539	рстоттадатссаррарадааданти	568
BNH GnRH III arah R.txt	443	SCTCTTAGGTCCBBBB	458
BNMG GnRH III arah R.txt	442	GCTCTTAGGTCCCACAGACGAGGAAGAGTGCTCGAGCCCACCAGGTTAGTCTTTGCCAGC	501
BNM_GnRH III_arah R.txt	441	GCTCTTAGGTCCABCABCAGGAGG	
BNBP GnRH III arah R.txt	568		568
BNH GnRH III arah R.txt			458
BNMG GnRH III arah R.txt		ACTGGTCATATGGTTGGCTTCCTGGTGGAAAAAGAAGTGTTGGTN	546
BNM GnRH III arah R.txt			462

Fig. 4. Alignment of the nucleotide bases of the coding sequence of GnRH III-reverse

The nucleotide base sequence of the GnRH coding sequence III reverse direction in green, mangot, and red strains has many similarities. The composition of beureum panon nucleotide bases at nucleotide positions 257 to 354 has different nucleotide variations from the three nilem (Figure 4). Mangot strains have different nucleotide variations in the nucleotide sequence to 470 to 531. The differences in nucleotide variations indicate differences in genetic relationships in the four strains.

Phylogenetic Analysis of Hard Lipped Barb Carp Strains

Phenogram kinship of hard lipped barb carp samples obtained from Genetyx R software version 7.0 using sequences of GnRH III-F encoding genes showed that the genetic distance between green and red nilem is very close at 0.0033 (from a scale of 0.00 to 1.00), kinship both are very close. In mangot strain has a genetic distance that is close to the red nilem and green strains that is equal to 0.0054. The relationship between mangot and the red and green strains is relatively close. The genetic distance of beureum panon with red, green and mangot strains of 0.0603 (Figure 5). Beureum panon strain showed a distant genetic relationship with red, green and mangot strains.

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[GENETYX : Evolutionary tree] Date : 2010.1.1 Method: UPGMA

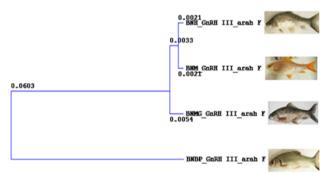


Fig. 5. Phenogram of the hard lipped barb carp strain using a III-forward GnRH primer

Phenogram analysis of the hard lipped barb carp strain using the GnRH III sequence reverse direction showed a pattern similar to the forward direction (Figure 6).

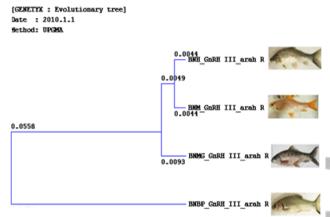


Fig. 6. Phenogram of the hard lipped barb carp strain using the GnRH III-reverse primer

The genetic distance between green nilem and red is very close at 0.0049 (close kinship). The results of this phenogram are consistent with the results of the nucleotide base alignment analysis using Genetyx R software (Figure 6). The genetic distance of red and green with mangot strains is 0.0093 indicating the third genetic kinship of hard lipped barb carp is close. Furthermore, the genetic distance of beureum panon with red, green and mangot strains of 0.0558 showed a distant genetic relationship.

Based on the alignment of the coding sequence of the GnRH III gene, the genetic kinship of red, green and mangot hard lipped barb carp is close, whereas bereum panon showed a distant genetic relationship .

Bioinformatic Analysis of GnRH III Encoding Genes

The results of GnRH III sequence of red, beureum panon, green and mangot hard lipped barb carp were then processed using bioinformatic analysis with the Blast-X program on-line to obtain the similarity of the gene encoding sequence from genbank.

Based on the alignment of the amino acid sequence of the GnRH III encoding gene four hard lipped barb carp strains contain the functional domain of the gene protein which consists of 10 amino acids with the code Q H W S Y G W L P G (Gln His Trp Ser Tyr Gly Trp Leu Pro Gly). This result is in accordance with that obtaoned on the hard lipped barb carp carried out by Prayogo *et al.* (2011). Blast-X analysis showed that the amino acids of the red, green, mangot and beureum panon hard lipped barb carp had homology to sGnRH *O. hasselti* in genbank (accession number

AFH41001.1) 98%, 98%, 98% and 96% respectively of the reverse direction.

Based on this, the encoding sequence of GnRH III can be used to determine the kinship of hard lipped barb carp strains, as shown in the phylogenetic analysis using GnRH III of the pejerrey (Silverside) fish, *Odontesthes bonariensis* (Atheriniformes) (Guilgur *et al.*, 2007).

4. Conclusion

Based on the results of the study, it can be concluded that the genetic distance of red and green hard lipped barb carp is 0.0033 and 0.0049, whereas red, green, with mangot hard lipped barb carp is 0.0054 and 0.0093 and the three hard lipped barb carps with beureum panon is 0.0603 and 0.0558. The coding sequence of GnRH III can be used to determine the phylogenetic strains of hard lipped barb carp.

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