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Molecular evolution of connective tissue growth factor in Cyprinidae (Teleostei: Cypriniformes)

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Abstract

Connective tissue growth factor (CTGF) plays an important role in regulation of cell growth, differentiation, apoptosis and individual development in animals. The study of sequences variation and molecular evolution of *CTGF* gene across various species of the cyprinid could be helpful for understanding of speciation and gene divergence in this kind of fish. In this study, 19 novel sequences of *CTGF* gene were obtained from the representative species of the family Cyprinidae using PCR amplification, cloning and sequencing. Phylogenetic relationships of Cyprinidae were reconstructed by neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian method. *Oryzias latipes* from the family Cyprinodontidae was assigned to be the outgroup taxon. Leuciscini and Barbini were clustered into the monophyletic lineages, respectively, with the high nodal supports. The estimation of the ratio of non-synonymous to synonymous substitution (dN/dS) for the various branches indicated that there stood the different evolution rates between the Leuciscini and the Barbini. With the ratio of dN/dS of the Leuciscini being lower than that of the Barbini, species within the Barbini were demonstrated to be subjected to the relatively less selection pressure and under the relaxable evolution background. A 6 bp indel (insertion/deletion) was found at the 5′ end of *CTGF* gene of Cyprinidae, and this 6 bp deletion only appeared in the Leuciscini, which is a typical characteristic of the Leuciscini and provides evidence for the monophylogeny of the Leuciscini. For the amino acid sequences of CTGF protein, the most variations and indels were distributed in the signal region and IGFBP region of this protein, implying that these variations were correlated with the regulation of the *CTGF* gene expression and protein activity.

Keywords: Connective tissue growth factor; Sequence variation; Molecular evolution; Cyprinidae

1. Introduction

Connective tissue growth factor (CTGF) secreted by multiple types of cells is one of the important members of the CCN family comprising CTGF, CYR61 (Cysteine-rich 61) and NOV (Nephroblastoma overexpressed proteins), which plays important roles in regulating cell growth, proliferation, apoptosis, adherence, transformation, and differentiation [1–3], and is involved in many biological processes such as angiogenesis, skeleton growth, embryogenesis, development, and wound healing [4,5]. It has been known that the expression of CTGF can be induced by transforming growth factor-β (TGF-β), which leads to epidermis fibrosis or fibrosis lesions [3]. However, the expression of *CTGF* gene can be suppressed by protein kinase C (PKC) and tyrosine kinase (TK) [6]. The N-terminal and C-terminal regions of CTGF protein were demonstrated to have the different biological functions [7]. The N-terminal region performs the important functional regulation in fibroblast differentiation and collagen synthesis, and the C-terminal region plays a curial role in regulating fibroblasts proliferation. *CTGF* gene is a discontinuous

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coding gene, consisting of a series of exons and introns. For example, the complete *CTGF* gene of *Danio rerio* (ENSDARG00000042934 from Ensemble) comprises five exons and four introns. CTGF protein in different species is highly conservative and the regions with the enriched cysteines were demonstrated to be involved in the biological function [8]. But few studies have been focused on phylogeny evolution of *CTGF* gene. To date, it is not known if variations of *CTGF* gene, especially functional regions, across the different fishes are correlated with the phenotypic characteristics of growth, development and fibrosis. To answer this question will help us understand the whole phylogeny evolution process and mechanism of species divergence.

The family Cyprinidae contains 122 genera and 600 species [9], with various morphology, niches and food habits. Therefore, the Cyprinidae is thought to be an ideal group for the study of the phylogenetic relationships and molecular evolution. Based on the morphological characters [10], Chen et al. classified Cyprinidae into two groups, Leuciscini and Barbini, and Gobiobotinae and Schizothoracinae were merged into the Gobioninae and Barbinae, respectively. Howes [11] grouped cyprinids into the subfamilies of Cyprininae, Gobioninae, Rasborinae, Leuciscinae, Acheilognathinae, Cultrinae, and Alburninae. In the recent revision based on the morphology, Cyprinidae was rearranged into 12 subfamilies by Chen [9] and Yue [12]. By molecular approaches, phylogenetic relationships of Cyprinidae in North America were reconstructed based on mitochondria 12S and 16S rRNA genes [13–15], and the phylogeny of Cyprinidae in East Asian inferred from cytochrome *b* and the 1st intron of S7 ribosome protein gene was achieved [16–19].

In this study, DNA sequences of *CTGF* gene were obtained from 19 representative species of the family Cyprinidae using polymerase chain reaction (PCR) and cloning, from which the coding DNA sequences (CDS) were retrieved and compared with that of *Danio rerio CTGF* gene. We aimed at (1) to analyze the difference of evolution rates for different species in the family Cyprinidae; (2) to characterize *CTGF* gene variation in each lineage within the Cyprinidae; and (3) to understand the correlations between *CTGF* gene variation and species divergence.

2. Materials and methods

2.1. Sample collection

In this study, 19 representative species of the family Cyprinidae were selected from the collections of the Freshwater Fish Museum of the Institute of Hydrobiology of the Chinese Academy of Sciences. One Cyprinodontidae species (ENSORLG00000018064, From Ensemble), *Oryzias latipes*, was assigned to the outgroup taxon. The locations of collection, deposited voucher and GenBank accession numbers of all species are listed in Table 1. Muscle or fin tissue preserved in 95% ethanol was used to extract the genome DNA.

2.2. Primer design

According to the conservative regions of *CTGF* gene sequences of *D. rerio* deposited in GenBank (NM_001 015041.1), primers were designed and optimized to amplify the objective *CTGF* DNA sequences. The forward primer: 5′AGCYTTCASHCAACAVVAHCAG3′; the reverse primer: 5′ATCRGGGCAYTTGAACTCC3′.

2.3. DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from muscle or fin tissues using standard phenol/chloroform extraction procedure. The *CTGF* gene was amplified from total DNA by PCR. PCR amplification mixtures contained 3 μl of DNA

Table 1
Species of the Cyprinid in this study

Species	Sampling location	Voucher	GenBank Accession No.
Hemiculter leucisculus	Wuhan, Hubei	IHBCY2603026	EF524096
Megalobrama amblycephala	Wuhan, Hubei	IHBCY0305004	EF524097
Aristichthys nobilis	Wuhan, Hubei	IHBCYK0411001	EF524098
Hypophthalmichthys molitrix	Wuhan, Hubei	IHBCYK0411002	EF524099
Pseudobrama simony	Taoyuan, Hunan	IHBCY0405361	EF524100
Ochetobius elongates	Tengxian, Guangxi	IHBCY0108003	EF524101
Elopichthys bambusa	Taoyuan, Hunan	NRMT2286	EF524102
Opsariichthys bidens	Taoyuan, Hunan	NRMT2358	EF524103
Gobiocypris rarus	Wuhan, Hubei	IHBCYK0411006	EF524104
Pseudorasbora parva	Mengla, Yunnan	IHBCY0312003	EF524105
Coreius heterodon	Wuhan, Hubei	IHBCY0312002	EF524106
Tanichthys albonubes	Guangdong	IHBCYK0411007	EF524112
Schizothorax waltoni	Tibet	IHBCY0380477	EF524107
Schizothorax oconnori	Tibet	IHBCY0510086	EF524108
Puntius semifasciolatus	Mengla, Yunnan	IHBCY0405496	EF524109
Cyprinus Carpio	Wuhan, Hubei	IHBCYK0411014	EF524110
Acrossocheilus monticola	Hechuan, Chongqing	IHBCYK0411016	EF524111
Rasbora trilineata	Wuhan, Hubei	IHBCYK0411012	EF524113
Danio rerio	Wuhan, Hubei	IHBCYK0411011	EF524114

template, 6 μl of 10× Ex *Taq* PCR buffer, 4.8 μl dNTPs (each at 2.5 mM, pH 8.0), 1.5 μl of each oligonucleotide primer (each at 15 μM), and 0.6 μl Ex *Taq* polymerase (5 U/μl). The reactions were carried out at an initial denaturation at 94 °C for 4 min, 32 cycles of denaturation at 94 °C for 50 s, annealing at 51 °C for 50 s and extension at 72 °C for 90 s, at last, a final extension at 72 °C for 6 min.

The amplified fragments were separated by 1.2% agarose gel electrophoresis, purified using an OMEGA kit (OMEGA Bio-tek) and ligated with a T-tailed pMD18-T vector (Takara). Transformation of the fragments into DH5α bacteria and sequencing of the inserted fragments were performed by routine methods.

2.4. Sequence analysis

The obtained *CTGF* DNA sequences were subjected to multiple alignments using CLUSTAL X [20] with a gap-opening penalty of 15.0 and a gap-extension penalty of 3.0. The aligned sequences with a manual correction were used to analyze gene characteristics. The *CTGF* CDS base composition and substitution were calculated by MEGA3.1 [21]. Mutation saturation analysis of nucleotide substitution was estimated by DAMBE (V4.1.33) [22] from the slope of a linear regression of transversions and transitions against F84 distance.

2.5. Phylogenetic analysis

Based on *CTGF* CDS, phylogenetic relationships of the family Cyprinidae were reconstructed using neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian method. NJ, MP, and ML were conducted with PAUP4.0b10 [23], and Bayesian analysis was performed with MrBayes3.1.2 [24,25].

For NJ analysis, the parameters were the following: Distance = ml, lscores (1/nst = 6, rmatrix = estimate, rates = gamma, shape = estimate), bootstrap (nreps = 1000, conlevel = 50, search = heuristic). The major-rule consensus tree was achieved from the equal parsimony trees produced by MP using the heuristic research. The parameters used to the 1000 bootstrap were: ConLevel = 50; search = heuristic; brlens = yes. The parameters for ML were estimated by Modeltest 3.7 based on AIC criterion, and the best model of *CTGF* CDS substitution was GTR+I+G (Base = 0.2244, 0.2800, 0.2871, $R_{mat} = 1.4129$, 3.1990, 1.3319, 0.2150, 6.4380, Shape = 0.5518, $P_{invar} = 0.3083$). Of the 100 bootstraps were performed with the following parameters: ConLevel = 50, search = heuristic, brlens = yes. Bayesian analysis was implemented under the following parameters: $N_{st} = 6$, rates = invgamma, $N_{gen} = 1{,}000{,}000$, $N_{runs} = 2$, $N_{chains} = 4$, Burnin = 600.

2.6. Molecular evolution analysis

The ratio of non-synonymous to synonymous substitutions (ω or dN/dS) was estimated by PAML3.14 [26]. The following three models were implemented to evaluate the variable ω rates: (1) One ratio model assigns the same ω rate for all branches; (2) two ratio model, with phylogenetic sense, specifies two or more than two branches to have independent ω rates; (3) free ratio model assumes that each branch was specified to have an independent ω rate.

2.7. Identification of indel sites in CTGF CDS

On the aligned *CTGF* CDS dataset, indel sites (insertion/deletion) were analyzed, by which the relationships among species with the similar indel characteristic were achieved.

2.8. Analysis of amino acids sequences

According to CTGF protein sequences of *D. rerio* deposited in GenBank (NP_001015041.1), the aligned *CTGF* CDS were translated into amino acid sequences. The parsimony informative sites were analyzed to search the similar characters of amino acid variations among the closely related species, and to reveal the role of amino acid mutation in species divergence and evolution process.

3. Results

3.1. CTGF gene sequence variations

A total of 918 sites were identified in the aligned *CTGF* CDS sequences. Of these sites, 583 were conserved across all the taxa analyzed, and 335 were variable with 137 sites being parsimony informative. Base compositions of T, C, A, and G were 21.6%, 27.6%, 21.8%, and 29.1%, respectively. The transition to transversion ratio (Ts/Tv) was 1.7. The saturation analysis of *CTGF* gene CDS indicated that base substitution did not get to saturation.

3.2. Phylogenetic analysis

Based on *CTGF* CDS, the consensus tree of the equal parsimony trees was constructed and is shown in Fig. 1, with tree length = 596, consistency index (CI) = 0.7148, retention index (RI) = 0.6074, and rescaled consistency index (RC) = 0.4341. Leuciscini and Barbini formed the monophyletic lineages, respectively, with the high nodal supports; *D. rerio* and *R. trilineata* were placed in the basal position of MP tree. The topology structure of Bayesian tree (Fig. 2) was similar to MP tree. In Bayesian tree, *D. rerio* and *R. trilineata* were clustered into the monophyletic lineage with 81% posterior probability. In addition, the topology structures of NJ and ML trees (not shown) were consistent with MP tree.

3.3. Indels in CTGF CDS

In the aligned *CTGF* CDS dataset, two deletions, one is 3 bp and the other is 6 bp in length, were identified. The

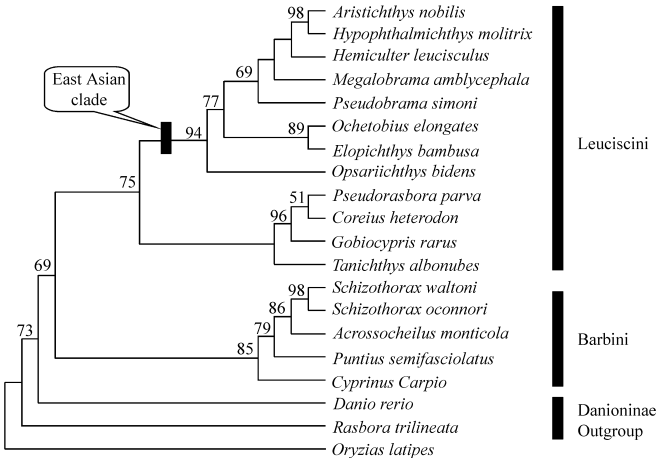

Fig. 1. The major-rule consensus tree achieved by heuristic research based on *CTGF* CDS. Tree length = 596, CI = 0.7148, RI = 0.6074, RC = 0.4341. The numbers above the branches represent the 1000 bootstrap scores.

3 bp insertion only appeared in the outgroup taxon (*Oryzias latipes*). In addition, we found the 6 bp deletion in all species of Leuciscini, and the 6 bp insertion (AGAGTG or AGGGTG, coding amino acid Arg and Val, respectively) in Barbini (Fig. 2) was similar to the 6 bp (AGAGTG) insertion in *R. trilineata*, but not to the 6 bp (AGTGTG, coding Ser and Val) insertion in *D. rerio*.

3.4. Evolution analysis

Based on the constructed MP tree and Bayesian tree, the ratio of non-synonymous to synonymous substitution was estimated using the software PAML3.14. The results are shown in Table 2. In one ratio model, the value of ω (dN/dS) in MP tree was similar to that in Bayesian tree. When the Leuciscini and the Barbini were specified to different ω values, the evolution rate of the Leuciscini ($\omega = 0.0532$) was lower than that of the Barbini ($\omega = 0.0850$). This result indicated that the species in Barbini were subjected to less selective pressure, and living in a more relaxable evolution environment. Compared with the one ratio model, the estimated likelihood value from the free ratio model was not significantly different, but when looking at the Bayesian tree, the likelihood value at the specified ω rate was significantly different ($p < 0.01$).

3.5. Variations of CTGF amino acid sequences

The deduced amino acid sequences for CTGF protein contained 306 amino acids. Of these, 19 amino acid

Table 2
The estimated likelihood values and ω rates in different evolution models

Model	Ratio	$-\ln L$	ω
MP Tree			
One ratio	1	3861.41	0.0555
Specified ratio	3	3859.52	0.0532/0.0850/0.0432
Free ratio	37	3841.54	–
Bayesian Tree			
One ratio	1	4097.84	0.0556
Specified ratio	4	3857.45[a]	0.0530/0.0850/0.0371/0.0544
Free ratio	35	4081.13	–

Note: When Leuciscini and Barbini were specified at different ω rates (dN/dS), the ω rate of leuciscini was 0.0532 (in MP tree) or 0.0530 (in Bayesian tree); barbini was 0.0850. Compared with one ratio model.
[a] The significant difference ($p < 0.01$).

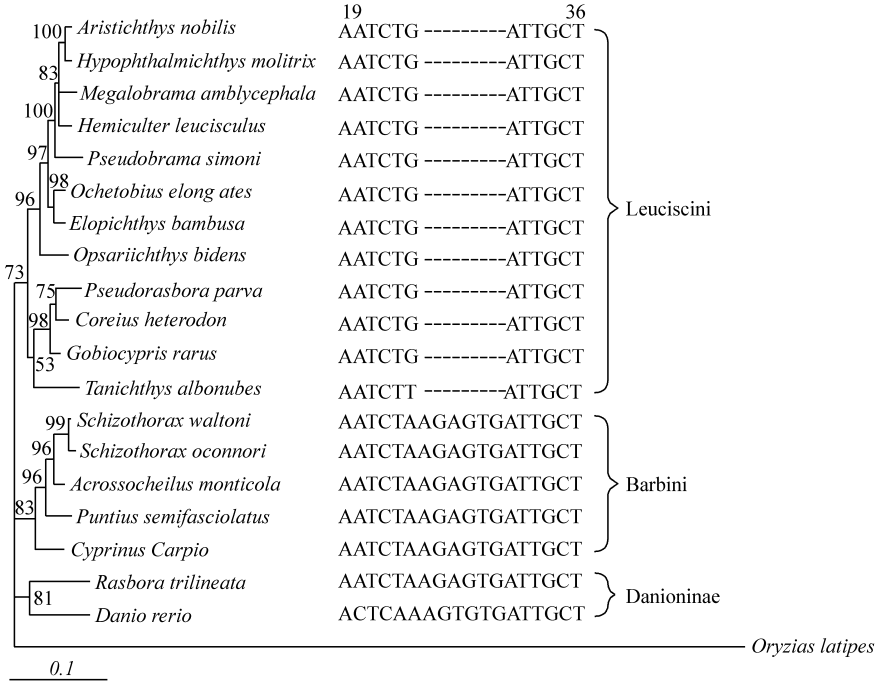

Fig. 2. Bayesian tree constructed based on *CTGF* CDS. The numbers near the nodes are Bayesian posterior probability.

variations with parsimony information (without considering gap) (Fig. 3) were found, which located at residues of 11, 17, 19, 20, 22, 32, 35, 36, 39, 48, 96, 103, 105, 109, 166, 167, 174, 175 and 223, respectively. Given only the ingroup species, there were 15 parsimony informative sites. At residues 19 and 20 in the Barbini were cysteine (Cys) and glycine (Gly), respectively, while in the other species of the Cyprinidae were leucine (Leu) and arginine (Arg). So Cys at site 19 and Gly at site 20 could be considered as the characteristics of the Barbini. At site 32, the amino acid Leu occurred in the Leuciscini; glutamine (Gln) in the Barbini and *R. trilineata*; and histidine (His) in *D. rerio*, therefore, Leu at site 32 might be a characteristic of the Leuciscini. Glutamic acid (Glu) at site 35 and Leu at site 48 occurred only in *D. rerio* and *R. trilineata*, so they are characteristics of the primitive Danioninae. Although amino acids parsimony informative sites were shown in five functional regions of CTGF protein, namely, signal region, IGF-binding proteins (IGFBP), von Willebrand factor type C (VWC), thrombospondin-1 (TSP-1) and cysteine-knot (CYS-KNOT), respectively [7], the most sites were located to the signal region and IGFBP region of the CTGF protein.

4. Discussion

CTGF protein plays an important role in regulating cell growth and individual development, which is highly conserved among different species of animals, therefore analysis of *CTGF* gene across the various lineages of Cyprinid will help us to estimate evolution rates for this kind of fish. Based on *CTGF* CDS, we reconstructed phylogenetic trees to estimate the ratio of non-synonymous to synonymous substitution for the different lineages. Leuciscini comprising Cultrinae, Xenocyprinae, Hypophthalmichthyinae, Leuciscinae, Cobioninae and some species of Danioninae, and Barbini which contains Barbinae, Schizothoracinae, and Cyprininae were recovered as separate monophyletic lineages with the high nodal supports. Our results also support that the East Asian Clade [17] include Xenocyprinae, Cultrinae, East Asian species of Leuciscinae and Danioninae. The phylogeny of the Cyprinidae based on *CTGF* CDS is consistent with other molecular phylogenies [16,17,19].

In this study, *CTGF* CDS variation was analyzed to reveal the evolution of nuclear gene among the various species in Cyprinid. The estimated dN/dS ratios among different branches reflected the different evolution pres-

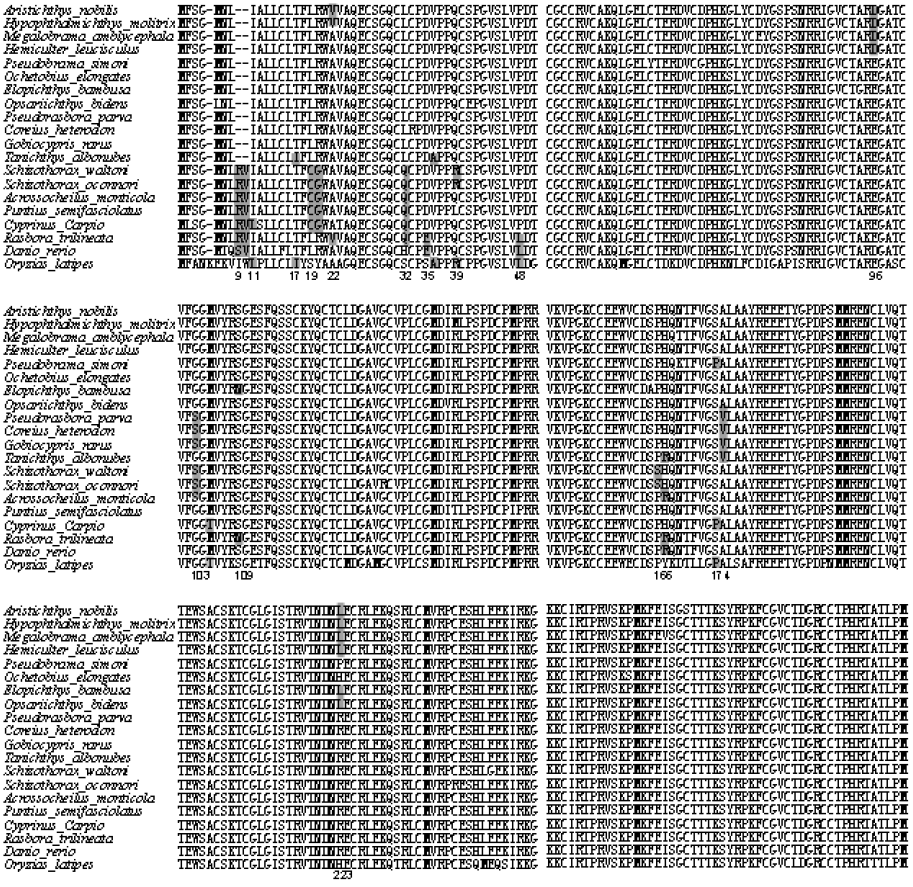

Fig. 3. Amino acid (aa) variations in CTGF protein sequences. The numbers represent the serial numbers of amino acids. Only the first 300 aa of the total 306 aa are presented. Amino acid variation sites with parsimony information are in grey. The 6 bp indel is located at sites 9 and 10.

sure for various groups in some degree [27]. The relatively more non-synonymous mutations were accumulated in the Barbini, implying that this species was living under less selection pressure and evolved in a more relaxable environment.

Indel sites in gene, especially in the functional regions of nuclear gene, could be considered as the important evolution events. In this study, the 6 bp insertion was identified in the Barbini, *D. rerio* and *R. trilineata*; while the 6 bp deletion was in all species of the Leuciscinis. Therefore, the 6 bp deletion was proved to be the typical characteristic of the Leuciscini, and it indicated an important event during phylogeny evolution and species divergence of the Cyprinidae. In addition, this 6 bp indel (insertion/deletion) sites in *CTGF* CDS was identified in signal region of *CTGF* gene, we assumed that this 6 bp insertion/deletion could be associated with the regulation of *CTGF* gene expression and protein activity. However, this must be proved by the further studies.

CTGF protein is a highly conserved sequence with the enriched cysteines. It plays a crucial role in regulating connective tissue growth in animals [4,7]. For the amino acid sequences of CTGF of the Cyprinidae, most of the 15 parsimony informative sites identified were situated in the signal regulation region and IGFBP region, both are involved in CTGF protein expression and activity, indicating the evolutionary significance of these informative sites in regulating muscle fibroblast differentiation and collagen production [7]. IGFBP region is involved in regulating individual growth and development mediated by insulin-like growth factor combining protein. Therefore, the most amino acid variation sites with parsimony information situated in N-terminal region of CTGF proteins should be correlated with tissue fibrosis and individual development. However, this hypothesis needs to be proved by study of CTGF protein function via gene rebuilding and other approaches.

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References

[1] Brigstock DR. The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. Endocr Rev 1999;20:189–206.
[2] Perbal B. NOV (nephroblastoma overexpressed) and the CCN family of genes: structural and functional issues. Mol Pathol 2001;54:57–79.
[3] Rachfal AW, Brigstock DR. Connective tissue growth factor (CTGF/CCN2) in hepatic fibrosis. Hepatol Res 2003;26:1–9.
[4] Ivkovic S, Yoon BS, Popoff SN, et al. Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. Development 2003;130:2779–91.
[5] Omoteyama K, Ikeda H, Imaki J, et al. Activation of connective tissue growth factor gene by the c-Maf and Lc-Maf transcription factors. Biochem Biophys Res Commun 2006;339:1089–97.
[6] Fan WH, Karnovsky MJ. Activation of protein kinase C inhibits the expression of connective tissue growth factor. Biochem Biophys Res Commun 2000;275:312–21.
[7] Grotendorst GR, Duncan MR. Individual domains of connective tissue growth factor regulate fibroblast proliferation and myofibroblast differentiation. FASEB J 2005;19:729–38.
[8] Bork P. The modular architecture of a new family of growth regulators related to connective tissue growth factor. FEBS Lett 1993;327:125–30.
[9] Chen YY. Fauna Sinica, Osteichthyes, Cypriniformes II (in Chinese). Beijing: Science Press; 1998, pp. 1–18.
[10] Chen XL, Yue PQ, Lin RD. Major groups within the family Cyprinidae and their phylogenetic relationships. Acta Zootaxon Sin (in Chinese) 1984;9(4):424–40.
[11] Howes GJ. Systematics and biogeography: An overview. In: Cyprinid Fishes: Systematics, Biology and Exploitation. London: Chapman and Hall; 1991. p. 1–33.
[12] Yue PQ. Fauna Sinica, Osteichthyes, Cypriniformes III (in Chinese). Beijing: Science Press; 2000, pp. 1–8.
[13] Simons AM, Mayden RL. Phylogenetic relationships of the creek chubs and the spinefins: an enigmatic group of north American Cyprinid fishes (Actinopterygii: Cyprinidae). Cladistics 1997;13:187–205.
[14] Simons AM, Mayden RL. Phylogenetic relationships of the western North American phoxinins (Actinopterygii: cyprinidae) as inferred from mitochondrial 12S and 16S ribosomal RNA sequences. Mol Phylogenet Evol 1998;9:308–29.
[15] Simons AM, Mayden RL. Phylogenetic relationships of North American cyprinids and assessment of homology of the open posterior myodome. Copeia 1999;1:13–21.
[16] He S, Chen Y, Nakajima T. Sequences of cytochrome *b* gene for primitive cyprinid fishes in East Asia and their phylogenetic concerning. Chin Sci Bull 2001;46:661–5.
[17] He S, Liu H, Chen Y, et al. Molecular phylogenetic relationships of eastern Asian Cyprinidae (Pisces: cyprinidformes) inferred from cytochrome *b* sequences. Sci China (Ser C) 2004;47:130–8.
[18] Wang X, He S, Chen Y. Sequence variations of the S7 ribosomal protein gene in primitive cyprinid fishes: implication on phylogenetic analysis. Chin Sci Bull 2002;47(19):1638–43.
[19] Wang X, Liu H, He S, et al. Sequence analysis of cytochrome b gene indicated that East Asian group of cyprinid subfamily Leuciscinae (Teleostei: Cyprinidae) evolved independently. Prog Nat Sci 2004;14(2):132–7.
[20] Thompson JD, Gibson TJ, Plewniak F, et al. The Clustal-X windows interface: flexible strategies for multiple sequences alignment aided by quality analysis tools. Nucleic Acids Res 1997;25(24):4876–82.
[21] Kumar S, Tamura K, Nei M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 2004;5:150–63.
[22] Xia X, Xie Z. DAMBE: data analysis in molecular biology and evolution. J Hered 2001;92:371–3.
[23] Swofford DL. PAUP: phylogenetic analysis using Parsimony (Ver 4). Sinauer Associates, Sunderland, Massachusetts, 1998.
[24] Huelsenbeck JP, Ronquist F. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 2001;17:754–5.
[25] Ronquist F, Huelsenbeck JP. MRBAYES 3: Bayesian phylogenetic inference under mixed model. Bioinformatics 2003;19:1572–4.
[26] Yang Z. PAML: a program package for phylogenetic analysis by maximum likelihood. CABIOS 1997;13:555–6.
[27] Baum DA, Yoon HS, Oldham RL. Molecular evolution of the transcription factor LEAFY in Brassicaceae. Mol Phylogenet Evol 2005;37:1–14.