Aquaculture 310 (2011) 298–304

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Growth hormone gene polymorphisms in tench, *Tinca tinca* L.

Martin Kocour [a,b,*], Klaus Kohlmann [a]

[a] Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 310, 12587 Berlin, Germany
[b] University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zátiší 728/II, 389 25 Vodňany, Czech Republic

ARTICLE INFO

Article history:
Received 27 January 2010
Received in revised form 1 October 2010
Accepted 7 October 2010

Keywords:
Cloning
Growth hormone
Haplotype
Polymorphism
Sequencing
Tench
Tinca tinca

ABSTRACT

Studies of the growth hormone (GH) gene have both fundamental and practical significance, but no data on intra-specific polymorphisms of the tench, *Tinca tinca*, GH gene has been available. In the present study, the complete GH gene was cloned from 17 tench individuals chosen from 10 European and two Asian populations, cultured as well as wild and sequenced from 28 recombinant plasmids (at least one per individual). The tench GH gene was found to be 1758–1763 bp in length. Altogether 14 polymorphic sites were observed, 12 located in introns (SNPs and very short indels) and two in exons (SNPs only). In non-coding regions, the highest polymorphism was found in intron 2 (five SNPs and two indels). Intron 4 was short (137 bp) and monomorphic. In coding regions, one polymorphic site was observed in exon 3 and one in exon 5. The detected SNPs in exons were synonymous, having no effect on the amino acid composition of the GH protein. Thirteen GH gene haplotypes were observed. The tench GH gene haplotypes clustered into two major classes with a mean genetic distance of 0.0037 ± 0.0014 and coefficient of GH gene differentiation of 0.67 ± 0.129 (mean $\pm$ S.E.). The two classes corresponded, based on phylogroup-specific PCR-RFLP, with previously determined two phylogroups, western and eastern. Although the described tench GH gene haplotypes might not include all the existing ones, all common polymorphic sites were identified. Polymorphisms in the tench GH gene, a representative of the five-exon type, are not as extensive as in fishes with the six-exon GH gene. However, GH gene polymorphisms in tench can still serve as suitable markers for population studies and conservation purposes. Investigation of growth performance differences in fish of different GH gene classes and/or haplotypes may be valuable.

1. Introduction

Growth hormone (GH), a single-chain polypeptide of ~22 kDa secreted by somatotrophs, is essential for growth, development, and metabolism in vertebrates. In fish, GH also has a role in seawater adaptation, reproduction, immune function, food conversion, and appetite (Canosa et al., 2007; Rajesh and Majumdar, 2007).

Sequence analysis of the GH gene has both fundamental and practical significance, so the GH gene structure (chromosomal and/or cDNA) was investigated in fishes. Polymorphisms in the GH gene, alone or together with other nuclear and mitochondrial markers, have been used for studying the genetic interrelationships and evolution of fish orders, genera and species (e.g. Bernardi et al., 1993; Marins et al., 2003; Mayden et al., 2009). Identified intra-specific allelic variations in the GH gene might be useful genetic markers for the selection of fish with desirable growth traits. However, observations of polymorphisms within the exon sequences of fish GH genes associated with growth are rare, and the majority of mutations in the GH gene occur within introns (De-Santis and Jerry, 2007). The relationship of GH gene genotypes with growth of fish has been studied, e.g., in Atlantic salmon, *Salmo salar* (Gross and Nilsson, 1999), Arctic char, *Salvelinus alpinus* (Tao and Boulding, 2003), and olive flounder, *Paralichthys olivaceus* (Kang et al., 2002).

The aim of the present study was to detect and characterize GH gene polymorphisms in tench, *Tinca tinca*. A complete sequence of tench GH coding region (mRNA) (GenBank accession number DQ980027) and a partial genomic GH gene sequence (GenBank accession number FJ265043) have been published, but no data on polymorphisms of the GH gene [single nucleotide polymorphisms (SNPs) and/or insertions/deletions (indels)] are available. We focused on tench, as the species has a potential to diversify the fish market and increase from its present production. Tench belongs to the family *Cyprinidae*. It has a small body size, slow growth, and a typical green-coloured body. Tench has been reared in polyculture ponds with common carp, *Cyprinus carpio*, in Central and Eastern European countries, and recently its culture has expanded to China (Wang et al., 2006). Global production of tench was 5044 t in 2007 (FAO, 2009). Reports have described considerable genetic differentiation among tench populations (Kohlmann et al., 2010; Lajbner et al., 2010) and

* Corresponding author. Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 310, 12587 Berlin, Germany. Tel.: +49 30 641 81 637; fax: +49 30 64 181 799.
E-mail address: kocour@vurh.jcu.cz (M. Kocour).

0044-8486/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2010.10.006

differences in growth performance of tench strains (Gela et al., 2010; Kocour et al., 2010). The present study was undertaken in an effort to extend our understanding of the genetic basis for such differences.

2. Material and methods

2.1. Selection of fish samples

A microsatellite-based Neighbour-Joining tree of 21 tench populations (Kohlmann et al., 2010) was used to select 12 representative tench populations for this study (Table 1). This collection was representative of the most distantly related populations, populations from different clusters, and populations of the same cluster. This should have ensured that most common GH gene polymorphisms would be discovered even with the relatively low number of sequenced individuals. The selected populations included 10 originating in European countries and two from Asia. An appropriate DNA collection from tench body tissues (blood, fin, or muscle) was available from the Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany.

2.2. Amplification and cloning of the genomic tench GH gene

Genomic DNA was isolated using the peqGOLD Tissue DNA Mini Kit (Peqlab Biotechnologie). The reverse primer used to amplify the complete genomic tench GH gene (including exons and introns) was taken from the study on tench GH mRNA (GenBank accession number DQ980027), while the forward primer was designed with Primer 3 plus software (Untergasser et al., 2007) using information from GenBank on genomic GH gene sequences of closely related cyprinids (Table 2). The optimized PCR reaction mix was composed of 5 μl of 10× PCR buffer with $(NH_4)_2SO_4$ (MBI-Fermentas), 5 μl of 25 mM $MgCl_2$, 2.5 μl of 1.25 mM dNTPs, 1 μl of each primer (10 pmol/mm^3), 2 μl of 2% BSA, 10 μl genomic DNA, 0.2 μl of *Taq* DNA-polymerase (5 units/μl; MBI-Fermentas), and sterile water to a final volume of 50 μl. Amplification of the GH gene by PCR was done on a Thermocycler (BIOMETRA) with an initial denaturation at 95 °C for 5 min, followed by 5 cycles consisting of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2.5 min, and a further 35 cycles consisting of denaturation at 90 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2.5 min, and a final extension step at 72 °C for 7 min. PCR products (5 μl) were separated by electrophoresis on 1.7% agarose gels (0.5× TBE buffer, staining with Ethidiumbromide) and visualized on the Molecular Imager Gel Doc XR System (Bio-Rad Laboratories). From each of the 12 selected populations, the GH gene of 4–6 randomly chosen individuals was amplified. Before cloning, several PCR products were sequenced on a CEQ 8000 (Beckman Coulter), and obtained sequences were BLASTed in GenBank to confirm GH gene.

After verification, the best one or two (Table 1) amplified PCR products of the GH gene from each population (45 μl) were separated by electrophoresis on 1.7% agarose gels (1× TAE buffer, staining with Ethidiumbromide), cut from the gel, purified using the peqGOLD MicroSpin Gel Extraction Kit (Peqlab Biotechnologie), and stored at +4 °C until cloning. Prior to cloning, 3′A-overhangs were added to the DNA template to increase the success. Cloning was performed using the TOPO TA Cloning® Kit for Sequencing (Invitrogen). Bacterial colonies, grown on Petri dishes with agar, were checked for the presence of the GH gene insert by colony PCR according to instructions in the TOPO TA Cloning® Kit. Subsequently, 1–6 positive bacterial colonies (according to the number of colonies available) from each population were grown in liquid culture overnight (16 h), and plasmids containing the tench GH gene insert were isolated using the peqGOLD Plasmid Miniprep Kit II (Peqlab Biotechnologie).

Table 1
Origin and number of tench samples sequenced to detect GH gene polymorphisms.

Country of origin	Name of population	No. of samples	No. of clones sequenced
China	Chinese	1	1
Czech Republic	Blue	1	3
	Hluboka	1	1
	Tabor	1	3
	Vodnany 96	2	3
Germany	Felchowsee	2	3
	Königswartha	2	3
Hungary	Hungarian	1	1
Italy	Italian	1	3
Romania	Romanian	1	1
Spain	Spanish	2	3
Turkey	Turkish	2	3
Σ		17	28

Table 2
Primers used for PCR amplification and sequencing of the tench GH gene in this study. FP—forward primer; RP—reverse primer; amp.—amplification; seq.—sequencing; pDNA—plasmid DNA; GHG—growth hormone gene; GHGI—growth hormone gene insert.

Primer label	Primer sequences (5′>3′)	Description and utilization
T-F	TACCCTGAGCGAAATGGCTA	FP, amp. of the whole GHG
TGH-R	CTACAGGGTGCArTTdGAATCC	RP, amp. of the whole GHG
T-1R	TGTTGAACACGGATGACTGC	RP, seq. of the 1st pDNA frag. with GHGI
T-2F	TAGTGCTGTTGTCGGTGGTG	FP, seq. of the 2nd pDNA frag. with GHGI
T-2R	GCTCTTCTGTGTTTCATCTTTCC	RP, seq. of the 2nd pDNA frag. with GHGI
T-3F	AGGACAACCTGTTGCCTGAG	FP, seq. of the 3rd pDNA frag. with GHGI
T-3R	CACGCTGATGCCCACTTT	RP, seq. of the 3rd pDNA frag. with GHGI
T-4F	ATCTCTTTCCGCCTCATCG	FP, seq. of the 4th pDNA frag. with GHGI
T3	ATTAACCCTCACTAAAGGGA	FP, colony PCR, seq. of the 1st pDNA frag. with GHGI
T7	TAATACGACTCACTATAGGG	RP, seq. of the 4th pDNA frag. with GHGI
T16	CGCTGCCTTTTGAGGATTTC	FP, re-seq. of the 3′ end of tench GHG
T16-R2	AGCACTCCChAATGAGAAGAAT	RP, re-seq. of the 3′ end of tench GHG

2.3. Sequencing of the plasmid DNA with GH gene insert

One or three tench GH gene inserts (Table 1) in bacterial plasmids were sequenced from each population (depending on the number of plasmids available) on a CEQ 8000 (Beckman Coulter) according to the methodology provided by the manufacturer. Due to the length of the tench GH gene (approx. 1750 bp), it was necessary to design internal primers in order to sequence the complete gene. Primers were designed using information from our own previous sequencing and from tench GH mRNA sequence (GenBank accession number DQ980027) and tench GH partial genomic gene sequence (GenBank accession number FJ265043). Primers were designed to be located in exon areas (providing higher probability of conserved sequence) and to have sufficient overlap with neighbouring fragments for easy restoration of the complete tench GH gene sequence. Finally, eight primer pairs (Table 2) were used to amplify and to sequence the complete GH gene from 28 clones (12 tench populations, with one or three GH gene clones from each). Sequencing was done in both directions (5′–3′ as well as 3′–5′). Due to problems with sequencing of the second fragment, DMSO was added to a final concentration of 5% in the cycle sequencing reaction mix for this fragment.

2.4. Re-sequencing of the 3′ end of tench GH gene

Because the reverse primer (TGH-R) used for amplification of the complete GH gene was degenerated (see Table 2), it was necessary to

re-sequence the 3′ end of the tench GH gene. For the re-sequencing, original genomic DNA samples were taken. Forward primer (T16-F) for the re-sequencing was within the GH gene; a new reverse primer (T16-R2) was downstream of the GH gene. Its sequence was derived from information in GenBank on closely related species (Table 2). The primer T16-R2 was not used for direct amplification and cloning of the GH gene, as PCR with T-F primer did not function well.

2.5. Sequence analysis

Obtained forward and reverse sequence fragments were edited with the CEQ 8000 Genetic Analysis System, Sequence Investigator module. Individual complete GH gene sequences were assembled from the edited fragments using the MEGA4 software (Tamura et al., 2007). Polymorphic sites which appeared only once in the set of the 28 sequences (46 observations) were checked again by sequencing the genomic DNA of the appropriate sample to exclude PCR amplification errors. MEGA4 was also used for calculation of the degree of tench GH gene divergence and for construction of a phylogenetic tree with inclusion of the grass carp, *Ctenopharyngodon idella*, GH gene (GenBank accession no. X60419) as an outgroup. The UPGMA method with bootstrap test (1000 replicates) and the Maximum Composite Likelihood (MCL) model were used for tree construction. NETWORK 4.5.1.6. software (www.fluxus-engineering.com) was used for graphical illustration of a phylogenetic network of observed GH gene haplotypes, also showing mutated positions and missing hypothetical haplotypes. This calculation was done by the Median-joining (MJ) algorithm (Bandelt et al., 1999) with the MP option (Polzin and Daneschmand, 2003).

2.6. Phylogroup-specific PCR-RFLP analysis

Based on the GH gene sequencing results, all 17 tench samples used were additionally examined for polymorphisms in the second intron of the actin gene and the first intron of the RPS7 gene by phylogroup-specific PCR-RFLP, as described by Lajbner et al. (2010). These two markers were found to be suitable to distinguish two distant phylogroups in tench, western and eastern. The aim was to analyse the relationships between both phylogroups and the two GH gene classes identified in the present study. Pearson's chi-square test (STATISTICA 6.0, StatSoft CR s.r.o., Czech Republic) was used to test the differences in allelic distribution of the actin and RPS7 genes in tench having different GH gene classes.

3. Results

The complete GH gene of tench was 1758–1763 bp in length. It was composed of five exons and four introns. Fourteen polymorphic sites (P) were observed, 12 located in introns and two in exons (Fig. 1). The polymorphic sites represented 0.8% (14/1766) of the tench GH gene sequence, of which 0.1% were located in exons and 0.7% were located in introns. In introns, both types of polymorphisms, SNPs as well as short indels, were observed. Intron 1 contained two polymorphic sites (one SNP and one indel), intron 2 had seven polymorphic sites (five SNPs and two indels) and intron 3 contained three polymorphic sites (one SNP and two indels). No polymorphic sites were observed in the relatively short (137 bp) intron 4. Exons displayed only SNPs, one observed in exon 3 and one in exon 5 (Fig. 1). Both detected SNPs were synonymous and had no effect on the amino acid composition of the GH protein.

Among 28 sequenced GH gene clones, 13 sequence types (haplotypes) were found (Table 3), and heterozygous genotypes were often observed. According to the structure of the sequences, two major GH gene classes could be distinguished. The coefficient of GH gene differentiation calculated by MEGA4 software for these two GH gene classes was 0.67 ± 0.129 (mean ± S.E.). The main difference between the GH gene classes could be seen in (1) the presence of an AAA nucleotide motif (P7-nucleotide position 515–517) and nucleotide G (P8-position 817) in class II and their absence in class I, together with (2) SNPs in P3 (nucleotide C in class I and T in class II), P4 (nucleotide C in class I and A in class II), P6 (nucleotide G in class I and A in class II), and P9 (nucleotide C in class I and A in class II) (Table 3, Fig. 2). Division of tench GH genes into two classes was also seen at the UPGMA phylogenetic tree, where haplotypes 1–8 clustered together (bootstrap value 88%) as well as haplotypes 9–13 (bootstrap value 92%) (Fig. 3). The mean genetic distance between the lineages was 0.0037 ± 0.0014. Within lineage distances were 0.0012 and 0.0006 for the GH gene classes I and II, respectively. Overall mean genetic distance was 0.0024 ± 0.0008. All other polymorphisms appeared across both tench GH gene classes, but sometimes showed clear prevalence either in class I or in class II (P1, P10). At some polymorphic sites, polymorphism was observed only within class I (P14) or class II (P5, P12), while in the other class no polymorphism was observed. The SNP at P13 was unusual as it was monomorphic in GH gene class II (nucleotide A only), but polymorphic in class I, having nucleotide T or C which are both transversion mutations to nucleotide A. The polymorphism at P11 combined variable numbers of nucleotide A (0–4 repetitions) and/or a microsatellite motif AT (1, 3 or 4 repetitions) (Table 3). A phylogenetic network (Fig. 2) showed hypothetical haplotypes required to connect the observed sequences within the network with maximum parsimony. These hypothesized haplotypes might have been found if higher numbers of tench samples had been sequenced.

The phylogroup-specific PCR-RFLP analysis of the actin and RPS7 genes revealed that individuals from the Spanish (two), Chinese (one) and Turkish (two) populations possessed alleles of the eastern phylogroup only. GH gene sequences of tench originating from these populations clustered together and possessed patterns of the GH gene class II. The other tench samples carried alleles of both phylogroups of the actin and RPS7 genes. However, individuals carrying a GH gene of class I only (Blue, Felchowsee, Hluboka, Königswartha, Romanian, Tabor) or both GH gene classes (Italian, Vodnany) had significantly higher frequencies of western phylogroup alleles in the actin and RPS7 genes. Conversely, the individual carrying the GH gene class II only (Hungarian) showed a higher frequency of eastern phylogroup alleles in actin and RPS7 genes.

4. Discussion

4.1. Inter-specific GH gene differences

The GH genes of tench and other members of the order *Cypriniformes* are composed of five exons and four introns, while most other fish orders possess a six-exon GH gene (De-Santis and Jerry, 2007). However, the length of the GH gene varies among closely related species. This inter-specific length variation is restricted to introns. For instance, tench had a first intron (I1) 266 or 269 bps in length, compared to an I1 of 239 bp in common carp (Chiou et al., 1990). Conversely, tench I1 was shorter than that in grass carp, *C.*

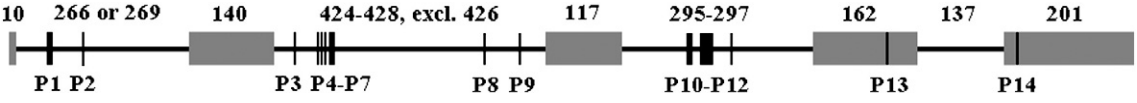

Fig. 1. Schematic diagram of tench GH gene with identified polymorphic sites (P). Grey boxes represent exons; black lines between exons represent introns. Polymorphic sites are indicated below the diagram. Length range (in bps) of individual exons and introns is indicated above the diagram.

Table 3
Summary of polymorphisms observed at individual nucleotide positions of the tench GH gene extracted from Fig. 1. Each row represents a haplotype (1–13). Division into the two GH gene classes is indicated, as well as individual polymorphic sites (P1–P14). Haplotype 1 (H1) was taken as a reference (in bold). (·) indicates identity with the reference haplotype, (–) represents indels.

GH gene haplotype		GenBank accession no.	Nucleotide position																											GH gene class
			75	76	77	125	462	498	506	511	515	516	517	767	817	1084	1085	1086	1097	1098	1099	1100	1101	1102	1103	1104	1150	1393	1596	
			A	**A**	**C**	**C**	**C**	**C**	**C**	**G**	–	–	–	–	**C**	–	–	–	**A**	**T**	**A**	**T**	**A**	**T**	**A**	**T**	**C**	**C**	**C**	I
	H1	GU205383	**A**	**A**	**C**	**C**	**C**	**C**	**C**	**G**	–	–	–	–	**C**	–	–	–	**A**	**T**	**A**	**T**	**A**	**T**	**A**	**T**	**C**	**C**	**C**	I
	H2	GU205385	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	
	H3	GU205387	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	T	·	
	H4	GU205390	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	
	H5	GU205391	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	T	·	
	H6	GU205392	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	T	·	
	H7	GU205394	·	·	·	·	A	·	·	T	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	T	·	
	H8	GU205396	·	·	·	·	A	·	·	T	·	·	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	T	·	
	H9	GU205398	·	·	·	·	A	T	A	·	A	A	A	G	A	·	·	·	·	A	·	·	·	·	·	·	·	A	·	II
	H10	GU205400	–	–	–	·	·	T	A	·	A	A	A	G	A	·	·	A	·	–	·	–	·	–	·	·	·	A	A	
	H11	GU205401	–	–	–	·	·	T	A	·	A	A	A	G	A	C	A	A	·	A	·	–	·	–	·	·	·	A	A	
	H12	GU205404	–	–	–	·	·	T	A	·	A	A	A	G	A	C	A	A	·	–	·	–	·	–	·	·	·	A	A	
	H13	GU205406	–	–	–	·	·	T	A	·	A	A	A	G	A	C	A	A	·	–	·	–	·	–	·	·	·	A	·	
						P1	P2	P3	P4	P5	P6	P7		P8	P9		P10					P11					P12	P13	P14	

idella, with an I1 of 272 bps (Zhu et al., 1992) and silver carp, *Hypophthalmichthys molitrix*, with 270 bps (Hong and Schartl, 1993). A similar situation was found in the second intron (I2) (424–428, excluding 426 bps in tench; 228 bps in common carp; and 471 bps in grass carp and silver carp) and the forth intron (I4) (137 bp in tench, 106 in common carp, 148 in grass carp, and 146 in silver carp). However, the length of the third intron in the tench GH gene (295 to 297 bps) was shorter compared to other carp (400 bps or more).

On the other hand, the length of the protein-coding areas (exons) is often consistent. The length of exons in the tench GH gene was identical to those in common carp (Chiou et al., 1990), grass carp (Zhu et al., 1992), silver carp (Hong and Schartl, 1993), and other species of the family Cyprinidae available in GenBank (E1-10 bp, E2-140 bp, E3-117 bp, E4-162 bp, and E5-201 bp). Generally, length uniformity of exons, with few exceptions, is observed within the group of fish possessing a six-exon GH gene and within the group of fish with a five-exon GH gene (Yowe and Epping, 1996). Despite the length uniformity of GH gene exons, synonymous as well as nonsynonymous nucleotide substitutions have been observed in closely related species, and divergence in exons is used as a tool for phylogenetic studies. (Bernardi et al., 1993; Marins et al., 2003; Mayden et al., 2009).

4.2. Intra-specific GH gene differences

Intra-specific GH gene differences might be useful for population studies, conservation, and breeding of tench. As growth hormone plays an important role in physiological processes connected to growth, finding of nonsynonymous polymorphisms would be of high importance for aquaculture. The amino acid sequence of tench GH observed in this study was monomorphic, since polymorphic sites P13 and P14 in exons were synonymous. An identical amino acid sequence of tench GH protein was found by Mayden et al. (2009). However, protein translated from mRNA (cDNA) (GenBank accession number DQ980027) has a different amino acid at the fifth position (M-methionine instead of L-leucine).

Regarding introns, length and nucleotide sequence variations were found among individuals, which is a common observation in all fishes (De-Santis and Jerry, 2007). Length variation was a characteristic of all introns, excluding the fourth, which was uniform in our study. The highest polymorphism was found in the second intron (P3–P9), representing two indels (three- and one-nucleotide) and five SNPs. In gilthead seabream, extensive size polymorphism was observed in the first (405, 424, 636, and 720 bp) and the third intron resulting from different numbers of minisatellite repeats (Almuly et al., 2000). Microsatellite di-, tri-, or tetra-nucleotide repeats were observed in all introns. Minisatellite and/or microsatellite repeats in introns of the GH gene were also found in *Nibea coibor* (Zhang et al., 2009); Nile tilapia, *Oreochromis niloticus* (Ber and Daniel, 1993); flounder (Tanaka et al., 1995); barramundi, *Lates calcarifer* (Yowe and Epping, 1996); and pufferfish, *Fugu rubripes* (Venkatesh and Brenner, 1997) which are all six-exon species. In our study, no minisatellite repeats were found in the tench GH gene, and only one microsatellite motif in the third intron with polymorphic (AT) repeats (1, 3 or 4) was observed. Similarly, no mini- or microsatellite motifs were detected in sea lamprey, *Petromyzon marinus* (Moriyama et al., 2006). Tench and sea lamprey possesses a five-exon GH gene. Thus, it seems that the exon type of GH gene determines many common characteristics within each group and differences between them.

4.3. Knowledge of tench GH gene polymorphisms—implications for breeding

Reports have indicated that tench may possess different GH proteins. It would be interesting to compare growth performance of these fish. Screening for mutation of tench genomic DNA would be possible with a restriction enzyme as derived from Webcutter (Haiman, 1997). The

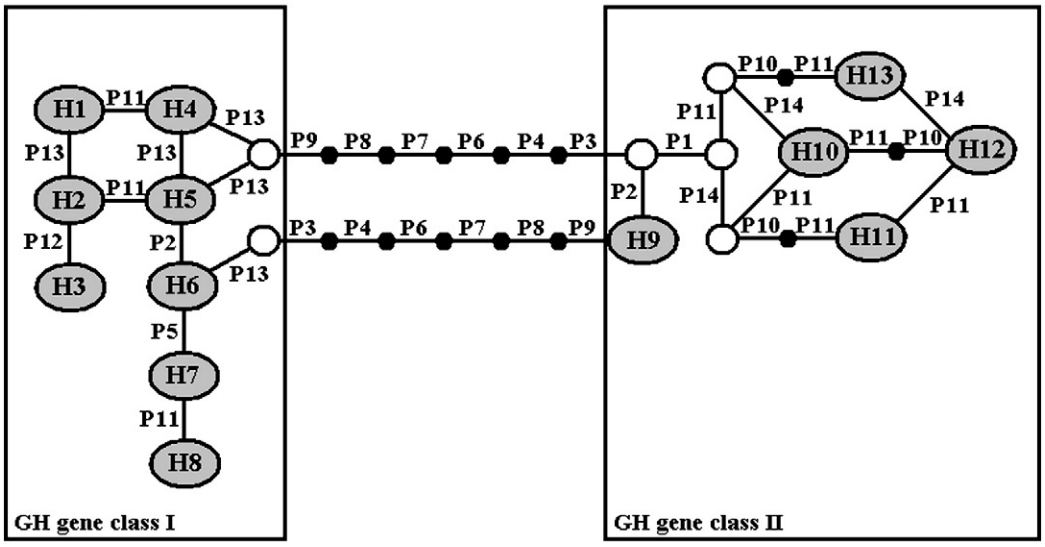

Fig. 2. Phylogenetic network of observed tench GH gene haplotypes. Lines connecting individual haplotypes represent differences in polymorphic sites (mutated positions). Open circles represent hypothesized sequences included by the NETWORK 4.5.1.6 software to connect existing sequences with maximum parsimony.

DNA samples sequenced in this study, from cultured as well as wild tench populations of different geographical origins, had identical protein. Thus, it is likely that there are no, or very few, tench with differing GH protein worldwide. Information on the GH gene mRNA at GenBank (accession number DQ980027) may be inaccurate. If so, screening of tench for this mutation would be pointless.

Recent studies have shown that protein is not the sole factor determining phenotype. Intron regions may play a role in the regulation of gene expression (De-Santis and Jerry, 2007) through microRNA (Ying and Lin, 2006) encoded in introns and/or by influencing the site of exon mRNA splicing (Soller, 2006). Polymorphisms in such introns resulting in different GH gene expression might thus influence growth performance. Hence, growth comparison of tench from the two GH gene classes and different haplotypes might be more useful. Intron polymorphisms associated with growth have been observed in Atlantic salmon (Gross and Nilsson, 1999), olive flounder (Kang et al., 2002) and Nile tilapia (Blanck et al., 2009). Discrimination of tench genotypes into five haplogroups (alleles) is possible when screening for indels at P1 and P7 by fragment length analysis (at a sequencer) and SNPs at P5 and P13 by RFLP (using one and two restriction enzymes at P5 and P13, respectively).

4.4. Knowledge of tench GH gene polymorphisms—implications for conservation and population studies

In this study, 13 haplotypes were found, and others may exist. Precise identification of haplotypes would be possible only by sequencing of the GH gene following cloning, but excessive costs make this impractical. As mentioned above, we found that simple

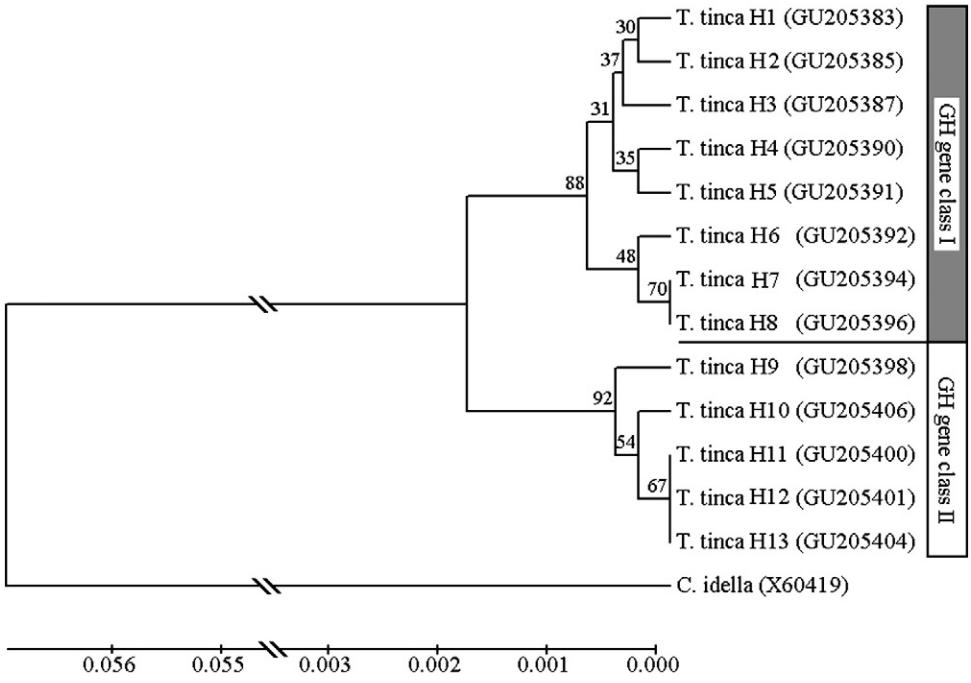

Fig. 3. UPGMA phylogenetic tree of observed tench GH gene haplotypes. GH gene of grass carp (*Ctenopharyngodon idella*) was used as an outgroup. Maximum Composite Likelihood model was applied together with bootstrap test of 1000 replicates. Bootstrap values are indicated.

fragment analysis and enzyme restriction at four polymorphic sites (P1, P5, P7, and P13) of the GH gene may distinguish five haplogroups, three of them from GH gene class I and two from GH gene class II. Screening for polymorphic sites P3, P4, P6, P8, and P9 would not add information, and screening for P10 and P11 is impossible without cloning and sequencing. Polymorphic sites P2, P12, and P14 would increase the number of haplogroups from five to eight, but screening for SNPs would require more complicated methods (e.g. DRMP-PCR, Berger et al., 2004; SNP-SCALE, Hinten et al., 2007). However, determining five haplogroups may be sufficient for population study and conservation when combined with information on other genetic markers. The GH gene can be easily used as a marker for determination of tench phyologroup (lineage) of an individual or stocks. Existence of the two tench lineages in our study is in accordance with conclusions of a microsatellite-based population study (Kohlmann et al., 2010) and a study based on mtDNA and introns of several nuclear genes (Lajbner et al., 2010). The latter authors distinguished two tench phylogroups, eastern and western. Our results indicated that fish of the pure western phylogroup carried GH genes of class I, and fish of the pure eastern phylogroup carried GH genes of class II. Observed mixing of GH gene classes with phylogroups inferred from actin and RPS7 genes within individuals indicates that natural and/or artificial mixing and hybridization of the two tench lineages occurred.

5. Conclusions

The present study contributes to the DNA sequence collection by inclusion of the complete tench GH gene sequence and to the determination of intra-specific GH gene polymorphisms. Sequencing of 28 single copies of the GH gene from 17 individuals across 12 populations from Europe and Asia revealed 14 polymorphic sites and 13 haplotypes. The described haplotypes may not include all existing haplotypes in tench worldwide, but most of the common polymorphic sites were identified. Polymorphisms in coding areas of the sequence were synonymous and had no effect on the composition of the GH protein. Due to the character of polymorphisms, the phylogenetic analysis divided the haplotypes into two major classes which corresponded with previously determined tench phylogroups, western and eastern. Polymorphism at the GH gene in tench, as a representative of the five-exon GH gene, is less extensive compared to fish species with six-exon GH genes. However, GH gene polymorphisms can be effectively used, optimally with other genetic markers, for population studies and conservation purposes. Present results open the possibility for future research on the effect of GH gene haplotypes on growth performance.

Acknowledgements

This study was supported by scholarship from the Humboldt Foundation, Germany (www.humboldt-foundation.de). Thanks also to the CENAKVA project, registration number CZ.1.05/2.1.00/01.0024 and Grant agency of University of South Bohemia no.046/2010/Z.

The authors also thank Petra Kersten from IGB, Berlin for her technical assistance during laboratory work; Juan M. Lara, professor at USAL and researcher of INCYL, Salamanca, Spain for providing us with primer sequences for amplification of tench GH mRNA and Dr. Jana Zrustova from MAFU Brno, Czech Republic for helpful information regarding sequencing of tench GH gene. The Lucidus Consultancy is gratefully acknowledged for English correction.

References

Almuly, R., Cavari, B., Ferstman, H., Kolodny, O., Funkenstein, B., 2000. Genomic structure and sequence of the gilthead seabream (*Sparus aurata*) growth hormone-encoding gene: identification of minisatellite polymorphism in intron I. Genome 43, 836–845.

Bandelt, H.J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16, 37–48.

Ber, R., Daniel, V., 1993. Sequence analysis suggests a recent duplication of the growth hormone-encoding gene in *Tilapia nilotica*. Gene 125, 143–150.

Berger, M., Zschemisch, S., Hocher, B., Mönks, D., Slowinski, T., Halle, H., Wanner, C., Walter, U., Lindner, T.H., 2004. Alternative approach for rapid and reliable single-nucleotide polymorphism typing with double restriction mutagenesis primer PCR. Clin. Chem. 50, 2376–2378.

Bernardi, G., Donofrio, G., Caccio, S., Bernardi, G., 1993. Molecular phylogeny of bony fishes, based on the amino-acid-sequence of the growth-hormone. J. Mol. Evol. 37, 644–649.

Blanck, D.V., Gasparino, E., Ribeiro, R.P., Marques, D.S., 2009. Polymorphism in the GH1-PstI gene associated to corporal characteristics in Nile tilapia strains. Pesqui. Agropecu. Bras. 44, 599–604.

Canosa, L.F., Chang, J.P., Peter, R.E., 2007. Neuroendocrine control of growth hormone in fish. Gen. Comp. Endocr. 151, 1–26.

Chiou, C.S., Chen, H.T., Chang, W.C., 1990. The complete nucleotide sequence of the growth hormone gene from the common carp (*Cyprinus carpio*). Biochim. Biophys. Acta 1087, 91–94.

De-Santis, C., Jerry, D.R., 2007. Candidate growth genes in finfish—where should we be looking? Aquaculture 272, 22–38.

FAO, 2009. Fishery Statistical Collections, Global production. http://www.fao.org/fishery/statistics/global-production/query/en, Accession date: February 10, 2009.

Gela, D., Kocour, M., Flajšhans, M., Rodina, M., Linhart, O., 2010. Comparison of performance of genome manipulated and standard tench, *Tinca tinca* (L.), groups under pond management conditions. Rev. Fish Biol. Fish. 20, 301–306. doi:10.1007/s11160-009-9142-1.

Gross, R., Nilsson, J., 1999. Restriction fragment length polymorphism at the growth hormone 1 gene in Atlantic salmon (*Salmo salar* L.) and its association with weight among the offspring of a hatchery stock. Aquaculture 173, 73–80.

Haiman, M., 1997. Webcutter 2.0. http://bio.lundberg.gu.se/cutter2/1997.

Hinten, G.N., Hale, M.C., Gratten, J., Mossman, J.A., Lowder, B.V., Mann, M.K., Slate, J., 2007. SNP-SCALE: SNP scoring by colour and length exclusion. Mol. Ecol. Notes 7, 377–388.

Hong, Y., Schartl, M., 1993. Sequence of the growth hormone (GH) gene from silver carp (*Hypophthalmichthys molitrix*) and evolution of GH genes in vertebrates. Biochim. Biophys. Acta 1174, 285–288.

Kang, J.H., Lee, S.J., Park, S.R., Ryu, H.Y., 2002. DNA polymorphism in the growth hormone gene and its association with weight in olive flounder *Paralichthys olivaceus*. Fish. Sci. 68, 494–498.

Kocour, M., Gela, D., Rodina, M., Flajšhans, M., 2010. Performance of different tench, *Tinca tinca* (L.), groups under semi-intensive pond conditions: it is worth establishing a coordinated breeding program. Rev. Fish Biol. Fish. 20, 345–355. doi:10.1007/s11160-009-9140-3.

Kohlmann, K., Kersten, P., Panicz, R., Memişc, D., Flajšhans, M., 2010. Genetic variability and differentiation of wild and cultured tench populations inferred from microsatellite loci. Rev. Fish Biol. Fish. 20, 279–288. doi:10.1007/s11160-009-9138-x.

Lajbner, Z., Kohlmann, K., Linhart, O., Kotlík, P., 2010. Lack of reproductive isolation between the Western and Eastern phylogroups of the tench. Rev. Fish Biol. Fish. 20, 289–300. doi:10.1007/s11160-009-9137-y.

Marins, L.F., Levy, J.A., Folch, J.M., Sanchez, A., 2003. A growth hormone-based phylogenetic analysis of euteleostean fishes including a representative species of the Atheriniformes Order, *Odontesthes argentinensis*. Genet. Mol. Biol. 26, 295–300.

Mayden, R.L., Chen, W.J., Bart, H.L., Doosey, M.H., Simons, A.M., Tang, K.L., Wood, R.M., Agnew, M.K., Yang, L., Hirt, M.V., Clements, M.D., Saitoh, K., Sado, T., Miya, M., Nishida, M., 2009. Reconstructing the phylogenetic relationships of the earth's most diverse clade of freshwater fishes order Cypriniformes (Actinopterygii: Ostariophysi): a case study using multiple nuclear loci and the mitochondrial genome. Mol. Phylogenet. Evol. 51, 500–514.

Moriyama, S., Oda, M., Takahashi, A., Sower, S.A., Kawauchi, H., 2006. Genomic structure of the sea lamprey growth hormone-encoding gene. Gen. Comp. Endocr. 148, 33–40.

Polzin, T., Daneschmand, S.V., 2003. On Steiner trees and minimum spanning trees in hypergraphs. Oper. Res. Lett. 31, 12–20.

Rajesh, R., Majumdar, K.C., 2007. A comparative account of the structure of the growth hormone encoding gene and genetic interrelationship in six species of the genus *Labeo*. Fish Physiol. Biochem. 33, 311–333.

Soller, M., 2006. Pre-messenger RNA processing and its regulation: a genomic perspective. Cell. Mol. Life Sci. 63, 796–819.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Tanaka, M., Toma, Y., Ohkubo, T., Sudo, S., Nakashima, K., 1995. Sequence of the flounder (*Paralichthys olivaceus*) growth hormone-encoding gene and its promoter region. Gene 165, 321–322.

Tao, W.J., Boulding, E.G., 2003. Associations between single nucleotide polymorphisms in candidate genes and growth rate in Arctic charr (*Salvelinus alpinus* L.). Heredity 91, 60–69.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., Leunissen, J.A.M., 2007. Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res. 35, W71–W74. doi:10.1093/nar/gkm306.

Venkatesh, B., Brenner, S., 1997. Genomic structure and sequence of the pufferfish (*Fugu rubripes*) growth hormone encoding gene: a comparative analysis of teleost growth hormone genes. Gene 187, 211–215.

Wang, J.X., Min, W.Q., Guan, M., Gong, L.J., Ren, J., Huang, Z., Zheng, H.P., Zhang, P., Liu, H.J., Han, Y.Z., 2006. Tench farming in China: present status and future prospects. Aquac. Int. 14, 205–208.

Ying, S.Y., Lin, S.L., 2006. Current perspectives in intronic micro RNAs (miRNAs). J. Biomed. Sci. 13, 5–15.

Yowe, D.L., Epping, R.J., 1996. A minisatellite polymorphism in intron III of the barramundi (*Lates calcarifer*) growth hormone gene. Genome 5, 934–940.

Zhang, D.C., Shao, Y.Q., Jiang, S.G., Li, J.Z., Xu, X.P., 2009. *Nibea coibor* growth hormone gene: its phylogenetic significance, microsatellite variation and expression analysis. Gen. Comp. Endocr. 163, 233–241.

Zhu, Z., He, L., Chen, T.T., 1992. Primary-structural and evolutionary analysis of the growth-hormone gene from grass carp (*Ctenopharyngodon idellus*). Eur. J. Biochem. 207, 643–648.