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Molecular evolution of growth hormone gene family in old world monkeys and hominoids

Chun Ye[a,1], Ying Li[a,b,c,1], Peng Shi[a], Ya-ping Zhang[a,b,*]

[a]*Laboratory of Cellular and Molecular Evolution, and Molecular Biology of Domestic Animals, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, China*
[b]*Laboratory for Conservation and Utilization of Bio-resources, Yunnan University, Kunming 650091, China*
[c]*The Graduate School, Chinese Academy of Sciences, Beijing 100039, China*

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Abstract

Growth hormone is a classic molecule in the study of the molecular clock hypothesis as it exhibits a relatively constant rate of evolution in most mammalian orders except primates and artiodactyls, where dramatically enhanced rate of evolution (25–50-fold) has been reported. The rapid evolution of primate growth hormone occurred after the divergence of tarsiers and simians, but before the separation of old world monkeys (OWM) from new world monkeys (NWM). Interestingly, this event of rapid sequence evolution coincided with multiple duplications of the growth hormone gene, suggesting gene duplication as a possible cause of the accelerated sequence evolution. Here we determined 21 different GH-like sequences from four species of OWM and hominoids. Combining with published sequences from OWM and hominoids, our analysis demonstrates that multiple gene duplications and several gene conversion events both occurred in the evolutionary history of this gene family in OWM/hominoids. The episode of recent duplications of CSH-like genes in gibbon is accompanied with rapid sequence evolution likely resulting from relaxation of purifying selection. GHN genes in both hominoids and OWM are under strong purifying selection. In contrast, CSH genes in both lineages are probably not. GHV genes in OWM and hominoids evolved at different evolutionary rates and underwent different selective constraints. Our results disclosed the complex history of the primate growth hormone gene family and raised intriguing questions on the consequences of these evolutionary events.

Keywords: Growth hormone; Primates; Gene duplication; Positive selection

1. Introduction

The mammalian pituitary growth hormone (GH) stimulates the growth and metabolism of muscle, bone, and cartilage cells and plays an important role in development. Most mammals only possess one GH gene in their genomes, while humans have five GH-related genes all located in chromosome 17q22–24 (Harper et al., 1982). These genes are the pituitary growth hormone gene (hGHN) and four placentally expressed genes, growth hormone variant (hGHV), chorionic somatomammotropin hormone A and B (hCSHA and hCSHB), and an hCSH-like gene (hCSHL). These five genes collectively make up the human growth hormone (hGH)/chorionic somatomammotropin hormone (hCSH) gene family. Functional members of the hGH/hCSH family have 217 amino acid residues (including signal peptide sequences) encoded by 5 exons, and these sequence

Abbreviations: GH, growth hormone; CSH, chronic somatommamotropin hormone; NWM, new world monkey; OWM, old world monkey; Mya, million years ago.

* Corresponding author. Laboratory of Cellular and Molecular Evolution, and Molecular Biology of Domestic Animals, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, China. Tel.: +86 871 5190761; fax: +86 871 5195430.
E-mail addresses: zhangyp1@263.net.cn, zhangyp@public.km.yn.cn (Y. Zhang).

[1] Those authors contributed equally to this work.

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similarity among the members displays a greater than 90% at DNA level (Chen et al., 1989). Members of the hGH/hCSH family work together to maintain the nutritional balance between the mother and fetus during pregnancy. As a research model of the endocrinology of human pregnancy, the rhesus macaque GH/CSH gene family has also been characterized, which contains five genes, mGHN, mGHV, mCSH1, mCSH2, and mCSH3, but how these genes are organized at the genomic level is not clear yet (Golos et al., 1993). Furthermore, the marmoset, a new world monkey, has been identified to have eight GH-like genes (Wallis et al., 2001; Wallis and Wallis, 2002). While in the bushbaby and slow loris (prosimians), a single copied GH gene was observed (Adkins et al., 2001; Wallis et al., 2001), thus the multiple gene duplications giving rise to a cluster of GH-like genes in higher primate start before the divergence of NWM from OWM/hominoids lineage and after the split of prosimians and simians.

GH sequence is quite conserved in most mammals with only a few amino acid substitutions. In primates, however, the human and rhesus macaque GHs differ from the inferred GH sequence of the common ancestor of eutherian mammals by about 60 amino acids, demonstrating a burst of rapid substitutions during primate evolution (Wallis, 1994). Recent studies further defined the date of the rapid evolution to a time after the divergence of simians and the tarsier but before the separation of new world monkeys (NWM) from old world monkeys (OWM) and hominoids (Adkins et al., 2001; Liu et al., 2001; Wallis et al., 2001). The temporal proximity of gene duplication events and rapid sequence changes has led to the hypothesis that the rapid evolution was due to relaxation of functional constraints or positive selection after gene duplication (Liu et al., 2001). Wallis (1996), however, believed that the rapid evolution predated gene duplication because the majority of primate-specific substitutions are shared among all the loci of higher primates. On the contrary, Liu et al. (2001) argued that the sequence similarity in duplicated genes could be the result of gene conversion.

To study the complex history of the GH/CSH gene family in OWM/hominoids in detail, we sequenced GH/CSH gene family from four species in Catarrhini. Our phylogenetic analysis suggested that the evolutionary mechanism of GH/CSH gene family in OWM/hominoids is consistent with the so-called birth-and-death process. Further more, our results show that different members of this gene family evolved differently, even homologous genes in different lineages evolved at remarkable different rates and underwent different selective constraints.

2. Materials and methods

Genomic DNA was prepared from four species of primates, including three OWMs, the Sub-nosed Golden Monkey (*Rhinopithecus roxellana*), the Douc Langur (*Pygathrix nemaeus*), and the Assamese Macaque (*Macaca assamensis*) and one ape, the White-cheeked Gibbon (*Hylobates leucogenys*). Members of the GH/CSH gene family in these four species were amplified using the following primers designed by Wallis et al. (2001): GH sense: 5′-TGGCTATCCTGACATCCTTTCCCGC-3′ and GH antisense: 5′-CCACCCCATAATATTAGAGAAGGACAC-3′. The pair of primers spans the full-length GH gene (including 5 exons and 4 introns) and some regulatory elements. In order to reduce the errors due to PCR recombination and mutation, we performed three times of independent PCR with one time using *pfu* polymerase (Takara, China) and the other two using taq polymerase (Takara, China). The PCR products obtained by using *taq* polymerase were cloned into PMD18-T vector (Takara, China) and transformed into an ultracompetent *E. coli* cell (Takara China). Those performed by using *pfu* polymerase were cloned into PCR-Script Amp Cloning Kit, according to the manufacturer's protocol (Stratagene, La Jolla, CA.), and transformed into the ultracompetent *E. coli* cell (Takara). Plasmids carrying PCR fragment were extracted and sequenced in both directions. For each PCR product, 15–40 clones were sequenced using ABI 3700 DNA sequencer (PE Biosystems, USA). Those sequences identified by at least two times of independent PCR were taken into consideration. Moreover, any single nucleotide variant that occurred uniquely in single clone was assumed to be PCR error and changed to match the consensus nucleotide found in other clones at that site. Thus, most nucleotide variants and recombination due to PCR error were removed from consideration.

Sequences were aligned using CLUSTAL X program (Jeanmougin et al., 1998) followed by manual adjustments. Other OWM and hominoid GH/CSH gene sequences retrieved from GenBank were also used in the analysis (Table 1). Kimura's (K80) (Kimura, 1980) model was used to infer neighbor-joining (NJ) (Saitou and Nei, 1987) tree implemented in the program MEGA 2.1 (Kumar et al., 2001). Then 1000 replications of bootstrap analyses were done. Statistical tests for gene conversion were performed using the program GENECONV 1.81 (Sawyer, 2000) implementing Sawyer's (1989) methods, with the full-length sequences as input data. Analyses were repeated where mismatches were either not allowed, or allowed but given a relative penalty of 1, 2, or 3. Ancestor sequences of all interior nodes of the GH gene tree were inferred by the distance-based Bayesian methods (Zhang and Nei, 1997). Numbers of synonymous substitutions (s), numbers of nonsynonymous substitutions (n), potential numbers of synonymous sites (S), potential numbers of nonsynonymous sites (N), pairwise nonsynonymous substitutions per nonsynonymous site (d_N), and synonymous substitutions per synonymous site (d_S) are all calculated by modified N-G method (Zhang et al., 1998) implemented in MEGA 2.1(Kumar et al., 2001).

3. Results

3.1. Sequences obtained

Twenty-one GH/CSH-like sequences were identified from the four species mentioned above by at least two independent PCR. Table 1 shows their GenBank accession numbers. The alignment of the full-length nucleotide sequences of these genes is shown in Appendix A. We revealed a 1-base deletion in sequence P.nem3 at site 783 of the alignment file (Appendix A), a 1-base deletion (site 1396) and a nonsense mutation (1422–1424) in M.ass3, these mutations (shaded in pink in Appendix A) may lead to changing of open reading frame and beforehand terminating, which indicate that the two sequences are probably pseudogenes. The alignment file also shows that the signal peptide region of the gibbon GH/CSH gene family presents unusual patterns. In the H.leu1 sequence, the first three codons are absent (including the starting codon), and no other starting codon was observed in the signal region or in the promoter region nearby, which indicates that this gene is probably a pseudogene. In addition, the starting codon ATG is replaced by AAG and an ATG appears at residue 7 in both the H.leu4 and H.leu5 sequence. Mutations in the signal peptide may affect secretion of protein from the cell, which may result in diseases (Zschenker et al., 2001). Although it is unclear whether these differences in the signal peptide would render the proteins nonfunctional, our analyses of the substitution pattern of the H.leu4 and H.leu5 genes do not show characteristics of pseudogenes, such as greater transition mutations rate than transversion rate and higher A and T frequency than C and G. (Gojobori et al., 1982; Li et al., 1984) (data not shown). We thus tentatively take them as functional genes.

3.2. Phylogenetic analysis

We constructed the phylogenetic tree of all available cDNA sequences of GH/CSH genes from OWM/hominoids (Table 1) based on NJ method by using four prosimians GH sequences as outgroups (Fig. 1). Furthermore, all available intronic sequences and full-length (include 5 exons and 4 introns) sequences are used to construct phylogenetic tree by using NJ and MP methods, and they all show similar topology when branches with low bootstrap support are collapsed (results are available upon request). NWM GH gene families were not used to reconstruct the phylogeny for the following reasons: (1) Independent gene duplication has been inferred to be a possible reason of high sequence similarity among different members of GH-like genes in marmoset (Wallis and Wallis, 2002). (2) Using relatively remote species as outgroup may decrease the affection of sampling errors, Zhang et al. (2002) use mice rather than primates as outgroup when comparing evolution rates of some genes from human and chimpanzee for this reason.

As Fig. 1 show, all sequences obtained in this study clustered with previously reported GHN, GHV, or CSH genes. However, some unexpected features are presented in the tree. Three CSH genes were identified in macaque placental, and the relationships of these genes were similar to human CSH genes. Thus, macaque CSH genes were considered to be homology to those of human (Golos et al., 1993). However, macaque CSH1, CSH2, and CSH3 do not cluster close to corresponding genes in human, instead they clustered by lineage-specific manner, all CSH genes from OWM and hominoids clustered together and formed two distinct clades with high bootstrap supports (83% and 99%, respectively). As for GHN and GHV genes, although the relationships of these two genes are unclear in the present

Table 1
Sequences used in this study

Gene	Accession no.	Gene	Accession no.
Human GH/CSH cluster	J03071	H.leu4 (Gibbon ghlp4)	AY621638
Chimpanzee GHN	AF374232	H.leu5 (Gibbon ghlp5)	AY621639
Chimpanzee GHV	AF374233	H.leu6 (Gibbon ghlp6)	AY621640
Chimpanzee PL-A	AY146625	H.leu7 (Gibbon ghlp7)	AY621641
Chimpanzee PL-B	AY146626	R.rox1 (Gold monkey ghlp1)	AY621642
Chimpanzee PL-C	AY146627	R.rox2 (Gold monkey ghlp2)	AY621643
Chimpanzee PL-D	AY146628	R.rox3 (Gold monkey ghlp3)	AY621644
Macaque GHN	L16556	R.rox4 (Gold monkey ghlp4)	AY621645
Macaque GHV	L16555	P.nem1 (Langur ghlp1)	AY621646
Macaque CS1	L16552	P.nem2 (Langur ghlp2)	AY621647
Macaque CS2	L16553	P.nem3 (Langur ghlp3)	AY621648
Macaque CS3	L16554	P.nem4 (Langur ghlp4)	AY621649
Western tarsier GH	AF339081	P.nem5 (Langur ghlp5)	AY621650
Philippine tarsier GH	AF339080	M.ass1 (Assamese Macaque ghlp1)	AY621651
Slow Loris GH	AJ297562	M.ass2 (Assamese Macaque ghlp2)	AY621652
Bush baby GH	AF292938	M.ass3 (Assamese Macaque ghlp3)	AY621653
H.leu1 (Gibbon ghlp1)	AY621635	M.ass4 (Assamese Macaque ghlp4)	AY621654
H.leu2 (Gibbon ghlp2)	AY621636	M.ass5 (Assamese Macaque ghlp5)	AY621655
H.leu3 (Gibbon ghlp3)	AY621637		

PL represents placental lactogen; ghlp represents growth hormone like protein.

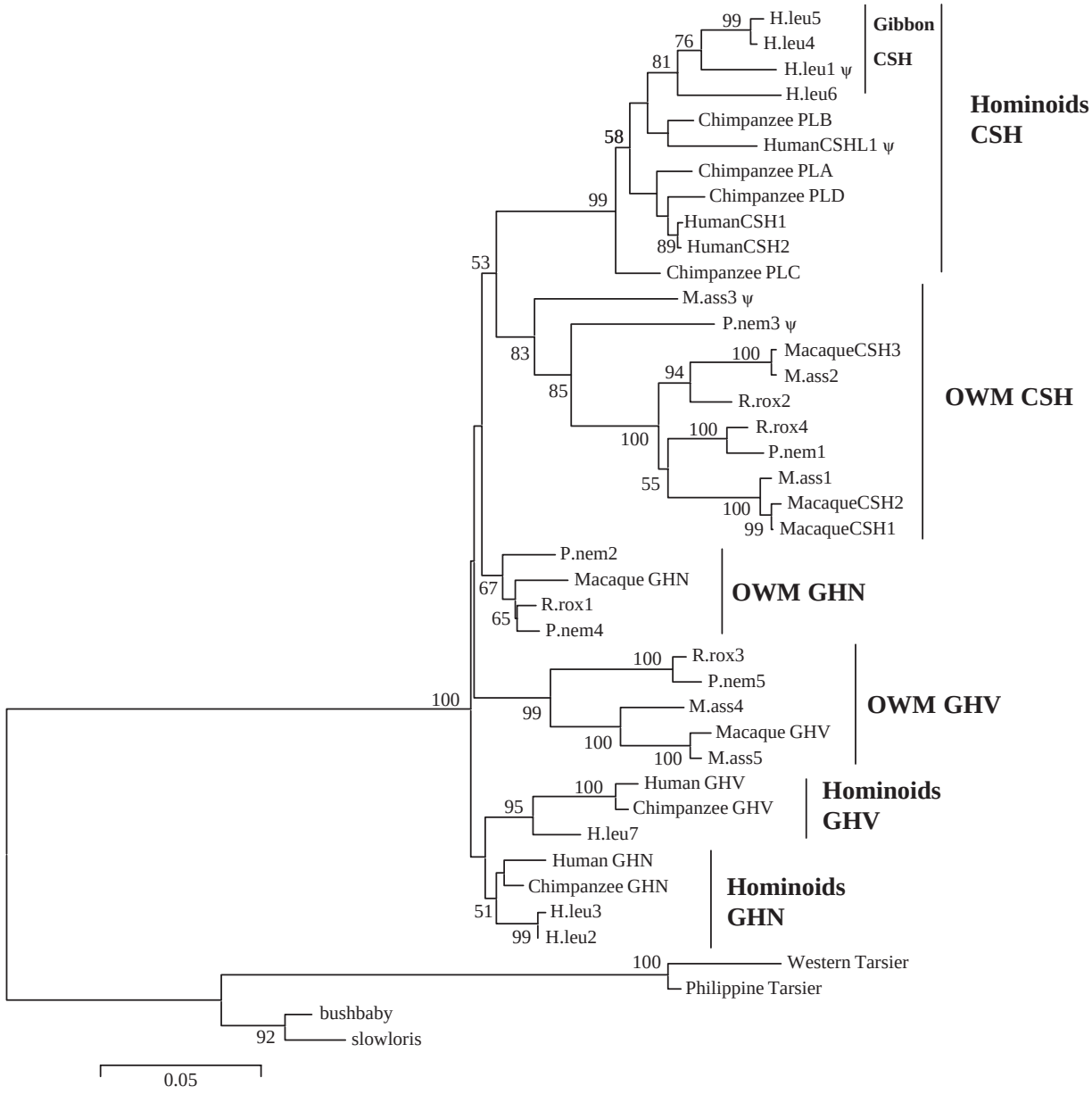

Fig. 1. Neighbor-joining tree based on coding sequence of GH/CSH genes from OWM/hominoids. We used four prosimians GH sequences (Bushbaby, Slow Loris, Western tarsier, and Philippine tarsier) as outgroups to locate the root of this tree. Kimura 2-parameter distances were used. Bootstrap percentages (> 50) by 1000 replications are shown. The sequences identified by our research were designated by the first one letter of the genus name and the first three letters of the species name added the clones' number. "ψ" represents pseudogene.

tree, it appears that OWM GHN genes are not very closely related to hominoids GHN as expected. A similar pattern is observed for GHV gene. It is reasonable to consider that new gene duplication frequently occurred after the split of hominoids and OWM. Furthermore, species-specific gene duplication seems to have occurred in this gene family. For example, four gibbon CSH-like genes clustered together with 81% bootstrap support. But other genes of this species are closely related to human and chimpanzee GHN or GHV genes, respectively, which suggested that recent duplication of the gibbon CSH genes occurred after the divergence between gibbon and other great apes. While, other evolutionary forces may lead to great similarity among paralogous genes (see below).

3.3. Tests for gene conversion in GH/CSH gene family

Concerted evolution always changes the record of molecular divergence during the evolution of paralogous sequences. Gene conversion is considered to be the most important mechanisms responsible for the occurrence of concerted evolution. It has also been reported as an important force in the evolution of GH gene family in OWM/hominoid (Hirt et al., 1987; Chen et al., 1989;

Table 2
Tests for gene conversion of GH/CSH gene family in OWM/hominoids

Sequence name	BC Sim	BC KA	Aligned			Num poly	Num dif	Tot difs
	P value	*P* value	Begin	End	Len			
P.nem2; P.nem4	0.0047	0.00001	378	1088	711	311	0	39
P.nem2; P.nem3	0.0047	0.00004	1158	1550	393	132	0	84
H.leu4; H.leu5	0.0047	0.04600	1	616	616	237	24	70

Note: Aligned "begin" and "end" are the estimated boundaries of the sequence fragment affected by gene conversion; Num poly is the number of polymorphic sites shared by the two sequences in the inferred conversion region; Num dif is the number of differences in the converted region; Tot difs is the number of total differences between the two compared sequences.

Giordano et al., 1997; Krawczak et al., 1999; Horan et al., 2003; Mendoza et al., 2004). Therefore, it is necessary to investigate gene conversion in those sequences obtained by our study. Two gene conversion events are evident when inspecting the full-length nucleotide sequences of all 21 genes from our study (Appendix A). The first gene conversion event took place at aligned nucleotide sequences from site 1158 to 1550 (include partial intron 4 and exon 5) between P.nem2 and P.nem3 (shaded in red in Appendix A), the P.nem 3 being the donor and the P.nem2 the acceptor. The other conversion event occurred at aligned site from 378 to site 1088 (include partial exon 2, entire intron 2, entire exon 3 and partial intron 3). Because the P.nem4 possesses characteristics of OWM GHN gene, we suppose that, in this gene conversion event, P.nem4 is the donor and the P.nem2 is the acceptor (shaded in green in Appendix A). Furthermore, a recent review (Drouin et al., 1999) found that the statistical method of Sawyer (1989) gives the most consistent results in detecting gene conversion events. Therefore, we employed the program Geneconv 1.81 based on this method for our data with sequences from the same species being defined as a group. The fragment pair with global permutation *P* values smaller than 0.05 was considered as being infected by gene conversion. Using four mismatch penalties (gscale=0, 1, 2, 3), statistically likely cases of gene conversion under any mismatch penalties were listed in Table 2. In addition to the two gene conversion events identified above, H.leu4 and H.leu5 are also inferred to subject to gene conversion by this method (shaded in yellow in Appendix A).

These gene conversion events identified here should not be artificial recombination by PCR errors, for the reason that all sequences determined by our study are obtained by at least two independent PCR. Previous studies have also identified several gene conversion events in GH gene family, such as the human CSHA vs. CSHB (Hirt et al., 1987), chimpanzee PLA vs. GHN and chimpanzee PLC vs. GHV (Mendoza et al., 2004). Therefore, gene conversion is an important evolutionary force in this gene family. However, gene conversion seems not the only force in the origin and diversification of GH/CSH gene family in OWM/hominoids. For example, for gibbon CSH genes, only one gene conversion event is detected within a short fragment (one third of the whole gene). It could not explain that all four CSH-like genes formed a single cluster on the phylogenetic tree (Fig. 1). So recent gene duplications might be an alternative and more reasonable explanation.

3.4. Evolutionary rates of different genes among different lineages

Our above analyses revealed very complex and unusual phylogenetic relationships among different members in different lineages. Thus, we are interested in the evolutionary rates of these genes after duplications. For hominoids lineage, the evolutionary rates for GHN, GHV, and CSH genes are estimated by averaging the rates of orthologous genes between chimpanzee vs. human and gibbon vs. other apes. And for OWM lineage, the evolutionary rates for the three genes are estimated by averaged rates of orthologous genes between golden monkey/Douc Langur, and macaque. The divergence times between those species used here are based on Goodman et al. (Goodman et al., 1998; Goodman, 1999) which suppose that chimpanzee and human diverged at 6 million years ago (Mya), gibbons diverged form other apes at 18 Mya, and Colobini separated from Cercopithecini at 14 Mya. Using those divergence times and the amino acids P-distance (using other distances, such as poisson-corrected or gamma model distance will slightly change the values of those rates but not change the main results), we calculated the amino acids substitution rates

Table 3
Evolutionary rates for members of GH/CSH gene family in hominoids and OWM lineages

Gene	AA rate ($x10^{-9}$) (AA substitutions/site/year)	*R*	d_N	d_S	d_N/d_S
O-GHN	0.66±0.000293	4.38	0.016±0.004	0.048±0.010	0.33
H-GHN	0.64±0.000246	1.79	0.011±0.004	0.054±0.013	0.20
O-GHV	6.14±0.000804	1.52	0.064±0.009	0.054±0.012	1.18
H-GHV	1.40±0.000349	2.17	0.026±0.006	0.046±0.013	0.57
O-CSH	3.31±0.000475	0.99	0.049±0.007	0.043±0.011	1.14
H-CSH	2.33±0.000883	1.56	0.045±0.007	0.051±0.010	0.92
G-CSH	2.70±0.000536	2.21	0.035±0.007	0.028±0.010	1.23

Note: O—OWM; H—hominoids; G—gibbon; R—the ratios of transition substitutions and transversion substitutions; AA—amino acid.

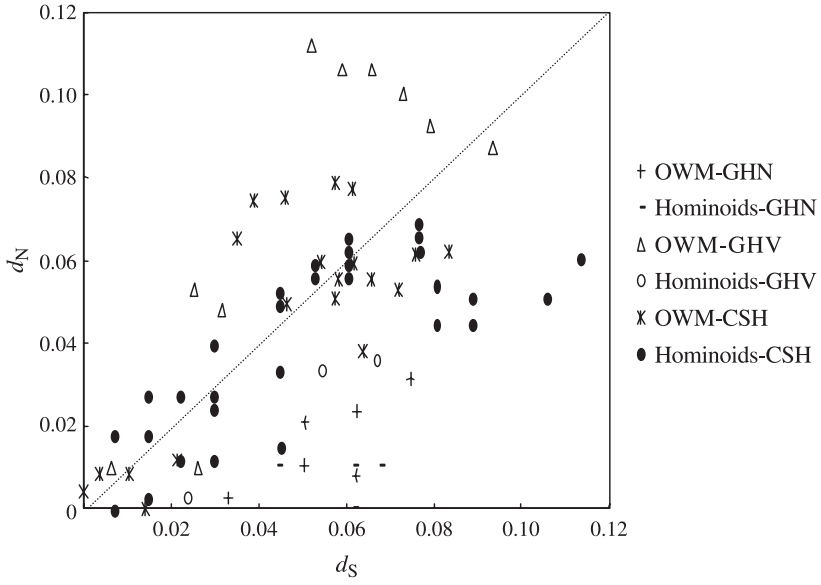

Fig. 2. Pairwise synonymous and nonsynonymous distance of different members of GH gene family among OWM and hominoids lineages.

for GHN, GHV, and CSH genes separately in OWM and hominoid (Table 3). Interestingly, different genes show different evolutionary rates. GHN gene in both OWM and hominoid evolved very slowly. The evolutionary rates for CSH genes in both lineages are significantly higher than those of GHN genes ($Z=4.74$, $P<0.001$ for OWM CSH compared to OWM GHN and $Z=1.81$, $P=0.03$ for hominoids CSH to hominoids GHN by one-tailed Z test). As gibbon CSH-like genes were possibly originated from independent gene duplication, we calculated the evolutionary rate among these newly duplicated genes. We used the divergence time of 18 Mya between gibbon and other great apes as the upper limit time of the duplications in gibbon CSH genes, because the duplication of these genes apparently occurred after the separation between gibbon and other great apes as mentioned above. Using such upper limit time and the amino acids P-distance between H.leu 4/H.leu 5 and H.leu 6, we estimated that the low limit rate of gibbon CSH-like gene is 2.7×10^{-9} substitutions/amino acid site/year (Table 3), which is slightly higher than the rate of hominoids CSH, but the difference is not statistically significant ($Z=0.39$, $P>0.05$ by one-tailed Z test). While, this rate of gibbon CSH genes is likely under estimated. For GHV genes, dramatically different evolutionary rates are observed in OWM and hominoids. The amino acid substitution rate for OWM GHV gene is 6.14×10^{-9} substitutions/amino acid site/year, which is significantly higher than that of hominoids GHV gene (1.40×10^{-9} substitutions/amino acid site/year) ($Z=5.40$, $P<0.001$ by one-tailed Z test). In short, our results suggested rapid evolution of CSH genes compared to GHN genes, and different rates of GHV genes in different lineages.

3.5. Different selective constraints act on different members of GH/CSH gene family in OWM/hominoid

Comparing synonymous and nonsynonymous substitution rate can separate the role of adaptive selection from neutral selection and purifying selection, as synonymous mutations are largely immune to selection. We tested the hypothesis of neutral evolution by computing the synonymous (d_S) and nonsynonymous (d_N) nucleotide substitutions per site for GHN-like genes, GHV-like genes and CSH-like genes (Fig. 1) separately in OWM and hominoids lineages. The average d_N and d_S values for each group of genes are shown in Table 3.

For GHN-like genes, all comparisons show d_N lower than d_S in both lineages (Fig. 2), the average d_N/d_S ratios are 0.20 and 0.33 for hominoids and OWM lineages, respectively, they are significantly lower than 1 ($Z=2.97$, $P<0.01$ and $Z=3.16$, $P<0.001$ by one-tailed Z test for hominoids and OWM lineages), which suggested that purifying selection acted on GHN-like genes in OWM/hominoids. As for CSH-like genes, most d_N/d_S ratios are greater than or close to 1 for both lineages (Fig. 2). The average d_N/d_S are 0.92 and 1.14 for hominoids and OWM lineages, respectively, but the average d_N is not significantly different from the average d_S values for both lineages ($Z=0.49$ and 0.46 for hominoids and OWM by one-tailed Z test). This suggests that positive selection or relaxation of purifying selection may be acting during the fast evolution of CSH genes in both lineages. As for GHV genes, the selective constraints imposed on hominoids and OWM lineages seem different. In hominoids lineage, all comparisons show $d_N<d_S$, but the average d_N is not significantly lower than d_S value ($Z=1.40$, $P=0.08$ by one-tailed Z test).

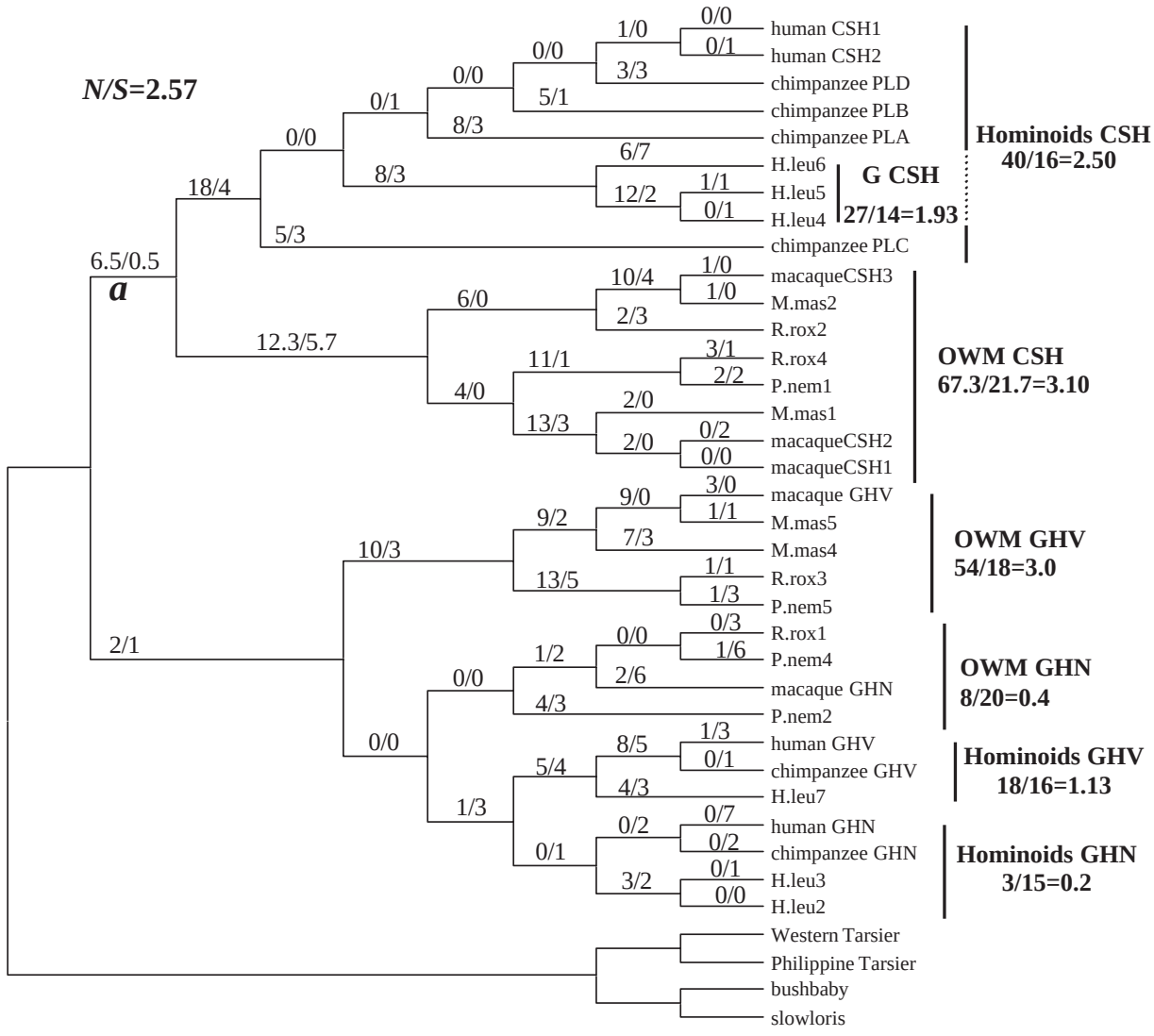

Fig. 3. Neighbor-joining tree based on coding sequence of putative functional GH/CSH genes from OWM/hominoids. Kimura 2-parameter distances were used. The numbers of nonsynonymous (n) and synonymous substitutions (s) are shown above the branches. Total numbers of n and s for each group of genes are shown below the group names. "G" represents gibbon.

In OWM lineage, most of comparisons reveal greater d_N than d_S, and the average d_N/d_S is 1.18, but this difference is still not significant (Z=0.67, P=0.25 by one-tailed Z test). Thus, whether the rapid evolutions seen in CSH gene and OWM GHV gene resulted from positive selection or relaxed functional constraint is unclear.

Taking the evolutionary features into account, we further test the evolutionary forces imposed on GH/CSH gene family using the phylogeny-based branch specific test (Zhang et al., 1998). We first inferred the ancestor GH sequences for every interior node by implementing the program Ancestor (Zhang and Nei, 1997) with NJ tree reconstructed by all putative functional cDNA sequences as user defined tree (Fig. 3). Our ancestor GH sequence estimates are reliable with the average posterior probabilities for all nodes being over 99%. Then we calculated the numbers of synonymous (s) and non-synonymous (n) substitutions for each branch and marked them onto the gene tree (Fig. 3). Considering that the orthologous genes of GHN, GHV, and CSH genes in OWM and hominoids are not clustered together, we counted the sum of n and s for all branches of the three members separately in hominoids and OWM lineages. And because gibbon CSH genes are probably derived from independent gene duplication event, we also counted the total s and n values for gibbon CSH genes. When calculating the sum n and s values for hominoids CSH gene, we do not include those occurred in gibbon (Fig. 3). With estimation of the average potential synonymous substitution sites (S) and nonsynonymous sites (N), we can test the neutral evolution hypothesis (Zhang et al., 1998). In the present case, n/s for GHN gene in hominoids and OWM are 0.2 and 0.4, respectively, which are significantly lower than N/S=350/136=2.57 (P<0.001 for both lineages by Fisher's Exact Test), suggesting the action of purifying selection for GHN

gene. The n/s values for gibbon CSH genes (1.93) and other hominoids CSH genes (2.50) are near to the average N/S, and the differences are not statistically significant (P=0.23 and 0.51, respectively by Fisher's Exact Test), suggesting possible action of near neutral evolution. However, these n/s values for CSH genes are significantly greater than those in hominoids GHN genes (P<0.001 for both comparisons by Fisher's Exact Test), suggesting different selective constraints for CSH and GHN genes in hominoids. For OWM CSH genes, the sum n/s=3.10 is greater than N/S, but the difference is not significant (P=0.27 by Fisher's Exact Test), providing no evidence of positive selection. While, this value is significantly higher than that of OWM GHN gene (P<0.001 by Fisher's Exact Test) suggesting variable selective constraints for CSH and GHN genes in OWM. For hominoids GHV genes, n/s (1.13) is significantly lower than the N/S value (P=0.01 by Fisher's exact test), supporting purifying selection as predominant evolutionary forces for hominoids GHV genes. While, for OWM GHV genes, n/s=3.0 is greater than the N/S, but the difference is not significant (P=0.32 by Fisher's Exact Test). However, the total n/s for GHV gene in OWM is significantly higher than that in hominoids (P=0.021, Fisher's Exact Test), suggesting different selective pressures imposed on different lineages as we have shown above. Furthermore, we also test the neutral selection hypothesis on branch a (Fig. 3), which leads to the entire CSH gene. As a result, the n/s=6.5/0.5=13 is higher than the N/S=2.57, but the difference is not significant (P=0.19 by Fisher's Exact Test). Whether positive selection or relaxed functional constraint acted on this branch is still uncertain.

4. Discussion

In the present study, we got totally 21 different GH-like sequences from four species of suborder Anthropoidea. Our further analysis revealed a complex and interesting evolutionary history of GH gene family in this lineage, in which gene duplication, gene deletion, and gene conversion all occurred. Phylogenetic analysis combining with other GH-like genes from GenBank revealed an erratic evolutionary pattern. Even though all CSH-like genes cluster together, CSH-like genes from OWM and hominoids formed separate clades with high bootstrap support. Furthermore, all the four gibbon CSH-like genes gathered together with high bootstrap support. Several gene conversion events in OWM/hominoids GH gene family are identified in our study and previous researches (Hirt et al., 1987; Mendoza et al., 2004). It seems that gene conversion is an important force in the evolution of CSH-like genes. However, it apparently could not explain the total evolutionary story of GH in OWM/hominoid. Multiple gene duplications must have occurred in the evolutionary history of CSH gene in OWM/hominoids. Similar to CSH genes, both GHN and GHV genes could be divided into OWM and hominoids clades, respectively (Fig. 1). However, we could not resolve the relationships of those four clades of GHN and GHV genes, as the bootstrap values in our tree are quite low. It should be noted that neither GHN nor GHV genes formed a well-supported monophyletic clade, respectively. It appears that the evolutionary pattern of this gene family in OWM/hominoids is in agreement with the so-called birth-and-death processes, which were envisaged for MHC, buiquitin, immunoglobulin, and histone H4 genes (Ota and Nei, 1994; Nei et al., 1997; Nei et al., 2000; Piontkivska et al., 2002). This model of evolution is characterized by frequent gene duplication; some of the duplicated genes are preserved as functional genes and others become pseudogenes or lost from genome (Nei et al., 1997). Existence of four potential pseudogenes further supports that this gene family in OWM/hominoids evolved by birth-and-death process.

Wallis (1997) proposed a "function switch mechanism": in a certain period of evolutionary time, the GH gene had to play a second role, lactogenic function, besides its main function in growth promotion. Fluctuations in the importance of this second role may result in many adaptive substitutions (Wallis, 1997). In our analysis, we did not find direct evidence of positive selection for branch a (Fig. 3), which represents emergence of the newly lactogen-like function, using the conservative method of Zhang et al. (1998). While, the possibility that CSH gene is subject to weak positive selection still cannot be excluded based on the present analysis. Moreover, researches on hormone function show that hGHV and hGHN have two bioactivities, somatogen and lactogen (Macleod et al., 1991). It is possible that this ancestral gene expressed both in the pituitary and placenta, which means that placental expression, had occurred before gene duplication. So the genes with new function in primate seem to have evolved in the process as suggested by Wallis (1997).

Our result revealed an interesting evolutionary phenomenon that different members of this gene family evolved under different rates and selective constraints. CSH genes evolved faster than GHN genes in both hominoids and OWM lineages. Our analysis based on d_N/d_S values and branch specific test of Zhang et al. (1998) suggested that GHN genes in both hominoids and OWM are under strong purifying selection. Our result also suggests significantly different selective constraints imposed on GHN and CSH genes, but whether the rapid evolution of CSH gene is caused by positive selection or relaxation of purifying selection is uncertain based on the present study. Most interestingly, GHV genes evolved at considerable different rates in hominoids and OWM lineages. Very conserved evolution by purifying selection was observed in hominoids, but not in OWM, which suggests the possible function difference of GHV genes in OWM and hominoids. Coincidentally, our phylogenetic tree shows that the macaque GHV may not correspond exactly to that in human, as they did not form a well-supported monophyletic clade. On the other hand, both human and macaque GHV genes are expressed in placenta

(Chen et al., 1989; Golos et al., 1993). Thus, what make such difference of GHV genes in OWM and hominoids lineages is unclear. Further studies of other species in OWM/hominoids with functional analysis and genome information are required.

Gene evolution is often accelerated following gene duplication, although it is not always clear whether positive selection or relaxation of purifying selection played a major role (Li, 1985; Zhang, 2003). Our study revealed independent gene duplication and rapid evolution of the gibbon CSH-like genes. Further analysis shows no evidence for positive selection among gibbon CSH genes. Considering the evidence of rapid evolution, and the similar d_N and d_S values and pseudogenizition of H.leu1 gene, we reckon that relaxation of purifying selection is the major force for the rapid evolution of gibbon CSH genes. Without more analyses of structure, function, and expression of the gibbon CSH-like gene, we are unable to tell whether these duplicated genes are differentially used in time and space. Interestingly, this species-specific gene duplication has also been reported in other genes such as bitter taste receptor (T2R) gene (Shi et al., 2003), suggesting that this evolutionary pattern may be more prevalent than expected. Although the biological significance of the new duplications in gibbon needs further investigation, the very recent duplications and the following fast evolution of the CSH-like genes in gibbon raised the question whether this unusual independent gene duplication is gibbon-specific or it is a common phenomenon during the evolutionary history of GH gene family in primate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2005.03.003.

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