RESEARCH NOTE

Purifying selection on leptin genes in teleosts may be due to poikilothermy

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[Wang S., Wang R. and Xu T. 2014 Purifying selection on leptin genes in teleosts may be due to poikilothermy. *J. Genet* **93**, 551–556]

Introduction

Leptin is a key harmone in regulating food intake, immune function and energy metabolism in vertebrates. Since it was first cloned in mouse (Zhang *et al*. 1994) , the leptin gene has received much attention and people started to research its function in energy homoeostasis and reproductive regulation (Campfiled *et al*. 1995). A large number of researchers have discovered that leptin is present in several fishes and it plays a similar role in regulation of food intake and energy metabolism in fishes as in mammals (Copeland *et al*. 2011). Although evidence of positive selection on the leptin gene has been documented in primates, seals and chiropterans (Yu *et al*. 2011), studies on evolution of leptin gene in fishes are fewer.

Leptin, the product of the *ob* gene, was discovered in 1950 (Coleman 2010). The discovery was followed by intense research into the properties and physiological action of leptin. In initial studies, people considered that the primary functions of leptin were its influence on metabolic rate and mobilization of fat stores (Minokoshi and Kahn 2003). With further research, we now know that leptin is pleiotropic, exerting effects on reproduction, immune function, capillary growth and bone remodelling (Friedman 2009). In mammals, leptin acts on the hypothalamus which sends further signals to the brain, leading the brain to regulate food intake and metabolism (Pelleymounter *et al*. 1995). To our knowledge, mutations in the leptin genes were associated with a large number of hormonal and metabolic alterations (Kennedy 1953). Deficiency of leptin can cause obesity and diabetes in humans and mice (Strobel *et al*. 1998). Leptin genes of some fishes have been cloned and people have started to study their structure and biological functions of the protein (Murashita *et al*. 2008; Aguilar *et al*. 2010). Although leptin genes of several several species of teleosts have been cloned (Kurokawa *et al*. 2005; Huising *et al*. 2006; Ronnestad *et al*. 2010), physiological function of the protein and molecular evolution of the leptin gene in teleosts have been poorly studied (Murashita *et al*. 2008; Aguilar *et al*. 2010).

The evolution of leptin genes has been studied in seals (*Halichoerus grypus* and *Leptonychotes weddellii*) (Hammond *et al*. 2005), beavers (*Castor Canadensis*), primates (Benner *et al*. 1998, 2000, 2001), and chiropterans (Geiser and Stawski 2011), which have high rates of leptin evolution. Hammond and Bennett (2005) proposed that positive selection in seal leptin may be driven by the aquatic environment. Leptin genes show different evolutionary patterns in marine mammals and land mammals. Like marine mammals, fishes also live in aquatic environment and they may face similar environmental stresses. Most studies on leptin genes of teleost fishes are about molecular cloning, structure and function, but not on their evolution. In this study, we looked at 55 leptin genes from gene sequence databases, and used a series of maximum-likelihood (ML) methods to construct a phylogenetic tree for this gene in teleosts. Our results show that leptin genes of mammals and teleost fishes have different evolutionary patterns, indicating that they may have faced different evolutionary pressures. Teleosts, being poikilotherms, need less energy than endotherms to maintain body temperature and they live in an aquatic environment which is relatively less variable than a terrestrial environment. Possibly owing to these reasons, the leptin genes of teleosts seem to have experienced purifying selection.

Materials and methods

Sequence and database searches

All the leptin gene sequences used in this study were from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and Ensemble (http://www.ensembl.org/) databases. The leptin gene sequences used in this study included 14 from species of teleosts, 35 from species of mammals and one from

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Keywords. leptin; evolutionary mechanism; poikilotherm; teleosts; energy metabolism; purifying selection.

an amphibian as the outgroup (see table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/).

Phylogenetic reconstruction

The gene sequences were aligned using MEGA software (Kumar *et al*. 2004). Before constructing the phylogenetic tree, jModeltest (Posada 2008, 2009) was used to select the optimal substitution model and then the GTR+I+G model was regarded as the best-fit model by Bayesian information criterion (BIC). A phylogenetic tree was constructed using MrBayes3.1.2 (Huelsenbeck and Ronquist 2001) by running 5,000,000 generations with 25% of trees burned. The deviation of split frequencies was below 0.01 when the run ended. Then the tree was visualized and edited using FigTree (Page 1996).

Molecular evolution analysis

Under neutrality, coding sequences are expected to present a ratio of nonsynonymous substitutions (d_N) over synonymous substitutions (d_S). The ratio ω (d_N/d_S) is a measure of natural selection acting on the encoded protein. Simplistically, $\omega=1$, $\omega<1$, and $\omega>1$ are indicative of neutral evolution, purifying selection and positive directional, respectively. In order to investigate the evolutionary process of leptin genes in mammals and fishes, PAML (Yang 2007) and the Hyphy package of Data monkey Web Server (http:\www.datamonkey.org; Pond and Frost 2005) were used to estimate the ratio of ω. The PAML package included a free-ratio model, a site-specific model, a branch-specific model (two-ratio) and a branch-site model (model A) (Yang 1997). The free-ratio model was used to evaluate, via the likelihood ratio test (LRT), whether this model fitted the data significantly better than the one-ratio model. The one-ratio model was used to identify the selective pressures in all leptin genes by the likelihood-ratio test (LRT). The branch-site model was used to detect positive selection on the ancestral foreground lineages. The site model included six models of codon substitution. The following codon-substitution models were utilized: M0 (one ratio), assuming a unique value of ω across all sites; M3 (discrete), which had three discrete classes of site with different ω values for sites in the phylogeny; M1a (nearly neutral model) with two site classes ($0<\omega<1$, $\omega=1$) estimating the frequencies p_0 and p_1; M2a (positive selection) which added a class of positively selected sites comparing with M1a: M7 (β) which did not allow positive selection, assuming the frequencies of different ω values ($0<\omega<1$) to follow a beta distribution whose shape was controlled by two parameters (p and q); and M8 (β and ω), which was similar to M7 by adding a proportion p_1 with $\omega > 1$ (Yang 1997). Then we carried out comparison between different models using LRTs. In all cases, significant differences between models can be evaluated by calculating twice the difference in log-likelihood following a χ^2 distribution, with the number of degrees of freedom equal to the difference in the number of free parameters between models (Yang *et al*. 2000). The Bayes empirical Bayes (BEB) were used in the M2a and M8 models to calculate the Bayesian posterior probability (BPP) of the codon sites under positive selection (Yang 1997).

To provide sufficient evidence to identify positively selected sites, we used the Data Monkey Web Server (http://www.datamonkey.org; Pond and Frost 2005) to test positive selection sites. In the Data Monkey Web Server, the best-fitting nucleotide substitution model was searched for by the automatic model selection tool available on the server. All leptin gene sequences were analysed under three distinct models: single-likelihood ancestor counting (SLAC), fixed-effect likelihood (FEL) and random-effect likelihood (REL) (Pond and Frost 2005). The SLAC model is based on reconstruction of the ancestral sequences and counts of d_N and d_S at each codon position of the phylogeny. Without assuming an *a priori* distribution across sites, we used the FEL model to estimate the ratio d_N/d_S on a site-by-site basis. Before inferring the substitution rate for individual sites, REL model first fits a distribution of rates across sites. Wlasiuk and Nachman (2010) used the same standard to identify codons under positive selection. With P values <0.1 for SLAC and FEL and Bayes Factor >50 for REL, sites were regarded as candidates for positive selection (Wlasiuk and Nachman 2010).

Results

We obtained the complete coding sequences of all leptin genes from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and Ensemble (http://www.ensembl.org/) databases. They all showed features typical of leptin, including an amino-terminal signal peptide of 21 amino acids and a mature protein of 146 amino acids. The mature protein included four α-helices (helices A-D) and a distorted helix E in the CD loop.

Site-model tests on mammals and teleosts

To explore whether differences in environments act on the evolutionary progress of extant leptin genes, we used the subsets of mammals and teleosts to analyse molecular evolution in extant leptin genes of mammals and teleosts. The site models allowed ω to vary among codons and treated the ω ratio for any site in the genes as a random variable from a statistical distribution (Nielsen and Yang 1998). Positive-selection site was defined as presence of some codons at which $\omega > 1$ (in model M2a or M8).

For mammals, no positive-selection site was detected by M2a and M8 models (table 1). But positive-selection sites were detected by other ML methods in this group (table 2). To identify candidate sites under positive selection, we considered that the site under positive selection should be detected in at least two of the ML methods. Thus two sites identified by SLAC and FEL were regarded as candidate positive-selection sites (table 2). In teleosts also, no positive-selection site was detected by M2a and M8 models

Table 1. Site model tests on leptin genes in subsets of mammals and teleosts by PAML.

Model	Np[a]	Parameter estimates	L_n likelihood	Models compared	Positive selection sites[b]	$2\triangle \ln L^c$ (P value)
Data set: mammals						
M0:one-ratio	72	$\omega = 0.36$	3994.69			
M3: discrete	76	$\omega_0 = 0.05, p_0 = 0.40,$ $\omega_1 = 0.59, p_1 = 0.58,$ $\omega_2 = 3.43, p_2 = 0.01$	3935.95	M3 vs M0	Not analysed	117.49 ($P = 0.00$)
M1a: nearly neutral	73	$\omega_0 = 0.16, p_0 = 0.62,$ $\omega_1 = 1.00, p_1 = 0.38$	3948.36			
M2a: positive selection	75	$\omega_0 = 0.16, p_0 = 0.62,$ $\omega_1 = 1.00, p_1 = 0.29,$ $\omega_2 = 1.00, p_2 = 0.09$	3948.36	M2[a] vs M1[a]	None	0.00 ($P = 1.00$)
M7: β	73	$p = 0.51, q = 0.79$	3937.86			
M8: β and ω	75	$p_0 = 0.99, p_1 = 0.01, \omega = 3.56,$ $p = 0.54, q = 0.87$	3935.86	M8 vs M7	None	4.01 ($P > 0.01$)
Data set: teleosts						
M0: one-ratio	36	$\omega = 0.41$	4282.64			
M3: discrete	40	$\omega_0 = 0.08, p_0 = 0.20, \omega_1 = 0.38,$ $p_1 = 0.53, \omega_2 = 0.87, p_2 = 0.27$	4239.59	M3 vs M0	Not analysed	86.09 ($P = 0.00$)
M1a: nearly neutral	37	$\omega_0 = 0.26, p_0 = 0.60,$ $\omega_1 = 1.00, p_1 = 0.40$	4249.74			
M2a: positive selection	39	$\omega_0 = 0.26, p_0 = 0.60,$ $\omega_1 = 1.00, \mathrm{p}_1 = 0.35,$ $\omega_2 = 1.00, p_2 = 0.05$	4249.74	M2 vs M1	None	0.00 ($P = 1.00$)
M7: β	37	$p = 1.19, q = 1.46$	4240.07			
M8: β and ω	39	$p_0 = 0.96, p_1 = 0.04, \omega = 1.18$ $p = 1.29, q = 1.73$	4239.87	M8 vs M7	None	0.39 ($P > 0.01$)

[a]Numbers of parameters
[b]only the sites with BPP > 0.95 were shown
[c]Twice the difference of ln[likelihood] between the two models compared

Table 2. Different methods test positive selection on leptin genes in mammals and teleosts.

	Sites under positive selection identified by different methods[a] Method				
Group	SLAC[b]	FEL[c]	REL[d]	Paml-M8[e]	Total no. of sites
Mammals	87, 95	15, 39, 87, 95	None	None	2
Teleosts	60	62, 92, 121, 173	None	None	0

[a]Codons identified by more than one ML method are underlined
[b, c]Codons with P values < 0.1
[d]Codons with Bayes factor > 50
[e]Only the sites with BPP > 0.95 are shown

(table 1). Although sites under positive selection were detected by SLAC and FEL (table 2), no site was detected by both. So there appears to have been no positive-selection site in leptin genes of teleosts. These results indicate that there are different selection pressures on mammals and teleosts.

Branch-site models on ancestors of mammals and teleosts

The phylogenetic tree (figure 1) of leptin genes of mammals and teleosts was used to detect evolution in ancestral lineages of mammals and teleosts. First, the one-ratio model assumed that there was a unique ω for all branches of the tree and

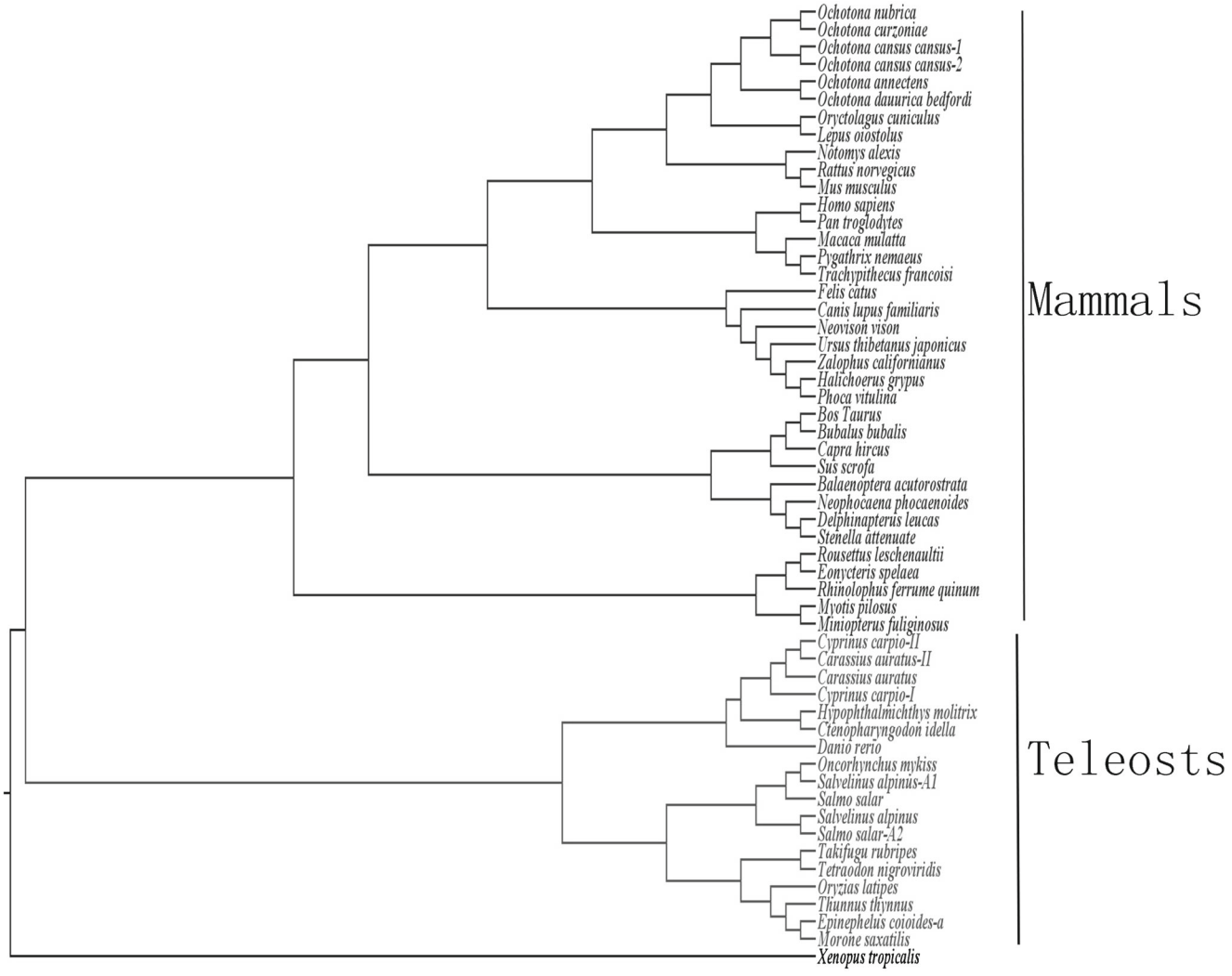

Figure 1. Phylogenetic tree of collected leptin genes was constructed using MrBayes with Bayesian method. The GTR+I+G model was selected for the Bayesian analysis and the consensus tree was built after burning 125,000 trees from 5,000,000 generations.

Table 3. Likelihood-ratio tests of branch models and branch-site models on leptin genes by PAML.

Model	Np[a]	Ln likelihood	Parameter estimates	Model compare	Positive selection sites[b]	2△lnL[c] (*P* value)
Branch model						
A: One-ratio	110	8287.26	ω=0.38		None	
B: Omega=1	109	8409.72	ω=1.00	B vs A		44.91 (*P*=0.00)
C: Free-ratio	217	8173.84	variable ω by branch	C vs A	n/a	226.85 (*P*=0.00)
Branch-site model						
1: Null-teleosts	112	8224.37				
2: Teleosts	113	8222.50		1 vs 2	None	0.00 (*P*=1.00)
3: Null-mammals	112	8225.02				
4: Mammals	113	8225.02		3 vs 4	None	0.00 (*P*=1.00)

[a]Number of parameters
[b]only sites with BPP > 0.95 are shown
[c]Twice the difference of ln[likelihood] between the two models compared

the value of ω was estimated to be ω=0.38, which is smaller than 1 (table 3). This result revealed that the entire leptin genes underwent purifying selection. Secondly, the free-ratio model allowed different ω values for each lineage in the tree compared with the one-ratio model, and the result showed that each branch had its own independent ω value (*P*=0.00, table 3). Lastly, the branch-site models were used to detect whether positive-selection sites occured in the ancestral lineages of mammals and teleosts. We found that no positive-selection site was detected in ancestral lineages of mammals and teleosts (table 3).

Discussion

Leptin is known as one of the key factors in the regulation of food intake and whole-body energy balance. To research the molecular evolution of leptin genes in teleosts, we surveyed 55 leptin genes from 50 species of mammals and teleosts to explore selection pressures on leptin genes and to examine their evolution in teleosts. No positive-selection site was detected in the ancestral lineages of mammals and teleosts, indicating that their ancestors probably underwent similar selection pressures. But two positive-selection sites were detected in extant mammals. When the ancestors of mammals left the water for land, they had to adapt to the new environment. Perhaps owing to the selection pressures caused by the change of environment, positive-selection sites exist in leptin genes in the extant lineages of mammals.

In previous studies, people looked at the leptin genes of marine mammals to examine whether leptin played an important role in body fat regulation and energy metabolism (Rea and Nagy 2000; Gurun *et al.* 2001; Ortiz *et al.* 2001, 2003; Ortiz and Wade 2001; Amould *et al.* 2002; Guilherme *et al.* 2004). However, all these studies found no correlation between fat mass and serum leptin levels in marine mammals. But, marine mammals had evolved a fat-based metabolism because fat was the main energy store and was essential for insulation from cold water (Young 1976; Whittow 1987). Like marine mammals, fishes also live in an aquatic environment, so we want to explore how leptin genes evolved in teleosts. In our study, we found that leptin genes were under purifying selection in teleosts. Teleosts are poikilotherms, which do not require high energy expenditure to maintain body temperature. Moreover, teleosts did not evolve a fat-based metabolism associated with a blubber layer to store energy for resisting the cold water, unlike marine mammals. In cold winter, teleosts do not hibernate. But when the temperature of water is not appropriate for teleosts to live, they may become torpid and even die. To survive, teleosts should swim to an area fit for them to live. Besides, teleosts use gills to breathe, where the utilization ratio of oxygen is lower than in the lungs of mammals, and they did not develop a new function to adapt to hypoxic conditions. Thus we infer that teleosts did not evolve new functions of energy metabolism to adapt to the aquatic environment.

In conclusion, although leptin plays an important role in regulation of food intake and energy metabolism in mammals and teleosts, leptin genes have different selection pressures in the two groups. In teleosts, leptin genes are under purifying selection. We speculate that poikilothermia in teleosts causes leptin genes to remain under purifying selection.

Acknowledgements

This study was supported by Zhejiang Provincial Natural Science Foundation of China (Y12C030014)).

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Received 16 October 2013, in final revised form 26 February 2014; accepted 3 March 2014
Published online: 27 August 2014