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Structural Characterization and Evolutionary Analysis of Toll-Like Receptor Gene Family in African Hunting Dog

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ABSTRACT

The innate system plays a major role in the recognition of microbial component. Mammalian toll like receptor (TLR) were evolutionary conserved protein and have contributed to the understanding of the host defense processes against infection. Researches have been performed on the evolution in many species, but the evolutionary characteristics of African hunting dog TLR genes are scared. The available genome sequence of African hunting dog offers us the way to examine the innate immunity of this endangered carnivore. 10 TLR genes (TLR1-10) were initially identified from the African hunting dog and lesser panda. The results showed that most of the TLR genes were very conservative in the African hunting dog. We found 6 of ten TLR genes had the evidence of positive selection, only a small proportion of sites show evidence of selection, ranging from 0 to 9. In the present study, only four and three common positive selection codons were identified in rabies virus associated TLRs (TLR3 and TLR7); we speculated that the virus associated TLRs were mainly under purifying selection. In conclusions, the TLR genes were mainly shaped by purifying selection, and the limit number of positive selection codons may be resulted from ancient functional adaptation in the carnivores.

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Authors' Contribution
XT and YW conceived and designed the work. HZ performed the experiments and wrote the article. HT and JC helped in performing the experiments. YZ helped in the data analysis.

Key words
***Lycaon pictus*, Innate system, TLRs, Evolutionary analysis.**

INTRODUCTION

Traditionally, the innate system can be divided into the adaptive immunity and innate immunity in mammals (Akira *et al*., 2001). As an ancient host-defense, the innate immunity can be activated by signaling through Toll-like receptor (TLR) (Kaisho and Akira, 2003). Mammalian TLRs belong to the type-I transmembrane proteins which contain three domains: an intracellular Toll/interleukin-1 receptor (TIR) domain, a transmembrane region and an extracellular leonine-rich repeat (LRR) domain (Kaisho and Akira, 2003). Mammalian TLR genes are previously classified into six subfamilies according to their phylogenetic relationship and recognition of microbial component: the TLR 1 subfamily (TLR1, TLR2, TLR6 and TLR10); the TLR 9 subfamily (TLR7, TLR8 and TLR9); the TLR 11 subfamily (TLR11, TLR12 and TLR13); the TLR3 subfamily; the TLR4 subfamily and the TLR5 family (Helena *et al*., 2011). TLRs can also be divided in non-viral TLRs (TLR3, 7, 8, 9) and viral TLRs (TLR1, 2, 4, 5, 6 and 10) according to their ligand recognition (Barton, 2007; Chaturvedi and Pierce, 2009; Carty and Bowie, 2010). At least fifteen TLR members were existed in the vertebrate ancestor, including TLR1-5, 7-9, 11-14, 19, 21 and 22 (Oshiumi *et al*., 2008; Temperley *et al*., 2008).

Previous study believed that the repertoire and functional diversification of TLR genes was shaped by episodes of gene duplication, gene loss and gene conversion appears (Hughes and Piontkivska, 2008; Roach *et al*., 2013; Ishengoma and Agaba, 2017). The TLRs are conserved protein and contributed to the understanding of the host defense processes against infection (Janssens and Beyaert, 2003). Many researches have been performed on the evolution of the TLR genes, including human (Barreiro *et al*., 2009), primate (Wlasiuk *et al*., 2009) and the cetacean (Shen *et al*., 2012). Areal found that all the TLR genes had positively selected codons, ranging from 2 to 26 codons in the mammals (Helena *et al*., 2011).

As an endangered carnivore, the African hunting dog (*Lycaon pictus*) formerly distributed most of sub-Saharan Africa (Stankowich, 2003). However, the wild dogs are currently restricted in fragmented pockets of eastern and southern Africa due to numerous threats including habitat

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loss, human persecution, prey by large carnivores and disease epidemics (such as rabies and canine distemper) (Woodroffe *et al*., 1997; Marsden *et al*., 2012; Campana *et al*., 2016). Rabies virus and canine distemper present so major risk that the infectious disease spread killed 49 of 52 African hunting dogs within 2 months in 2000 (Mw *et al*., 2002). In 2006, the rabies virus was considered to have killed 25 of 26 African hunting dogs (Flacke *et al*., 2013). The functional and evolutionary characteristics of TLR genes have been analyzed in many species, but still remaining a limit understanding in the African hunting dog. The available genome sequence of African hunting dog offers us the way to analyze the innate immunity of this endangered carnivore (Campana *et al*., 2016). Thus we search against the African hunting dog' genome to identify the TLR genes of this species.

METHODS

Identification of TLR sequences

The TLR gene sequences of the African hunting dog (PRJNA304992) used in this study were identified from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The identification of TLR genes was followed previous methods (Wang *et al*., 2016). Meanwhile, we also obtained the TLR genes from other carnivores, including the cat (*Felis catus*), leopard (*Panthera pardus*), cheetah (*Acinonyx jubatus*), tiger (*Panthera tigris altaica*), lesser panda (*Ailurus fulgens*), giant panda (*Ailuropoda melanoleuca*), walrus (*Odobenus rosmarus divergens*), ferret (*Mustela putorius furo*), wolf (*Canis lupus*), badger (*Meles meles*), Hawaiian monk seal (*Neomonachus schauinslandi*), and North American Mink (*Neovison vison*). For each TLR genes, a subset of at least 9 carnivores was used, and the sequences of TLR genes used in this study are presented in Supplementary Table I.

Phylogenetic analysis and identification of domains

The nucleotide sequences of TLR genes were translated into protein sequences using Mega version 6 software (Tamura *et al*., 2013). Then the intact protein sequences were aligned using the Multiple Sequences Comparison by log-Expectation (MUSCLE) (http://www.ebi.ac.uk/Tools/msa/muscle) (Edgar, 2004), which was also used to generate the percent identity matrix of these TLR genes. After removed of the gaps sites, the aligned protein sequences of TLR genes were then used to build the phylogenetic trees in Mega 6.0. We used the maximum likelihood method to build the tree, and the best model was built by using the ProTest server (version 3.0) (Darriba *et al*., 2011). The SMART was used to predict the domains in the proteins (Letunic *et al*., 2015).

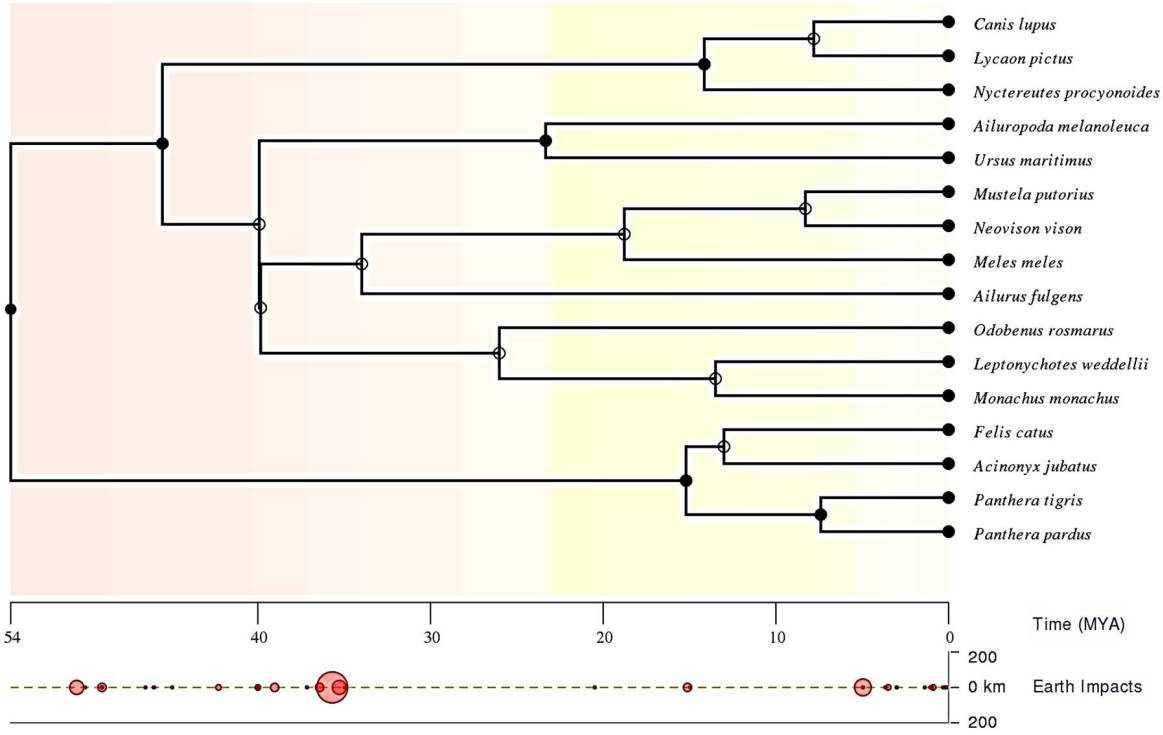

Fig. 1. The divergence time of 16 carnivorous species studied in this research.

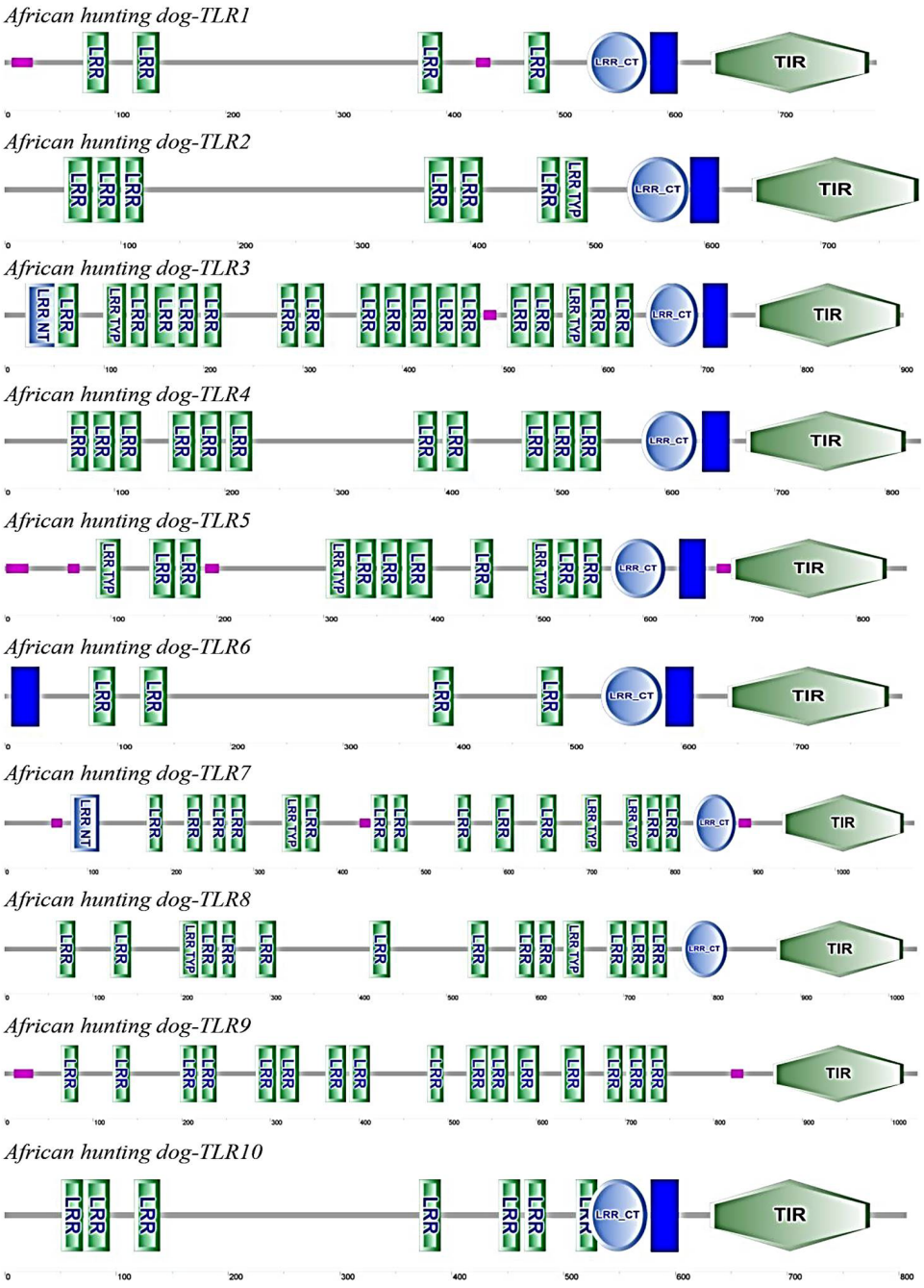

Fig. 2. Comparison of domain architecture of TLRs in the African hunting dog.

Evolutionary analysis

In order to test the selective pressure in individual site of TLR genes, we used the branch model and site model in the Codeml program in PAML 4 (Yang, 2007). In this model, the nucleotide substitutions rates (ω) of nonsynonymous/synonymous (d_N/d_S) with more than 1, less than 1 and equal to 1 represent positive selection, purifying selection and neutral selection, respectively. Two alternative models M7 and M8 were used to detect the selection of each TLR genes, and the likelihood ration test (LRT) was used to compare with 2 degrees of freedom (Yang *et al*., 2000). Meanwhile, the Datamonkey Web Server was used to analyze the selection of these TLR genes (Pond and Frost, 2005). Three distinct models,

including the fixed-effect likelihood (FEL), the single likelihood ancestor counting (SLAC) and the random effect likelihood (REL) were used to analyze all sequences of each TLR genes. The FEL model is the best overall method overall to estimate site-by-site substitution rates (Kosakovsky-Pond and Frost, 2005); the REL model is a codon-based selection analyses to infer the substitution rate for individual sites (Pond and Muse, 2005); the SLAC model estimate the d_S, d_N based on the reconstruction of the ancestral sequences (Kosakovsky-Pond and Frost, 2005). The sites with Bayes Factors larger than 50 for REL, or the P values less than 0.1 for FEL and SLAC were considered to be under positive selection (Helena *et al*., 2011).

RESULTS

Identification of TLR genes and motif analysis

TLRs are comprised of LRRs, a single membrane spanning helix and a TIR. In the present study, through screening the genome of African hunting dog and lesser panda, 10 TLR genes (TLR1-10) were first identified from two species, respectively. The sequence similarity of the African hunting dog TLR coding sequences which compared with TLRs from wolf and other carnivores is shown in Supplementary Table II. There were small degree of sequences difference between dog and African hunting dog, the similarity of TLR genes was ranging from 88.46% - 99.89%, and except the TLR6 and the TLR7, the similarity was ranging from 98.94% - 99.89%. The 10 TLR genes of wolf were obtained from our previous study (Liu *et al*., 2017). The TLR genes of other carnivores (cat, giant panda, leopard, cheetah, ferret, polar bear, tiger, Hawaiian monk seal, weddell seal, walrus, raccoon dog, badger and American mink) were retrieved from GenBank, and the accession number and TLR gene sequences were supplied in Supplementary Table I. In all, 118 TLR genes from 16 carnivorous species were obtained in this study (Fig. 1). The sequence similarity of the same TLR genes was ranging from 71.68-100% (Supplementary Table II).

The species tree (Fig. 1) is obtained from TimeTree v3.0 (http://www.timetree.org/). The divergence time of 16 carnivores was shown above.

Schematic representation of TLR genes domains of African hunting dog was predicted by SMART (Fig. 2). According to SMART predictions, the patterning of the large extracellular domain, the single-pass transmembrane and the TIR domains among these carnivores revealed no significant differences. However, the number of domains was also different among different TLR genes in African hunting dog which was ranging from 4 to 16 (Fig. 2). The results also showed that the viral TLR genes tended to have more numbers of LRRs than the nonviral TLR genes.

Phylogenetic relationships of TLR genes

We aligned the amino acid sequences of all 118 intact TLR genes from 16 carnivorous species. The ML tree of these TLR genes was constructed with the best fit model Jones-Taylor-Thornton (JTT) +G+I. The partial genes were not obtained in the phylogenetic analysis. The phylogenetic relationships of 10 TLR genes among carnivorous species were shown in Figure 3. The result showed that the TLR1, TLR2, TLR6 and TLR10 tended to cluster together; the TLR7, TLR9 and TLR8 tended to cluster together; the TLR3 closed to the TLR5; the TLR4 formed a clade. Then we compared the species tree (Fig. 1) with the TLR genes tree (Fig. 3). The result showed that the phylogenetic proximity of the TLR genes among different species corresponded to traditional taxonomic groups, thus we speculated that the TLR genes were very conservative among carnivores.

The phylogenetic tree was built by Mega 6 with best fitting model. Different TLR genes were marked in different colors. The bootstrap of the ML tree was marked in blue circle.

Evolution of TLR genes among carnivora

In the present study, four methods (PAML, SLAC, FEL and REL) were used to identify the evidence of positive selection at individual codons of 10 TLR genes among carnivorous species. We addressed the common positively selected codons which identified more than one method (Table I). The number of positively selected codons observed for each TLR genes ranged from 0 to 9. Six of ten TLR genes (TLR1, TLR3, TLR4, TLR 6, TLR7 and TLR9) fit the data significantly better than a neutral model (Table I). This group of six genes contains three viral TLR genes and three non-viral TLR genes. The number of identified positively selected codons by more than one ML method was different from the TLR genes, ranging from 1 to 9. The non-viral TLR 4 gene was observed to have the high number of positively selected codons in the present study, and then was the TLR1 and TLR3 gene (Table I), and the viral TLR9 gene was identified only one positively selected codon (Table I). Our results showed that most positively selected sites of the six TLRs were located in the unknown region, and then was located in the extracellular LRRs (Table II).

In the present study, we used the branch model to analyze whether the African hunting dog faced different selective pressure with other carnivorous species. The result showed that only the TLR5 gene in African hunting faced different selective pressure with other carnivorous species (Table III). The result also showed that the ω of each TLR genes' branch was smaller than 1, we speculated that the TLR genes were very conservative among carnivores.

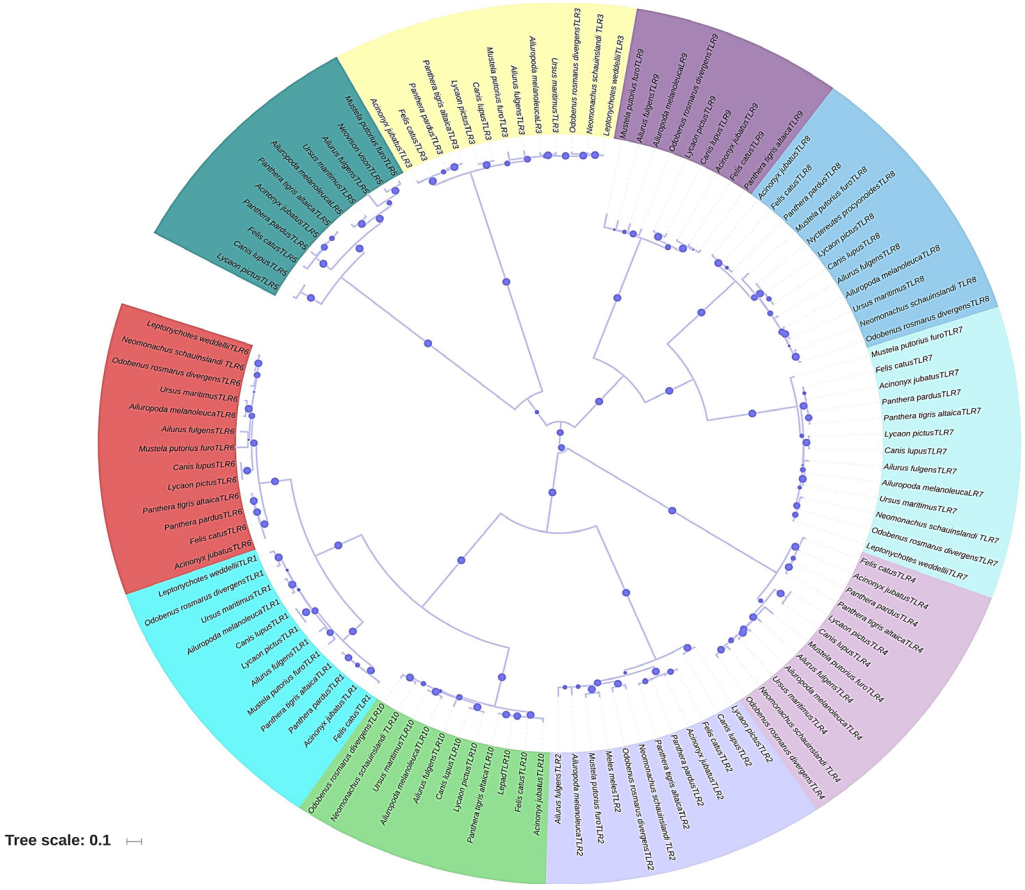

Fig. 3. Phylogenetic tree of 10 TLR genes among carnivores.

Table I.- Tests for positive selection at carnivorous species using site models.

Gene	Species	Lnl M7	Lnl M8	2lnΔL	P-values	M8	SLAC	FEL	REL	Total No. of site
TLR1	12	-5904	-5896.2	14.83	0.0006**	291 324 562	630	291 324 562 630	630	4
TLR2	12	-7443	-7437.7	10.26	0.00592**	338	0	101 212 262 270 342 487 511 537 555 577 609	0	0
TLR3	13	-7469	-7467.7	1.838	0.3988	0	0	26 270 293 587	13 24 26 69 121 146 259 270 293 344 346 484 491 496 587 609 776	4
TLR4	12	-7416	-7401.3	29.69	3.58E-07**	193 295 341 514 564		46 76 193 319 341 544 562	46 58 76 175 193 295 298 306 308 319 323 341 347 364 369 393 394 395 505 510 544 562 564 578 620 636 832	9
TLR5	11	-8434	-8433	1.672	0.433	0	0	63 137	0	0
TLR6	13	-5804	-5800.2	7.546	0.02298**	63 236	0	236 391 563	90 109 236 391 563 631	3
TLR7	13	-7624	-7619.1	9.676	0.0792	0	0	113 606	113 606	2
TLR8	12	-8702	-8697	9.204	0.01**	0	0	235 437 458 684	0	0
TLR9	9	-8542	-8530.5	21.93	1.72E-05**	5 19	0	5	0	1
TLR10	11	-5758	-5757.8	0.938	0.6256	0	0	3 797	0	0

The common predicted positive selected sites were underline. *, means the significant level; **, mean highly significant level.

Table II.- The domain location for each positively selected sites.

Gene	Species	Total No. of sites	Domains						
			Signal	Unknown region	LRR-NT	LRR	LRR-CT	Transmembrane	TIR
TLR1	12	4	0	2	0	0	1	0	0
TLR3	13	4	0	1	1	2	0	0	0
TLR4	12	9	0	6	0	3	0	0	0
TLR6	13	3	0	0	0	2	1	0	0
TLR7	13	2	0	0	0	1	0	0	0
TLR9	9	1	0	1	0	0	0	0	0

Table III.- Positive selection used Branch-model of 10 TLR genes among carnivores.

Gene	Model	np	Ln L	Estimates of parameters	Model compared	LRT P-value	Omega for Branch
TLR1	Two ratio Model 2	25	-5928.570340	ω: 0.51004 0.52379			0.51004
	Model 0	24	-5928.576182	ω= 0.51218	Model 0 vs. Model 2	0.913922275	
TLR2	Two ratio Model 2	25	-7510.915256	ω: 0.33212 1.61229			0.33212
	Model 0	24	-7510.915054	ω= 0.33213	Model 0 vs. Model 2	0.983963799	
TLR3	Two ratio Model 2	27	-7511.896237	ω: 0.28839 0.22189			0.28839
	Model 0	26	-7512.512443	ω= 0.27855	Model 0 vs. Model 2	0.266938474	
TLR4	Two ratio Model 2	25	-7483.909632	ω: 0.47332 0.51123			0.47332
	Model 0	24	-7483.982504	ω= 0.47962	Model 0 vs. Model 2	0.702636182	
TLR5	Two ratio Model 2	23	-8536.027952	ω: 0.20424 0.02238			0.02238
	Model 0	22	-8544.722994	ω= 0.17822	Model 0 vs. Model 2	0.000030441**	
TLR6	Two ratio Model 2	27	-5844.434481	ω: 0.43975 0.52755			0.43975
	Model 0	26	-5844.693071	ω= 0.45135	Model 0 vs. Model 2	0.472047099	
TLR7	Two ratio Model 2	27	-7648.961942	ω: 0.15884 0.09783			0.15884
	Model 0	26	-7650.788660	ω= 0.14782	Model 0 vs. Model 2	0.055953667	
TLR8	Two ratio Model 2	25	-8771.114745	ω: 0.19723 0.18931			0.19723
	Model 0	24	-8771.116115	ω= 0.19715	Model 0 vs. Model 2	0.958253790	
TLR9	Two ratio Model 2	19	-8649.647917	ω: 0.10793 0.07876			0.10793
	Model 0	18	-8650.648968	ω= 0.10367	Model 0 vs. Model 2	0.157081244	
TLR10	Two ratio Model 2	23	-5763.615442	ω: 0.48938 0.30703			0.48938
	Model 0	22	-5765.186279	ω= 0.45252	Model 0 vs. Model 2	0.076315445	

DISCUSSION

The TLR genes were previously reported to belong to a very ancient receptor family (Voogdt and Putten, 2016). This is the first study presenting sequence analysis of 10 TLR genes from the African hunting dog. The similarity of TLR genes was ranging from 88.46%-99.89%, and except the TLR6 and the TLR7, the similarity was ranging from 98.94%-99.89% between the wolf and African hunting dog. Thus, the new identified TLR genes were reliable. The comparison of African hunting dog LRR motif was similar to the LRR motifs in the wolf. And the high nucleotide and amino acid similarities of African hunting dog in comparison to wolf is indicative of general conservation of TLR sequences among vertebrates in general (Roach *et al.*, 2005; Ishengoma and Agaba, 2017). Despite the high degree of conservation, the number of LRRs was still existed differences between species; this supported the importance of LRRs in species-specific ligand recognition.

In the present study, we found 6 of ten TLR genes had the evidence of positive selection, only a small proportion of sites show evidence of selection, ranging from 0 to 9, which was not in line with the previous study on primates that a signature of positive selection in most TLR genes and mammals that 10 TLR genes were observed to have the evidence of positive selection (Wlasiuk and Nachman, 2010; Helena *et al.*, 2011). We speculated that the differences may be due to the different species used to analyze. Previously researches believed that the viral TLR genes recognized viral nucleic acids and target self-components, thus the viral TLR genes were under a stronger purifying selection than non-viral TLR genes (Finberg and Kurt-

Jones, 2004; Barreiro *et al*., 2009; Wlasiuk and Nachman, 2010; Alcaide and Edwards, 2011; Helena *et al*., 2011). In order to adapt to coevolving microbial danger signals, the TLR genes underwent both purifying and diversifying selection (Voogdt and Putten, 2016). In the present study, we found that the non-viral TLR genes had equal positive selection to viral TLR genes, but the total positive selected codons in the nonviral TLRs (16) were larger than that in the viral TLRs (7). This may be due to the nonviral TLR genes located in the cell surface which had the higher tolerance of non-synonymous substitutions and the function of non-viral TLR genes were more redundant than that of viral TLR genes (Wlasiuk and Nachman, 2010; Helena *et al*., 2011). Thus the viral TLR genes were not expected to accumulate non-synonymous substitutions as this might affect their functional integrity. In addition, the non-synonymous mutation in one TLR might not mean the extinction of the function and did not compromise immunity.

Traditionally, rabies virus and canine distemper present a significant risk to the African hunting dogs' survival. Previous study investigated the role of TLRs in rabies virus infection, and found that TLR3-positive human neuronal cell line upregulates genes associated when infected with rabies virus *in vitro* (Ménager *et al*., 2009). Recently study identified TLR3 as the component of Negri bodies; TLR7 and 8 in humans are more likely to be activated during rabies virus infection (Préhaud *et al*., 2005). In addition, TLR2 and TLR4 were reported to contribute to the induction of antirabies viral immunity through recognition of host cell components (Prosniak *et al*., 2001). In the present study, only four and three common positive selection codons were identified in rabies virus associated TLRs (TLR3 and TLR7); we speculated that the virus associated TLRs were mainly under purifying selection. TLR4 was observed to have high number of positive selection codons in several researches among different species (Wlasiuk and Nachman, 2010; Alcaide and Edwards, 2011; Helena *et al*., 2011). Our result also showed the TLR4 gene had the largest number of positive selection in carnivores. The TLR4 was previously considered as the most versatile member of the TLR family which can respond to Gram-negative, yeast, *Trypanosoma* and viruses, resulting in a variety of putative ligands (Kumar *et al*., 2009; Döring *et al*., 2017). Thus, we speculated that the variety of functions lead to the high number of positive selection codons in TLR4 gene. The viral TLR 8 gene was previously considered to be under purifying selection (Wlasiuk and Nachman, 2010), while Helena *et al* indicated that the TLR8 gene had a similar level of positive selection as in the non-viral TLR4. In the present study, we also found that the TLR8 gene had a high level of positive selection among carnivores. We speculated that the presence of the positive selection may be resulted from ancient functional adaptation in the carnivores (Jann *et al*., 2008; Helena *et al*., 2011).

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Supplementary material

There is supplementary material (S1 and S2) associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20180503070501

Statement of conflict of interest

Authors have declared no conflict of interest.

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