Alternative Splicing of RNF180 Genes in Different Species Based on Comparative Genomics Analysis

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Research Article

Keywords: RNF180, alternative splicing, comparative genomics analysis, evolution

Posted Date: December 21st, 2020

DOI: https://doi.org/10.21203/rs.3.rs-129993/v1

Abstract

Alternative splicing (AS) influences gene regulation, cell differentiation, and tissue development and is involved in many human diseases. The ring finger protein 180 (*RNF180*), a tumor suppressor gene, has four transcripts and seven predicted transcripts. However, the role of alternatively spliced products in the function and regulation of the *RNF180* gene remain unknown. We used a comparative genomics approach to investigate *RNF180* AS in different species. Conserved coding sequences, alternative splicing expression profiles and intron sequences were compared, and evolutionary selection pressure analyses of exons were performed. We found that the *RNF180* zinc finger structure, which was related to major ubiquitination functions, was highly conserved and exon 5 was absent in many species. Comparisons with the corresponding intron revealed that exon 5 possessed high similarity. In exon pressure selection analysis, exon 6 was in the purifying selection, which corresponded to the zinc finger domain, while exons 7 and 8 faced positive selection evolution. Finally, there were multiple alternatively spliced forms of *RNF180* in four transcripts. These results suggested that complex alternative splicing of the *RNF180* gene occurred in multiple species. Partial splicing variants had evolutionarily conserved regions and functional region deletions.

Introduction

Alternative splicing (AS) is an important basic regulation mechanism in eukaryotes, whose spliceosomes recognize different splicing sites on pre-mRNA. The same gene pre-mRNA is then processed to produce different pattern combinations, resulting in different mature transcripts and proteins. Recent studies have shown that AS has an important influence on gene regulation, cell differentiation, and tissue development and is closely involved in many human diseases. Although AS patterns differ in different species, splicing patterns are largely uniform in conserved regions. Through comparing the gene sequences of different species, the role of conserved sequences and AS sequences in the evolutionary process may be elucidated.

The *RNF180* (ring finger protein 180) tumor suppressor gene is a member of the RNF/Rines gene family and is located at position 5q12.3[1]. *RNF180* expression has been detected in the endoplasmic reticulum membrane of cultured mammalian cells. *RNF180* product (Rines) is an E3 ubiquitin ligase with protein ubiquitination activity. Rines contains a RING finger domain (431–472), a basic coiled-coil domain (351–400), a novel conserved domain DSPRC (83–132) and a C-terminal hydrophobic region that is predicted to be a transmembrane domain (564–586)[2]. The ubiquitin-proteasome system (UPS) is a highly specific enzyme cascade involving the E1 ubiquitin-activating enzyme, E2 ubiquitin-binding enzyme, and E3 ubiquitin-protein ligase and plays a vital role in cell proliferation, differentiation, apoptosis, and other cellular processes[3, 4]. The *RNF180* encoded protein is expressed in many tissues, with the highest expression reported in brain and lowest expression in lung and thymus tissues. Methylation of the *RNF180* DNA promoter is also involved in cell proliferation, cell, the cell cycle, tumor invasion, and tumorigenicity[5]. There are four known *RNF180* transcripts (Fig. 1) and seven predicted *RNF180*

transcripts in the NCBI database(www.ncbi.nlm.nih.gov). Transcripts 2, 3, and 4 have different exon deletions than does transcript 1. Comparison of *RNF180* gene sequences from different species allows us to analyze AS evolution. Moreover, clearly defining the conservation of deleted exons throughout the evolution of an entire species can provide insight into gene function, tissue distribution, and differential expression in normal and tumor tissues. These comparisons may also provide clues for elucidating the molecular mechanisms of RNF180-related tumor development and progression.

Comparative genomics is a new approach for studying AS. Comparing exon sequences, AS type, relative expression of variants, and potential variable splice variants in two or more genomes may lead to the identification of variable splice regulatory elements and uncover the evolutionary conservation of spliced exons. Alternative splicing information and conserved RNA sequence could be integrated by comparative genomics to reveal RNA motifs that might have possible functions[6]. In this study, we used genomic data including multiple sequence alignment and selective pressure of evolution analyses to explore the evolution of *RNF180* AS in different species. Using this approach, we elucidated the AS function of *RNF180* and its alternative splicing function. Here, we describe the relationship between *RNF180* splicing, gene regulation, and resulting functional changes.

Materials And Methods

Comparison of the amino acid sequences of RNF180 genes from different species.

Ten evolutionary representative species were selected from the 73 species of vertebrates included in the UCSC Genome Browser (http://hgdownload.soe.ucsc.edu/downloads.html), and the amino acid sequences of their RNF180 proteins were downloaded. Interspecies evolution was analyzed using ClustalX[7] and MEGA7[8] software.

The evolutionary selection pressure (dN/dS) of RNF180 exons.

The exon sequences of 23 vertebrate *RNF180* genes were downloaded from the UCSC genomic database (http://genome.ucsc.edu) (Table.1). Each exon sequence was re-aligned based on a codon using MEGA7 software and manually corrected based on the corresponding encoded amino acid sequence. Using an online server (http://www.datamonkey.org/), we performed exon-selective pressure analysis by SLAC (single-likelihood ancestor-counting).

Table 1
Comparing of 23 vertebrates.

Common Name	Scientific Name	Common Name	Scientific Name
Human	Homo sapiens	Zebra finch	Taeniopygiaguttata
Chimpanzee	Pan troglodytes	Alpaca	Vicugnapacos
Gorilla	Gorilla gorillagorilla	Dolphin	Tursiopstruncatus
Orangutan	Pongo pygmaeusabelii	Cow	Bostaurus
Bushbaby	Otolemurgarnettii	Horse	Equuscaballus
Marmoset	Callithrix jacchus	Cat	Feliscatus
Mouse	Mus musculus	Dog	Canisfamiliaris
Rat	Rattusnorvegicus	Elephant	Loxodontaafricana
Kangaroo rat	Dipodomysordii	X. tropicalis	Xenopustropicalis
Guinea Pig	Caviaporcellus	Fugu	Takifugurubripes
Armadillo	Dasypusnovemcinctus	Zebrafish	Danio rerio
Chicken	Gallus gallus		

The relative rates of synonymous (dS) and non-synonymous substitutions (dN), ω = dN/dS, has been widely adopted as a measure of selective pressure[9]. This is defined as the average number of synonymous substitutions (dS) at each synonymous site and the average number of non-synonymous substitutions (dN) at each non-synonymous site. An excess of non-synonymous substitutions (dN/dS>1) can be interpreted as positive selection, suggesting that replacement substitutions increase fitness. A paucity of replacement changes (dN/dS<1) indicates that negative selection is working to remove such substitutions from the gene pool. dN/dS=1 indicates neutral selection.

Analysis of RNF180 AS products in different species.

The NCBI (http://www.ncbi.nlm.nih.gov) and Ensembl genome databases (http://asia.ensembl.org/index.html) were searched for *RNF180* AS variants and splice sites and splice modes were compared and analyzed.

RNF180 intron sequence comparison in different species.

Based on the *RNF180* gene annotation in the UCSC genome database (http://genome.ucsc.edu), *RNF180* genomic sequences from various species were download from the Ensembl genome database. Exons were compared and the corresponding intron sequences were obtained from each species and compared using Clustalx (Table.1).

Results

Comparison of RNF180 amino acid sequences in different species.

Ten evolutionary representative organisms from lower vertebrates to higher mammals were used for RNF180 amino acid sequence comparisons. These species included *Zebrafish*, *Xenopus tropicalis*, *Chicken*, *Zebra finch*, *Cow, Rat*, *Mouse*, *Orangutan*, *Chimpanzee* and *Human*. Our results showed that conserved *RNF180* gene sequences gradually increased from lower vertebrates to primates, and the *RNF180* sequence similarity between *Humans* and *Chimpanzees* is 99.0% (Table.2). With the exception of *Cow*, the species *RNF180* sequence alignment results revealed an evolutionary relationship consistent with that described by the evolutionary tree (Fig. 2). The RNF180 C-terminal amino sequence was highly conserved as were those of exons 3, 6, and 7. Exon 1 and exon 8 were not translated most of the examined species. Exon 2 (1–45) was missing in *Zebra finch* and us partially deleted in *Chicken* and *X. tropicalis*. Exon 4 (78–398) was not well conserved in *Zebrafish* and *Chicken*, *X. tropicalis*, and *Zebrafish* were devoid of exon 5 (399–411) (Supplementary Fig. S1). Further analysis using the COBALT online multiple sequence alignment analyzer revealed considerable variability at amino acid sites 151, 175, 221, 254, 361, 400, and 531.

Table 2
Human *RNF180* sequence similarity comparison with other species.

Species	Length	Species	ID	Length	Score(%)
Human	592	chimpanzee	ENSPTRP00000028966	592	99.0
Human	592	orangutan	NP_001125710.1	592	98.0
Human	592	cow	NP_001192746.1	592	87.0
Human	592	rat	NP_001128458.1	592	83.0
Human	592	mouse	NP_082210.1	575	80.0
Human	592	chicken	ENSGALP00000023728	571	51.0
Human	592	zebra finch	ENSTGUP00000002858	532	51.0
Human	592	x.tropicalis	NP_001107726.2	558	43.0
Human	592	zebrafish	NP_001103874.1	458	34.0

Evolutionary selection pressure (dN/dS) analysis.

dN/dS analysis was performed on *RNF180* exons 2–8 (Exon 1 is only present in individual higher mammals, and the sample size was insufficient for comparison). The results showed that the dN/dS values of exons 7 and 8 are > 1 (1.49825 and 1.23845), suggesting positive selection. Exons 1–6 with dN/dS < 1 were stably selected, and exon 6 was selected for purification (dN/dS = 0.160536 < 0.25) (Fig. 3 and Table.3).

Table 3
dN / dS analysis of eight exons of the *RNF180* gene.

Exon	dN / dS
Exon2	0.334887
Exon3	0.38672
Exon4	0.372754
Exon5	0.447605
Exon6	0.160536
Exon7	1.49825
Exon8	1.23845

Analysis of alternative splicing of the RNF180 gene in different species.

We downloaded the standard reference *RNF180* genes from a range of species from lower vertebrates to higher mammals using the NCBI database and the Ensembl Gene Browser. From these data, we predicted AS isoforms in *Human*, *Chimpanzee*, *Mouse*, *Rat*, *Cow*, *Chicken*, *X. tropicalis* and *Zebra finch*. The *Orangutan*, *Guinea pig*, and *Zebrafish* sequences did not match our query transcripts (Table.4). The *Human RNF180* genome annotation was provided by the UCSC Genome Browser. The longest transcript, transcript 1, had eight exons. For transcript 2, the TGA premature termination codon was introduced in exon 5. As for transcript 3, a jump of exon 4 to exon 3 was observed and a premature TGA termination codon was introduced in exon 5. A premature TAA termination codon was also observed in exon 7 of transcript 4. The CDS sequences of human *RNF180* gene were used as a reference. Comparison with *RNF180* sequences on other species revealed that the main splicing patterns in these species are consistent with that of human. Exons 7 and 8 terminate prematurely, exon 4 jumping only was observed in human, and exon 5 was missing in multiple species (Supplementary Fig. S2 and Table.5). Additionally, *RNF180* splicing in *Chicken*, *X. tropicalis* and *Zebra finch* was complicated and requires further study.

Table 4
RNF180 gene alternative splice in species.

Species	Transcript number	Total	Database
human	NM_001113561.2; NM_178532.4; NM_001323291.1; NM_001323292.1; XM_017009383.1; XM_017009384.1; XM_017009385.1; XM_017009386.1; XM_017009387.1; XM_017009388.1; XM_017009389.1	11	NCBI
chimpanzee	XM_009449113.2; XM_527201.6; XM_009449114.2	3	NCBI
mouse	NM_027934.2; XM_006517761.3; XM_011244696.2; XM_006517756.3; XM_006517757.2; XM_006517759.3; XM_006517760.2; XM_006517762.2; XM_017315607.1	9	NCBI
Rat	NM_001134986.1; XM_008760714.1	2	NCBI
cow	NM_001205817.1; XM_010816737.2	2	NCBI
chicken	XM_015277600.1; XM_015277601.1; XM_004937275.2; XM_004937273.2; XM_015277602.1	5	NCBI
X. tropicalis	NM_001114254.2; XM_012954387.2; ENSXETT00000032412.3	3	NCBI Ensembl
Zebra finch	XM_012577664.1; ENSTGUT00000002887; ENSTGUT00000002889	3	NCBI Ensembl

Table 5
Splicing methods of different species.

Species	Transcript number	Alternative method
human	XM_017009384.1; XM_017009385.1; NM_001323291.1 NM_178532.4; NM_001323291.1; XM_017009387.1; NM_001323292.1; XM_017009386.1	exon2 skip; exon4 skip; exon5 introduces the forward-termination codon TGA; alternative poly-A site; exon7 introduces the forward-termination codon TAA
chimpanzee	XM_009449114.2; XM_009449113.2	alternative promoter; exon2 skip
mouse	XM_006517762.2; XM_006517761.3; XM_006517757.2; XM_017315607.1; XM_006517759.3; XM_006517756.3 XM_006517760.2; XM_011244696.2; NM_027934.2	alternative promote; exon8introduces the forward-termination codon TGA
Rat	XM_008760714.2	exon3 skip
cow	XM_010816737.2; NM_001205817.1; XM_010816737.2	exon2 skip; exon5 skip

RNF180 intron sequence comparison in multiple species.

The UCSC Genome Browser provides human *RNF180* genome annotation and reference sequences for other species, including *Cow*, *Chimpanzee*, *Mouse*, and *Rat*. *Cow*, *Chimpanzee*, *Mouse*, and *Rat* lack exon 5, while *Cow*, *Mouse*, *Rat* also lack exon 1, and exon 8 is missing in *Mouse*. These results were consistent with those of previous *RNF180* gene sequence comparisons (Figs. 4 and 5). With reference to the *Human RNF180* intron sequences, we analyzed *RNF180* from multiple species (*Chimpanzee, Cat, Dog, Chicken, X. tropicalis, Zebrafish, Zebrafish*, and other species) using Clustalx. We found that *Human* and *Gorilla* had the most conserved intron sequences, and that exon 5 was absent in *Chimpanzee, Cow, Cat, Dog, Chicken, X. tropicalis, Zebrafish, Armadillos, Elephant*, and *Guinea pig*. Therefore, we sought to determine the evolutionary origin of the human exon 5 sequence by comparing it with the corresponding intron region sequences in other species. Our results showed that the conservation between *Human* and *Gorilla*, *Cow*, *Cat*, *Armadillo*, *Elephant*, and *Dog* were 100%, 96%, 94%, 91%, 88%, and 86%, respectively (Table.6). *In silico* analysis revealed that most of the frequent mutations were predicted to be deleterious (Supplementary Table S1).

Table 6
Contrast between Exon5 and corresponding Intron.

Name	Length	Name	Length	Score(%)
exon5	36	Gorilla	112248	100
exon5	36	Cow	135704	96
exon5	36	Cat	87272	94
exon5	36	Armadillo	147264	91
exon5	36	Elephant	149778	88
exon5	36	Dog	87296	86

Discussion

AS is a tightly regulated process whereby a single gene can encode multiple distinct transcripts, and provides an essential means of expanding the proteome[10]. Comparative genomics is an indispensable approach for studying AS[11]. More recently, investigations of new transcription products rely on high-throughput sequencing and EST sequencing technologies[12]. Here, we used comparative genomics to explore *RNF180* and found that the functional domain (zinc finger structure) was highly conserved. We also found that exon 5 was absent in many species. Comparison with the corresponding intron in other species revealed high similarity to *Human* exon 5. Exon pressure selection analysis revealed that exon 6, which corresponds to the zinc finger domain, was subjected to purifying selection. This analysis also showed that exons 7 and 8 were subjected to positive selection. Meanwhile, different splicing patterns were observed in the four human *RNF180* transcripts, which resulted in different mature transcripts, with transcripts 2 and 3 being shorter. In conclusion, this work is helpful to further understand the structure of human *RNF180* gene and the effect of AS on its function.

In this study, we compared RNF180 amino acid sequences form orthologous species and discovered that the zinc finger and the C-terminus of the basic coiled-coil domain were highly conserved. Furthermore, exon 5 was absent in *Chimpanzee, Cow, Cat, Dog, Chicken, X. tropicalis, Zebrafish, Armadillo, Elephant*, and *Guinea pig*, while exons 1 and 2 were deleted in lower vertebrates, suggesting that AS is closely related to *RNF180* gene evolution.

AS is an important mechanism for accelerating genome evolution[13, 14] and plays a prominent role as a source of functional innovation[15]. Synonymous amino acid substitutions have no impact on protein composition, but non-synonymous substitutions may directly affect protein function. Xing exon analysis of AS in human and mouse transcripts revealed that a variable splice exon had a higher dN/dS value than did the constitutive exon[16–19]. These findings suggest that AS plays a role in promoting gene evolution[20]. Lu et al. predicted a strong selectable AS event for 345 human genes and 262 mouse genes by combining multiple genomic alignments with RNA selective stress analysis. Here, dN/dS analysis

showed that *RNF180* exons 7 and 8 had high dN/dS values (1.49825 and 1.23845, respectively), suggesting that they are undergoing adaptive selection. It is not clear which domain exon 7 belongs to. Exon 8 is located in the transmembrane domain and may be related to the subcellular localization of RNF180 protein or may act as a signal peptide. Exons 7 and 8 are undergoing positive selection to accelerate *RNF180* evolution and may acquire new functions. The dN/dS value of exon 6 was 0.160536 (< 0.25), indicating purification selection. The deduced amino acid sequence of exon 6 is located in the *RNF180* ring finger domain (431472). The RING domain is a conserved domain rich in Cys residues, which binds two zinc ions through a highly conserved Cys or His residue. It mediates ubiquitin transfer of substrates (including itself), and plays a major role in ubiquitination as a support for bringing E2-Ub and substrate proteins into close proximity[21, 22]. These data suggest that exons 7 and 8 splicing may represent functional AS.

Exons and the AS patterns of orthologous genes in different species are in constant evolution[23, 24], mainly involving the deletion and retention of exons. Differences in exon splicing patterns and gene expression levels have been reported between species[25]. Potential variable splice variants can be identified by comparisons between two or more genomic sequences[26]. In this study, we identified the *RNF180* variable splice isoforms and compared them with those of the human *RNF180* reference sequence. Our results showed that all species examined have corresponding *RNF180* transcripts. Longitudinal comparison between AS in different species revealed *RNF180* exons 3, 6, and 7 are highly conserved, and exon 5 is absent in various species. Moreover, we found that exons 7 and 8 frequently have introduced termination codons, exon 2 is skipped in lower vertebrates skips, and human exon 4 is jumped. Transverse comparisons of transcripts from multiple species revealed specific splicing patterns in different species. Exon splicing in *Chicken*, *Zebra finch*, and *X. tropicalis* (1–5) is complex and requires further study. These results suggest that *RNF180* AS is much more complex than previously known. There are eleven types of human *RNF180* genes identified with predicted transcripts in the NCBI database. These specific splice variants are generally expressed in low-abundance and may not have a major effect on the physiological function of the gene. However, the accumulation of specific spliceosomes may have a subtle effect on gene function and may indirectly affect the interspecific characteristics of the gene. A comparison of four human transcripts has shown that transcripts 2 and 3 do not contain conserved exons 6 and 7. This would result in deletion of the ring finger structure and may affect *RNF180* E3 ubiquitin ligase function, leading to protein degradation. E3 ubiquitin ligase is involved in specific binding to ubiquitin, which is a key step in identifying the proteasome substrate and can be investigated by studying different AS forms. COBALT online multiple sequence alignment analyses of RNF180 amino acid sequences from 10 species, showed that amino acid sites 400 and 531 loci were located at the edge of splicing. Many exon-intron boundaries contain auxiliary splice recognition site cis-acting elements, and amino acid changes in these regions may affect splice site recognition.

Species evolution is both a simple and highly complex process. The information carried by the genome continues to change through selection and elimination leading to the gain of new features. The evolution of AS introns plays an important role in gene function gain[27, 28]. Comparisons of *RNF180* gene

sequences from different species revealed that exon 1 is located in the 5 ' untranslated region (5’-UTR) and is absent in lower vertebrates. This exon emerged through evolution, but is not translated. Approximately 30% of young cassette exons appear in the 5'-UTR. The new exon may be stored in the non-coding region to avoid the impact of translation and may be accessed in the open reading frame with the gradual emergence of function[29, 30]. *RNF180* exon 5 is deleted in many species, especially in lower organisms, and comparison of exon 5 with corresponding introns in species lacking exon 5 revealed that exon 5 sequence conservation in *Gorilla, Cow, Cat, Armadillo, Elephant*, and *Dog* reached 100%, 96%, 94%, 91%, 88%, and 86%, respectively. These data suggest that exon 5 may be derived from the original intron sequences. Smart and Uniprot databases suggest that exon 5 is an unknown region and does not match other domains in the NCBI blast tool. Therefore, the effect of exon 5 on *RNF180* gene function remains unclear and required further study. The functional evolution of *RNF180* is affected by AS, and new exons are introduced through AS. Frequently, these exons are derived from the original intron sequences, and these novel exons may be involved in the translation of protein sequences, thereby increasing the function of the original protein. To understand *RNF180* gene structure and the effect of AS on its function, we used bioinformatics methods including multiple sequence alignment and selective pressure analysis to explore *RNF180* AS in different species.

In summary, our findings showed that complex *RNF180* AS occurred in different species, including species-specific AS. Future well designed investigations are required to elucidate the function and regulatory mechanisms of AS in *RNF180* and thus develop the clinical utility.

Declarations

Data availability

All the datasets and software used in this work are publicly available.

Acknowledgements

This work was supported by grants from The National Key R&D Program (2016YFC1303200) and the Liaoning Provence Key R&D Program (2020JH2/10300063).

Author contributions

L.S. conceived and designed the research. Y.Z., Q.Z. and H.L. collected and analyzed the data. performed data interpretation and statistical analysis. Y.Z wrote the main manuscript text. L.S. and Y.Y. contributed to revising the manuscript. All authors read and approved the final version of the report.

Competing interests

The authors declare no competing interests.

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Figures

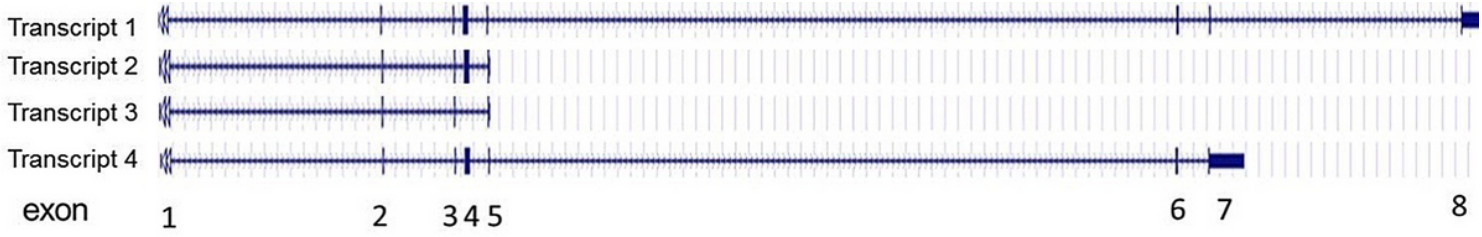

Figure 1

Human RNF180 transcripts Reference Sequence.

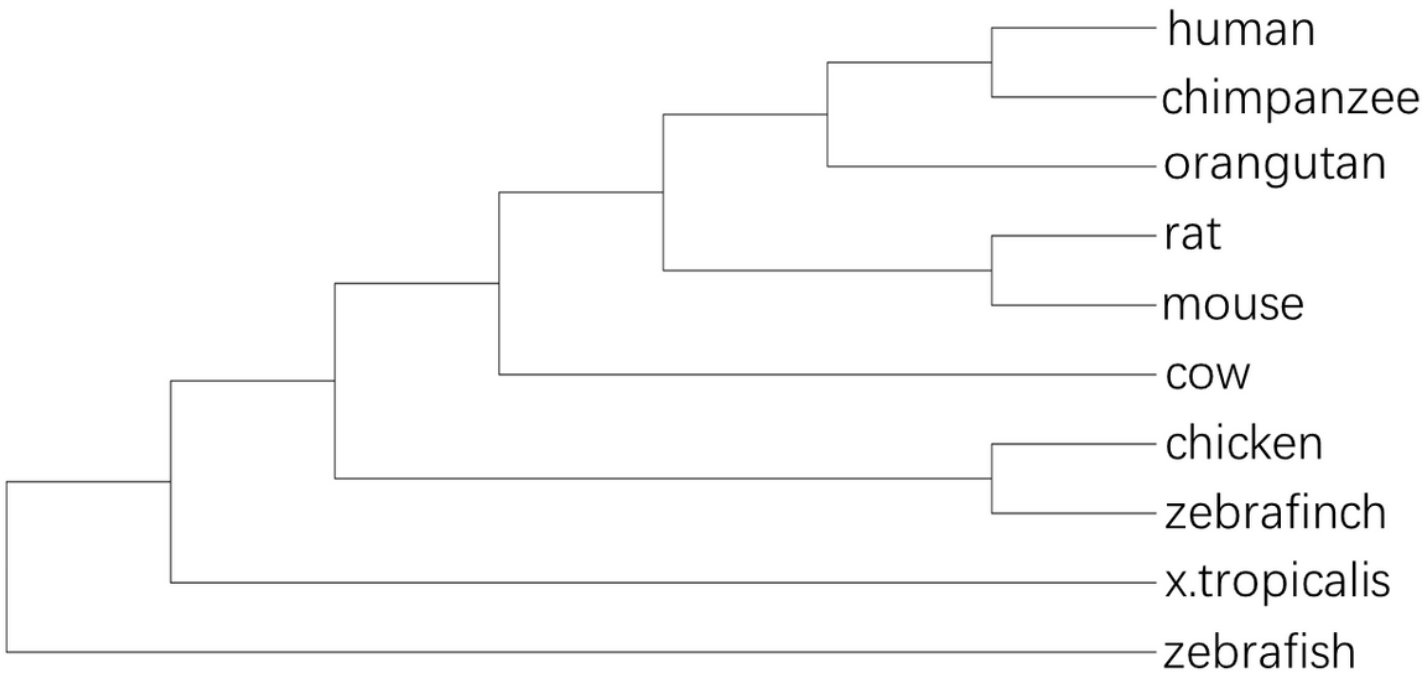

Figure 2

Phylogenetic tree of different species RNF180.

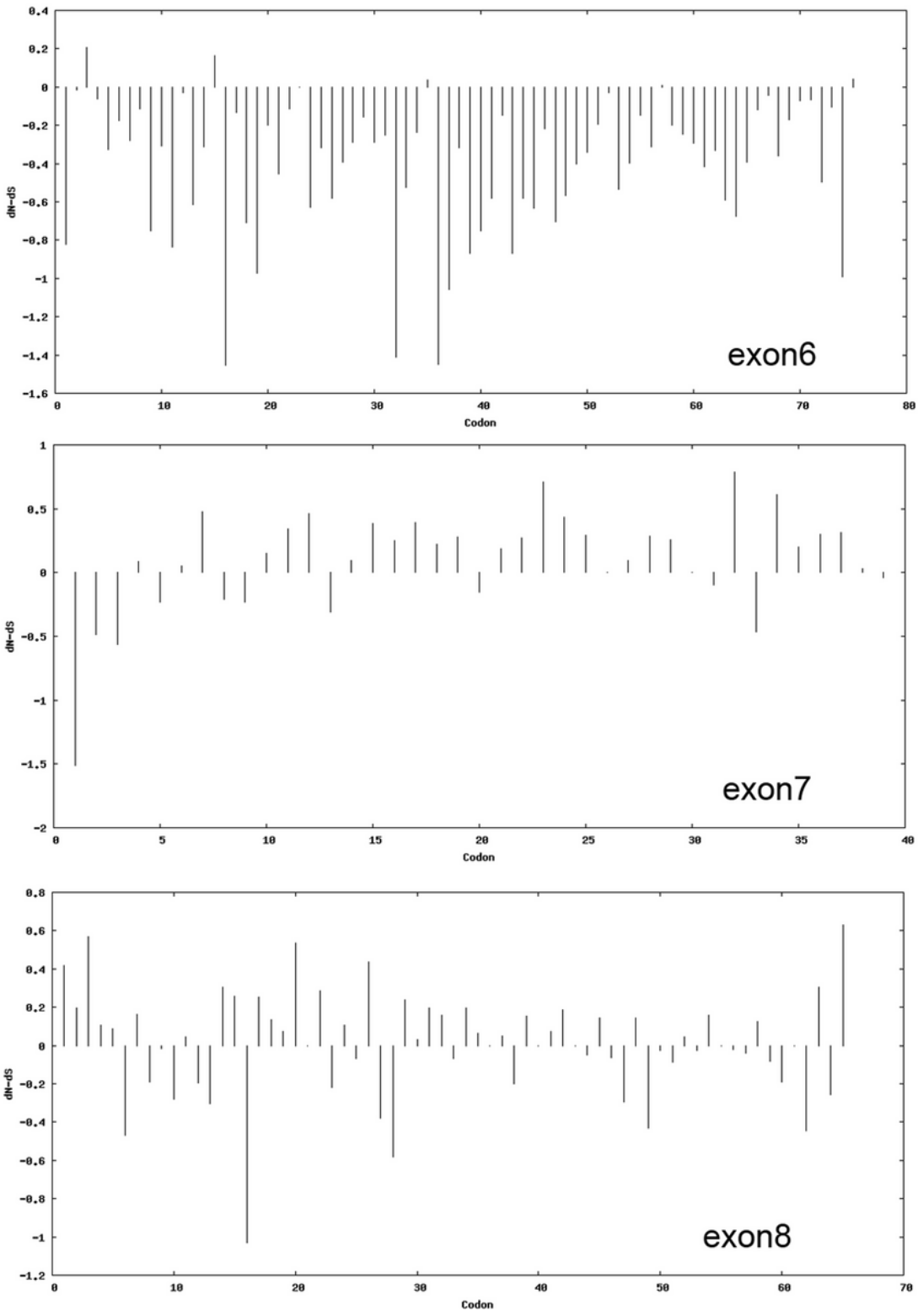

Figure 3

Exon dN / dS analysis. Abscissa for the codons of exons, ordinate as dN / Ds.

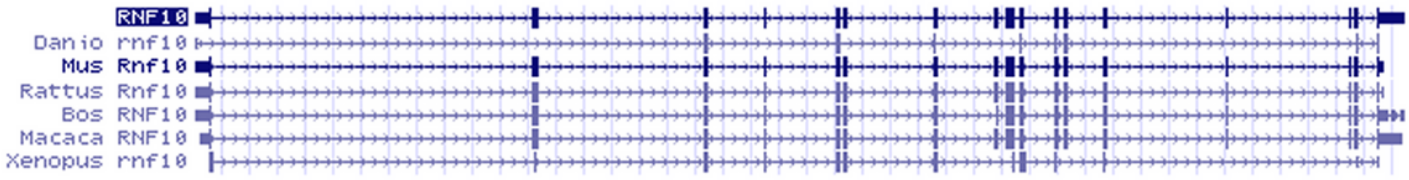

Figure 4

Other species reference sequence of RNF180 gene.

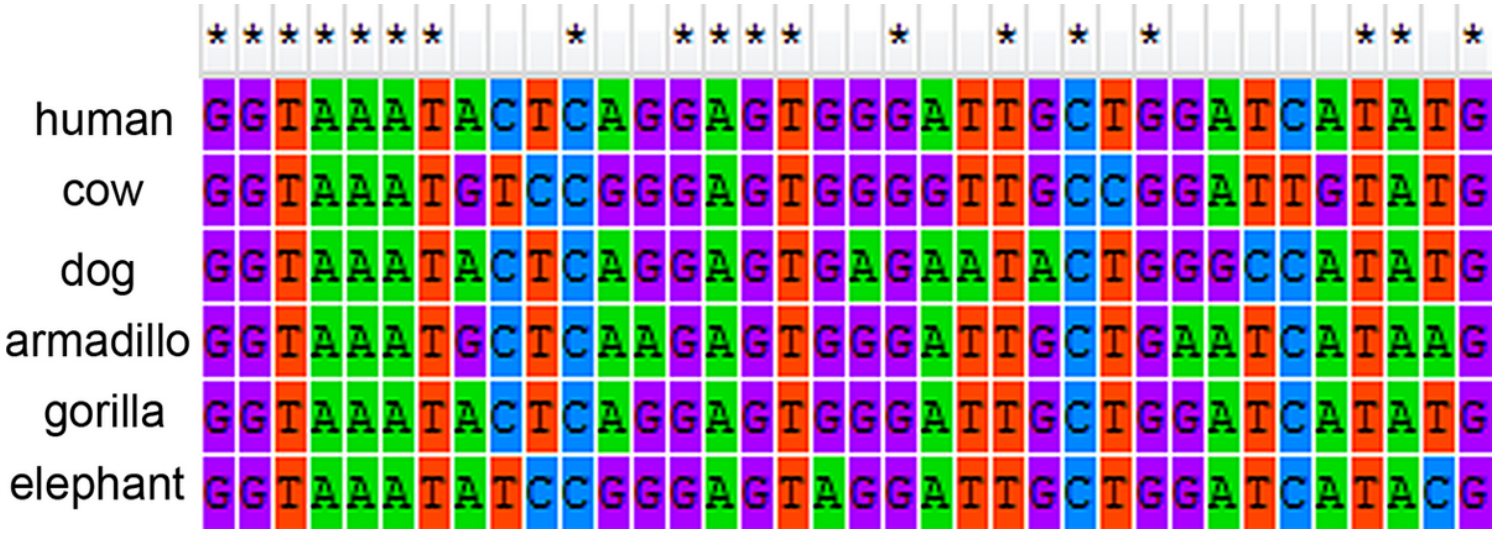

Figure 5

Results of clustering analysis of Exon5.

Supplementary Files

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- Supplementaryinformation.pdf