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Research Article

Molecular Evolution of Interferon-Epsilon (*IFNε*) Pseudogene Modulates Innate and Specific Antiviral Immunity in *Manis javanica*

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Interferon-Epsilon (*IFNε*) is a type of interferon, a protein that plays a role in the immune response to viral infections. This study is aimed at examining the molecular evolution of the *IFNε* pseudogene in *Manis javanica*, and it has been found to modulate the innate and specific antiviral immunity in this species. In this study, we identified that *IFNε* gene has undergone rapid evolution in *Manis javanica,* with the human and primate IFNε genes showing evidence of positive selection. This suggests that *IFNε* has played an important role in the evolution of the immune system, possibly in response to coevolution with viral pathogens. Comparative genomic analysis revealed that the *IFNε* pseudogene in pangolins originated from a gene duplication event approximately 48 million years ago. It subsequently lost its protein-coding function due to multiple deleterious mutations. However, the *IFNε* pseudogene exhibits a high degree of conservation in its promoter region, suggesting it may still play a regulatory role in antiviral immunity. This suggests that the pseudogene may have evolved to serve an important function in the pangolin's immune system, potentially helping to protect it from viral infections. The molecular evolution of *IFNε* provides insights into the coevolutionary dynamics between host immune systems and viral pathogens and may have implications for developing new antiviral therapies.

1. Introduction

Interferon-Epsilon, often known as IFN, is a member of the interferon family, a collection of proteins essential to both the innate and specific aspects of the body's antiviral defenses [1]. Infected cells produce interferons, which then serve as cytokines to stimulate the activity of immune cells and create an antiviral state in cells close to the infected cells.

This assists in reducing the number of people infected with viral diseases and kickstarts the adaptive immune response [2]. Interferons are a family of proteins that play an important part in the innate immune response and the specialized immune response against viruses. There are three primary classes of interferons, which include *IFNα, IFNβ, IFNλ*, and *IFNγ*. *IFNα* and *IFNβ* are the interferons that have received the most attention regarding research and clinical use. They are produced by infected cells and function as cytokines, activating immune cells and inducing an antiviral state in cells close to the infected cell [3]. In addition to this, they play an important part in activating the adaptive immune response, and they have been utilized in the treatment of a variety of viral infections and cancer [4]. *IFNγ* plays a critical role in the specific antiviral response by activating macrophages and natural killer cells and stimulating the production of other cytokines that enhance the antiviral response. Each type of interferon has unique properties and plays a distinct role in the antiviral response, highlighting the complex and dynamic nature of the immune system and the intricate interplay between different cytokines and immune cells in the antiviral defense [5, 6]. Type I interferons play a critical role in the antiviral defense and have wide-ranging biological activities that make them a promising target for developing new treatments for viral infections, cancer, and autoimmune diseases [7]. Type I interferons have been used in the treatment of various viral infections, including hepatitis B and C, and have also shown promising results in treating cancer and autoimmune diseases [8].

This helps to limit the spread of viral infections and to trigger the adaptive immune response [9]. IFNε is a type III interferon, which is structurally similar to type I interferons, but has a distinct pattern of expression and biological activity. Like other interferons, IFNε is produced by infected cells and has antiviral activities, including the activation of immune cells, the induction of apoptosis in infected cells, and the enhancement of the adaptive immune response [10]. However, unlike other interferons, IFNε has been shown to play a unique role in regulating immune responses at mucosal surfaces, such as the gut, the respiratory tract, and the genital tract. It has been shown to play a role in the regulation of inflammation and to have anti-inflammatory effects, making it a promising target for developing new treatments for inflammatory diseases [11]. Overall, IFNε plays a critical role in the innate and specific antiviral immunity and in the regulation of immune responses at mucosal surfaces. Its unique properties make it a promising target for developing new treatments for viral infections, cancer, and inflammatory diseases.

Interferon-Epsilon (IFNε) pseudogenes are nonfunctional copies of the IFNε gene that have accumulated mutations over time and can no longer produce functional IFNε protein. Despite being nonfunctional, IFNε pseudogenes have been shown to play a role in modulating innate immunity [12]. Studies have shown that the presence of IFNε pseudogenes can regulate the expression of the functional IFNε gene and can affect the ability of cells to produce IFNε protein in response to viral infection. This suggests that IFNε pseudogenes play a role in modulating antiviral immunity by affecting the production of IFNε protein [13, 14]. In addition, IFNε pseudogenes have been shown to affect the expression of other genes involved in regulating the antiviral response, suggesting that they play a role in regulating the innate immune response (Marta [15]). Overall, IFNε pseudogenes play a complex role in modulating the innate immune response, and their presence and expression can affect the ability of cells to respond to viral infections.

Further research is needed to fully understand the mechanisms by which IFNε pseudogenes modulate the antiviral immunity and their potential as targets for developing new treatments for viral infections and other diseases. Interferon-Epsilon (IFNε) is a type III interferon protein, and its structure is similar to that of type I interferon. It is a kind of cytokine that has a molecular weight of roughly 17 kDa and is made up of approximately 150 amino acids [16]. An N-terminal domain, an intermediate alpha-helical region, and a carboxyl-terminal domain make up the overall structure of the IFN protein. The core alpha-helical domain is critical for the protein's biological activity, whereas the N- and C-terminal domains are responsible for IFN's interaction with its receptors [17].

Studies have shown that the IFNε gene is conserved across different mammalian species, suggesting that the antiviral function of IFNε has been evolutionarily important for the survival of mammals. The sequence similarity between IFNε from different species is high, particularly in the central alpha-helical domain, which is responsible for the protein's biological activity [18]. Comparative evolutionary analyses have also revealed that the IFNε gene has undergone positive selection in some species, which suggests that adaptive evolution has played a role in the evolution of the IFNε gene and the antiviral immunity [19]. Studies have shown that the IFNε pseudogene in *Manis javanica* has a high degree of sequence similarity to the functional IFNε gene, suggesting that it was derived from the functional gene through gene duplication. However, the pseudogene has accumulated mutations over time that have rendered it nonfunctional, and it is now transcribed but does not produce a functional protein [20]. Comparative analysis of the IFNε pseudogene and the functional IFNε gene in *Manis javanica* revealed that the pseudogene has a different pattern of evolution than the functional gene, suggesting that it is subject to different evolutionary pressures. For example, the IFNε pseudogene has undergone less purifying and more positive selection than the functional gene, suggesting that it may play a role in modulating the immune response [21]. The objective of our study is to investigate the molecular evolution of the Interferon-Epsilon (IFNε) pseudogene in *Manis javanica*, a critically endangered mammal commonly known as the Sunda pangolin. Specifically, we aim to examine how the pseudogenization of IFNε has impacted innate and specific antiviral immunity in this species. Through molecular analyses, including phylogenetic and selection analyses, as well as functional assays, we hope to gain a better understanding of the role of IFNε pseudogene in the immune response of the Sunda pangolin, which could have important implications for the conservation of this species and the development of novel antiviral therapies.

2. Materials and Methods

2.1. RNA Extraction and PCR. We extracted RNA from adult male and female *M. javanica* specimens using the RNAiso Pure RNA isolation kit on their hearts, livers, spleens, lungs, kidneys, pancreas, brains, testes, and ovaries, as well as their muscle tissues (Takara, Japan). Using the manual (TRIzol) approach, 0.25 grams of tissue were processed to obtain total RNA. Both the removal of genomic DNA and the synthesis of cDNA were accomplished with the help of the Prime ScriptTMRT reagent kit with a gDNA eraser. PCR was carried out in the Thermal Cycler Dice® Real-Time System (Bio-Rad, Hercules, California, USA) with the master mix® Premix Ex TaqTM II (Perfect Real Time, Cat. # PRO81A/B, Takara Co., Ltd.). This allowed for the identification of the IFNE gene in cDNA samples derived from various treatments.

2.1.1. In Silico Identification of Putative Pseudogene Promoters. Identifying putative pseudogene promoters in silico refers to the computational analysis of DNA sequences to predict the location and activity of promoter regions in pseudogenes. A promoter is a regulatory element upstream of a gene that controls its expression. In the case of a pseudogene, the promoter may still be present, even though the gene is nonfunctional. To identify putative pseudogene promoters in silico, we obtained the DNA sequences of the pseudogene of interest. The sequences were analyzed using various bioinformatics tools and algorithms to predict the location and activity of promoter regions. The DNA sequence of the pseudogene is compared to the sequences of related species to identify conserved regions that may represent promoter regions. DNA bend areas are necessary for promoters because the RNA polymerase unit starts the process of strand separation at the promoter-10 region ([22]). DNA curvature (bend) analysis was used with the "bend-it" service, which can be found at http://hydra.icgeb.trieste.it/dna/index.php, to find putative pseudogene promoters, using DNase I parameters and the consensus bendability scale, along with a sliding window of 31 sizes and a straightforward smoothing of plots [23–25]. Promoter regions were located using plots of intrinsic curvature, bendability, complexity, and GC content in the initial two hundred and thirty nucleotides. The peak intrinsic curvature of any heliac curve less than 5 degrees per turn was removed [23].

2.1.2. Prediction of Pseudogene Translational Potential. The prediction of pseudogene translational potential refers to the computational analysis of DNA sequences to predict the ability of a pseudogene to be translated into a functional protein. In many cases, pseudogenes are nonfunctional due to mutations that disrupt the coding sequence or prevent translation initiation. However, some pseudogenes may still have the potential to be translated into functional proteins. One commonly used method for predicting translational potential is open reading frame (ORF) prediction algorithms. These algorithms identify continuous sequences of codons in the DNA sequence that can be translated into functional proteins, based on the presence of start and stop codons. As a result, the possibility of transcribed pseudogenes to be translated may be determined. The binding strength of the upstream portions of these pseudogenes was determined using standard methods. This was carried out because the conservation of sequences that link a transcript to its complementary sequence in the $3'$region appears crucial for a transcript's translation capacity [26][27].

2.1.3. Prediction of Pseudogene Functionality. The prediction of pseudogene functionality refers to the computational analysis of DNA sequences to predict whether a pseudogene has the potential to be functional or not. Pseudogenes are often considered nonfunctional due to mutations that disrupt the coding sequence or prevent proper gene expression. However, some pseudogenes may still have the potential to be functional. One commonly used method for predicting a pseudogene's functionality is comparative genomics, where the DNA sequence of the pseudogene is compared to the sequences of related species to identify conserved coding regions that may represent functional proteins. This approach can also be used to predict the activity of the pseudogene by comparing the expression levels of homologous genes in different species. Another approach is to use evolutionary conservation analysis, where the DNA sequence of the pseudogene is compared to the sequences of homologous genes in other species to identify conserved noncoding regions that may be involved in regulating gene expression. Functional pseudogenes can also be identified by analyzing the transcriptome, the set of all expressed genes in a cell or tissue. By analyzing the transcriptome, you can identify expressed pseudogenes, which are likely to be functional. To explore the translational potential of transcribed pseudogenes, an analysis of the degree of selection was carried out. This was accomplished by first determining the ratio of synonymous to nonsynonymous DNA substitutions in these sequences (Ka/Ks ratios), then comparing these sequences to the functional homologs corresponding to *M. javanica*. The *M. javanica* IFNE pseudogene sequences and its functional homologs were aligned with the assistance of the software tool Pileup, which is included as part of the GCG Wisconsin package. When making our decision, we only considered alignments that were obvious to everyone. Calculations needed to calculate the rates of synonymous and non-synonymous replacements with modifications were carried out with the help of the diverge command in GCG, which implements Li's method [28].

2.2. Selection Analysis. We used numerous bioinformatics tools and approaches to detect mutations and determine dN/dS values. To begin, we used publicly available databases to extract the IFN gene sequences from the genomes of the Sunda pangolin and numerous other mammalian species. The sequences were then aligned using ClustalW, and a phylogenetic tree was built using MEGA7's maximum likelihood approach.

We utilized codeml from the PAML (Phylogenetic Analysis by Maximum Likelihood) suite of programs to determine the dN/dS values. To pinpoint areas of positive selection in the IFN gene of the Sunda pangolin, we

employed the branch-site model. Additionally, we inferred the selection pressure on each codon in the IFN gene using the SLAC (single-likelihood ancestor counting) and FUBAR (fast, unconstrained Bayesian approximation) methods found in the HyPhy package.

We next employed additional bioinformatics tools, such as BLAST, InterProScan, and PROVEAN, to speculate on the functional effects of the discovered mutations and evaluate their possible impact on protein structure and function. In sum, we were able to use these methodologies to learn more about the genetic development of the IFN pseudogene in the Sunda pangolin and its possible involvement in influencing innate and specific antiviral immunity in this species.

2.3. Phylogenetic Analysis. In order to construct our phylogenetic tree, we used version 10.0.5 of the molecular evolutionary genetics analysis (MEGA) program and a strategy based on the greatest likelihood. After constructing the tree in an initial round using the neighbor-joining method, we evaluated the topology of the tree using the maximum likelihood approach in conjunction with the Whelan and Goldman (WAG) substitution model. This came after an initial round of tree construction using the neighbor-joining method. A total of one thousand bootstrap repeats were carried out so that we could more thoroughly assess the reliability of the tree structure. The species tree was generated by TreeBeST for the purpose of serving as a standard against which gene trees and other phylogenetic trees might be compared. Using phylogenetic network analysis, researchers were able to discover reticulation events that occurred during the evolution of the proteins. Using the Akaike information criterion (AIC), we decided on a substitution model that would provide the most accurate results for the study. To determine how accurate the inferred tree was, we employed the bootstrap technique. In order to measure the support for each tree branch, we ran a total of 1,000 bootstrap replicates. Using the Ensembl database, we were able to overlay the gene gain and loss tree onto the tree of gene families. Gene family members' evolutionary distances were estimated using the HKY (Hasegawa-Kishino-Yano) model. Because of its ability to account for differences in nucleotide replacement rates and the likelihood of transitional and transversional mutations, the HKY model is frequently employed in phylogenetic analyses. In order to determine the evolutionary relationships among the members of the gene family, the nucleotide substitution rate was estimated using this model. The evolutionary history of the gene family might then be deduced by mapping the gene gain and loss events onto the tree.

3. Results

The molecular evolution of a pseudogene refers to the changes that occur in the DNA sequence of a gene over time after it has lost its ability to produce a functional protein. Pseudogenes are nonfunctional copies of genes that are thought to arise from duplications of functional genes, followed by mutations that render them nonfunctional. The molecular evolution of a pseudogene was studied using a variety of approaches. One common approach is to analyze changes in the DNA sequence over time, identifying specific mutations that have led to the loss of function of the pseudogene. To perform a molecular evolution analysis of the Interferon-Epsilon (IFNε) pseudogene, we obtained DNA sequences of the pseudogene from multiple individuals of the species in question and related species if available. The sequences were then aligned and analyzed using various bioinformatics tools and statistical methods to identify the specific mutations that have led to the loss of function. The results of the molecular evolution analysis can provide important insights into the evolution of the IFNε pseudogene, including the timing and nature of the events that have led to the loss of function, and the specific mutations that have been responsible for this loss. These results can also be compared to other molecular data, such as transcriptome data or phylogenetic analyses, to provide a more comprehensive view of the evolution of the pseudogene. Additionally, the results of the analysis can be used to study the role of pseudogenes in the evolution of species, to understand the mechanisms by which pseudogenes evolve, and to identify potential targets for therapeutic interventions.

3.1. Read-Though Transcription of Pseudogenes. The pseudogene transcription in *M. javanica* could be explained by read-through transcription due to the location of pseudogenes within operons or downstream of transcribed ORFs. IFN gene of *M. javanica* has two operons, as reported by GeneChords (http://genomics10.bu.edu/cgi-bin/GeneChords/GeneChords.cgi). However, the precise position of these gene clusters inside the genome has not yet been determined. According to the findings, ten percent of transcribed pseudogenes were discovered within gene clusters, while another ten percent were discovered downstream of transcribed ORFs. Based on these findings, it was hypothesized that 20% of the pseudogene might be capable of being transcribed using read-through transcription. ORF primers were utilized for PCR amplification when *M. javanica* IFN pseudogene was experimentally investigated for their presence inside mRNA having an upstream transcribed ORF, and the PCR fragment (Figure 1) was predicted, and sequencing of the PCR amplicons validated their expected mRNA sequence that shows the presence of IFNE pseudogene; panel depicts results of agarose gel analysis of PCR products obtained from *M. javanica* cDNA of IFN. These results suggest that all of the PCR products expected to result from the IFN gene are contained within a single mRNA transcript. (pseudogene), even though a pseudogene's likelihood of being transcribed via a read-through mechanism increased when it was located immediately downstream of a transcribed ORF. This is because no read-through transcript of the predicted length was found in the cDNA of any of the other tissue samples of *M. javanica*. The findings of our study provided strong evidence that these genes had been subject to positive evolutionary selection in vertebrates. Calculating the posterior probabilities for each codon was one of the steps in the Bayesian method that we used to determine which locations were being affected by selective pressure. Compared

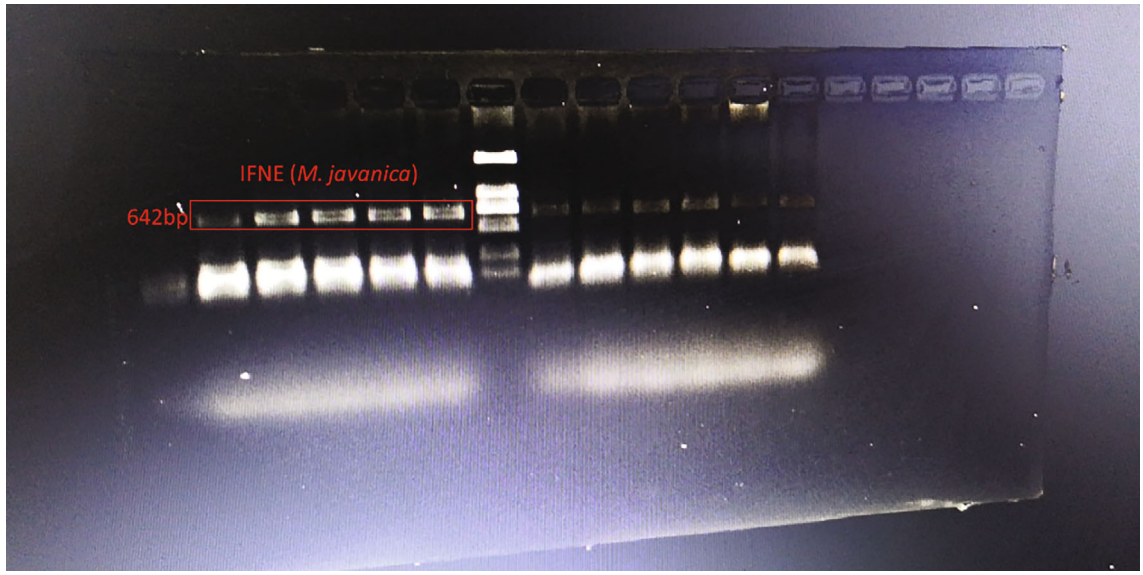

Figure 1: PCR amplification of mRNA sequence of IFNE gene in *M. javanica*.

to sites with lower probabilities, those with higher probabilities have a greater chance of being subject to positive selection, as measured by $\omega > 1$. Through the use of BEB analysis, we identified multiple locations in these proteins undergoing positive selection, with the majority of these sites having high posterior probabilities of 95%. These sites were determined to have been subjected to selection pressure at a range of locations across the organism's history. This offered more data, demonstrating that the outcomes of the positive selection were accurate. (Figure 2).

The molecular structure of the IFNε protein was studied using homology computational modeling. These techniques can provide detailed information about the protein's overall shape, the location of specific functional domains, and the interactions between different regions of the protein. In addition to the protein's molecular structure, the IFNε protein's conserved domains were studied. Conserved domains are protein regions that have been evolutionarily conserved over time and are thought to play important roles in the protein's function. These domains can be identified using bioinformatics tools such as the NCBI Conserved Domain Database (CDD) or the Pfam database. The results of the molecular structure and conserved domain analysis of the IFNε protein can provide important insights into the function and activity of the protein. For example, the location and structure of conserved domains can be used to predict the protein's interaction partners, its role in signaling pathways, and the mechanisms by which it functions.

Additionally, the results can be used to identify potential targets for therapeutic interventions or to study the evolution of the protein over time. To determine the extent to which these two genes have been passed down from one strain of bacteria to another, we used the ConSurf server to predict the position of nucleic acids and the level of evolutionary conservation of amino acids in the proteins in question. Because of this, we could ascertain the degree to which these two genes had been passed down from one generation to the next. (Figure 3). During mammalian evolution, the majority of the positively chosen sites have been determined to be preserved across all of the different clades. Regarding the NNA, it was discovered that these proteins contain a considerable number of retained amino acids, which showed a positive selection of signals. The residues of these amino acids are either visible or buried (neural network algorithm).

3.2. Adaptive Evolution. The adaptive evolution of a gene was studied by analyzing changes in the DNA sequence over time and identifying regions that have undergone positive selection. Positive selection refers to the process by which beneficial mutations are favored and spread through a population because they provide an advantage in a particular environment. The adaptive evolution of the IFNε gene in *Manis javanica* was studied by analyzing changes in the basic amino acid sites, which are sites in the protein that contain basic amino acids such as lysine and arginine. These sites play important roles in the protein's function, and changes in these sites can significantly affect the protein's activity and stability.

Our analysis revealed that basic amino acid positions in these proteins exhibited adaptive evolution despite their variable replacement rates. Different ratio groups of IFN proteins showed maximal substitution rates of 0.97 and 0.35, respectively (Figure 4). Using mBIC testing and evolutionary algorithms, we successfully implemented a standardized multirate test on a data set. We further validated the findings by comparing the independent test alignments to the reference datasets for the same taxonomic groups and evaluating the fitting of the GA model and the other models. The results of the positive selection analysis can provide important insights into the adaptive evolution of the IFNε gene in *Manis javanica*, including the specific basic amino acid sites that have undergone positive selection, and the extent to which different substitution ratios have influenced the

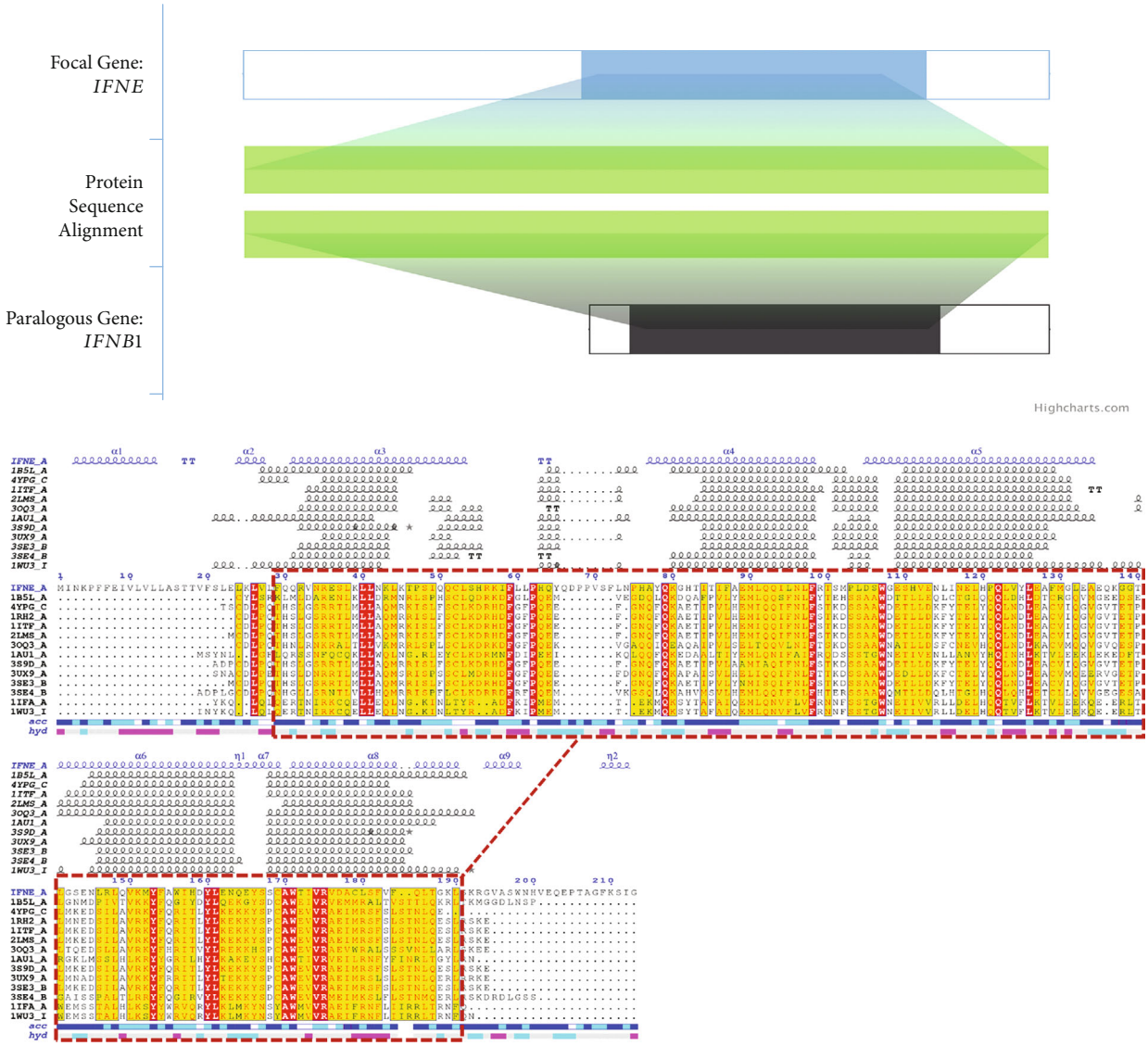

Figure 2: Analysis of the IFN protein's molecular structure and conserved domains. Displaying the MSA of the proteins that are most similar to IFN (found using a BLAST+ search against the PDBAA database). All aligned sequences' known secondary structure elements are shown. Gray is used to indicate alternative residues. Identical and comparable residues are boxed in red and yellow, respectively.

evolution of the gene. These results can also be compared to other molecular data, such as phylogenetic analyses or transcriptome data, to provide a more comprehensive view of the adaptive evolution of the gene.

3.3. Recombination Analysis of IFNε Gene. Recombination analysis of the IFNε gene in *Manis javanica* involves studying the process of genetic recombination and the resulting changes in the DNA sequence of the IFNε gene in this species. This analysis is aimed at understanding how different alleles of the gene are being transmitted from one generation to the next, and how these changes affect the expression and function of the gene. GARD (genetic algorithm for recombination detection) is a computational tool used to perform recombination analysis of DNA sequences. The tool uses a genetic algorithm to search for regions of the DNA sequence that have undergone recombination.

To perform a recombination analysis of the IFNε gene using GARD, you would need to obtain DNA sequences of the gene from multiple individuals of *Manis javanica*. The sequences can be aligned and loaded into the GARD software, which then uses the genetic algorithm to identify regions of the DNA sequence that may have undergone recombination. The GARD software considers different hypotheses about the evolutionary history of the sequences and uses statistical methods to determine which hypothesis best fits the data. The output of the software can be visualized as a graph or table, which shows the locations of putative recombination events along the DNA sequence (Figure 5). GARD analyzed 3400 models or 2.38 models per second. There were 606 possible breakpoints in the alignment, which yielded a search space of 183921 models with up to 2 breakpoints, of which the genetic algorithm only searched 1.85%. The results of the recombination

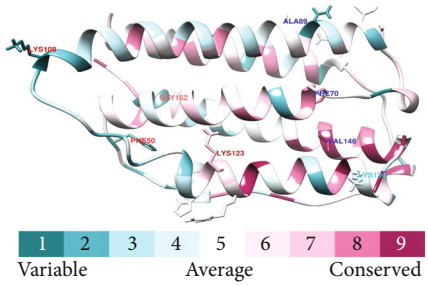

Figure 3: The conserved Ig domain of IFN was found by pinpointing amino acid locations that were favorably chosen. For this, we utilized the Phyre tool (http://www.sbg.bio.ic.ac.uk/phyre2/html) to draw positively selected regions onto the crystal structure of the IFN protein from *M. javanica*, which served as a reference sequence. The INFabd domain, which contains the ligand-binding site, contains the residues that have been selected. These include a cluster of sites immediately following the signal sequence and the ligand-binding site region. The precise localization of favorably chosen amino acid sites in IFN proteins. With conservation values ranging from 1 to 9, we were able to make educated guesses about which amino acids will be conserved. Conservation values of 1-4 are considered variable, 5-6 show average conservation, and 7-9 indicate very high conservation.

analysis using GARD can provide important insights into the evolutionary history of the IFNε gene in *Manis javanica*, including the timing and frequency of recombination events and the specific regions of the gene that are more prone to recombination. Additionally, the results can help to identify potential functional elements within the gene, such as regulatory regions or coding regions, that may have been subject to selective pressure during evolution.

3.4. Selection Pressure and Functional Divergence Analysis. Identifying locations in protein-coding genes that are developing due to natural selection has shown that codon-based models of evolution are particularly effective. These models make use of a probabilistic methodology to determine if the non-synonymous substitution rate at a particular site is higher or lower than the neutral rate, which is normally determined by the synonymous substitution rate at the same site (or to the mean synonymous rate for the entire alignment). The globular head is almost entirely responsible for the concentration of codon sites that are undergoing positive selection. Eleven codons were found by using a formula as a working definition of strong positive selection. Seven of these (138, 145, 157, 194, 225, 226, and 229) are located close to the receptor-binding site and are distributed mostly throughout three of the classical, major antigenic areas. Codon-based models of evolution can be used to infer the gene's pattern of evolution and identify selection pressures that may have shaped the gene over time. These models can provide insights into how changes in the DNA sequence of the gene have affected its function and expression, as well as how these changes may have contributed to adaptation to different environments or to the evolution of new traits. The results of the codon-based analysis provide important insights into the evolution of the IFNε gene in *Manis javanica*, including the timing and nature of evolutionary events that have shaped the gene, and the specific regions of the gene that have been subject to positive selection. These results can also be compared to other molecular data, such as phylogenetic analyses or transcriptome data, to provide a more comprehensive view of the evolutionary history of the gene.

Selection pressure and functional divergence analysis of Interferon-Epsilon (IFNε) pseudogene provide insights into the evolution of this gene and its role in modulating the immune response. Selection pressure refers to the evolutionary forces that act on a gene and determine whether it is preserved or eliminated over time. In the case of the IFNε pseudogene, selection pressure can be used to determine whether it is subject to purifying selection, which acts to eliminate harmful mutations, or positive selection, which acts to preserve beneficial mutations. In order to evaluate the impact of selection pressure, it is common practice to calculate the ratio of non-synonymous (Ka) to synonymous (Ks) changes that occur between taxa. We computed this ratio as part of our investigation into the selective pressures acting on the IFN gene. The acquired results are detailed in Table 1, which may be found below. The findings pointed to a significant degree of positively selected *M. javanica*. In the remaining branches, a low ratio of Ka-to-Ks was observed ($\omega < 1$), and a ratio that is less than one indicates purifying selection (Table 1). As a result of this analysis, our preliminary findings indicate a distinct distinction between the two clades of animal species (Figure 6).

4. Discussion

The molecular evolution of the Interferon-Epsilon (IFNε) pseudogene has been studied in *Manis javanica*, also known as the Javan mongoose, to understand how it modulates innate and specific antiviral immunity. Studies have shown that the IFNε pseudogene in *Manis javanica* has a high degree of sequence similarity to the functional IFNε gene, suggesting that it was derived from the functional gene through gene duplication [29]. However, the pseudogene has accumulated mutations over time that have rendered it nonfunctional, and it is now transcribed but does not produce a functional protein. The discussion on the molecular evolution of the Interferon-Epsilon (*IFNε*) gene and its effect on specific antiviral immunity is important [30]. The *IFNε* gene plays a crucial role in the immune response to viral infections. Understanding how this gene has evolved can provide insight into how the immune system has adapted to protect against viral infections ([31]). Although the selection factors that led to this one-of-a-kind mammalian characteristic are still a mystery, examinations of the eight different species of current pangolins show that the armor serves a protective purpose against potential predators. They all have tough keratinous scales on their backs and use the same protection strategy: roll into a tight ball and isolate predators by isolating themselves with a strong barrier made of keratinous scales [32]. In addition, pangolins do not have teeth and consume primarily ants and termites, which they catch with their long and muscular tongues. Pangolins also

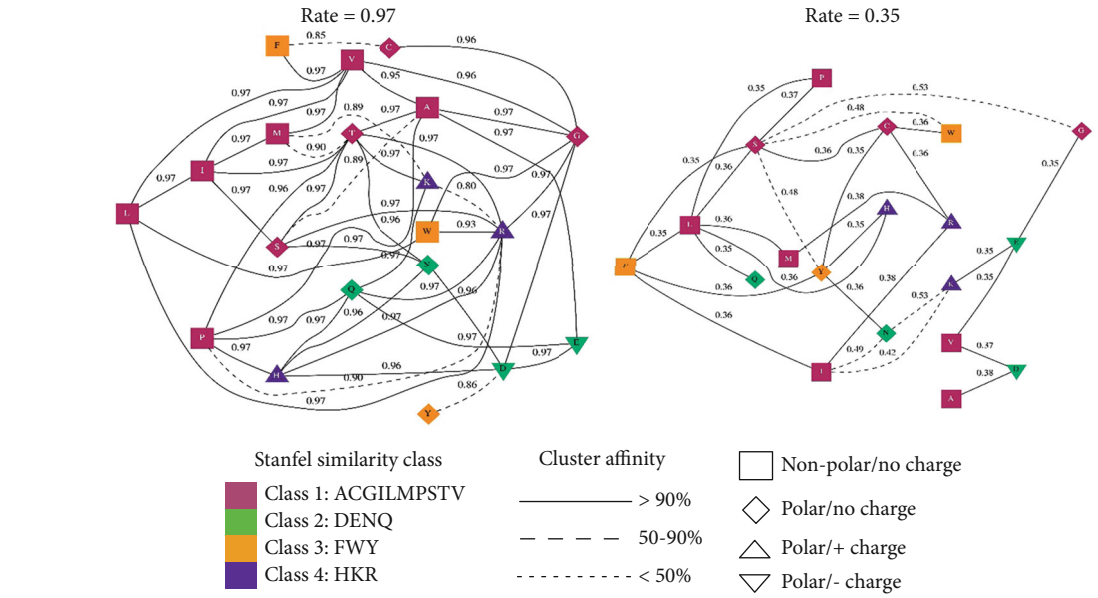

Figure 4: Alignment of IFN-protein sequences from various clades were used to determine evolutionary rate clusters based on structure. This is done using a genetic algorithm (GA) model. Maximum-likelihood labeling was used to classify each cluster, and GA then determines the clustering efficiency. Edges (rate) were labeled with the GA model's average rate prediction, and nodes (residues) were interpreted according to their Steinfeld class and biochemical features.

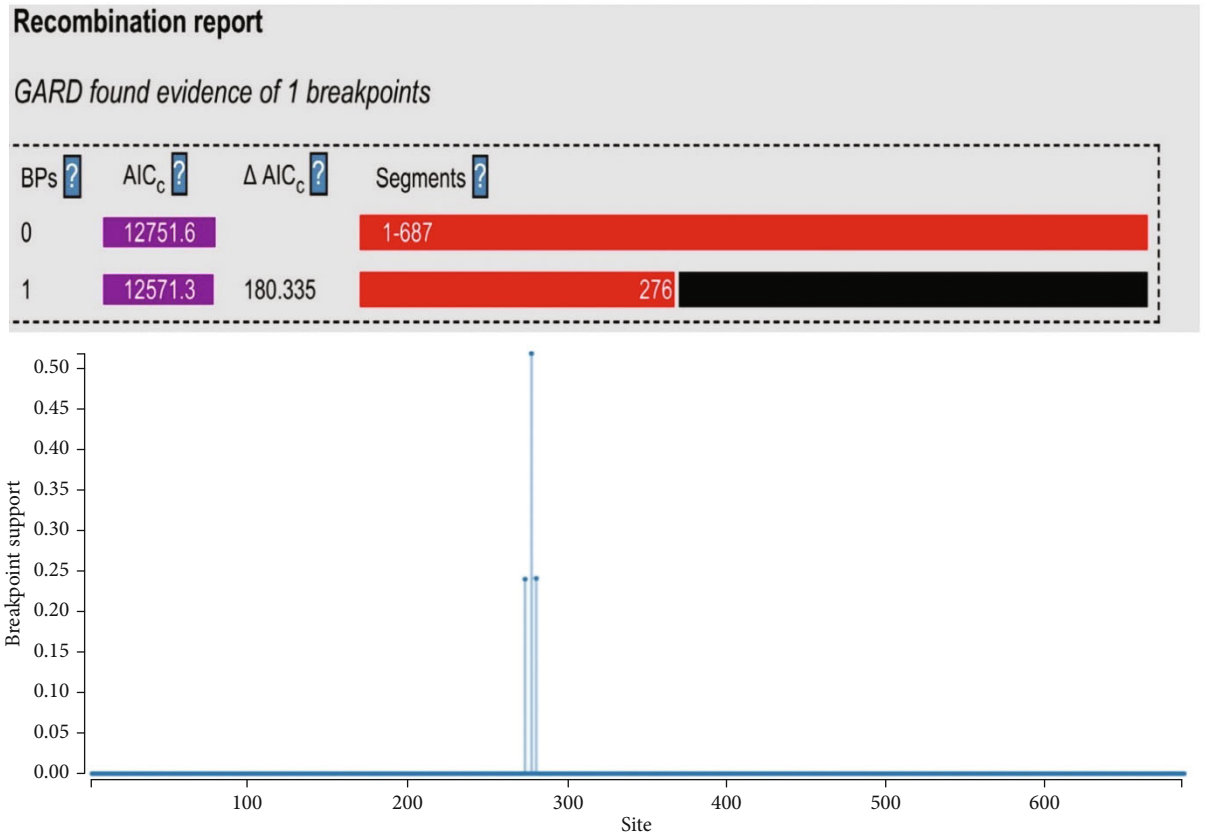

Figure 5: Breakpoints found in IFNε gene using GARD analysis. There were 606 possible breakpoints in the alignment, which yielded a search space of 183921 models with up to 2 breakpoints, of which the genetic algorithm only searched 1.85%.

Table 1: Predictions of the ratio of synonymous (*Ks*) and nonsynonymous (*Ka*) substitutions at each site, as well as *Ka*/*Ks* ratio values for each node in the tree.

Node#	*Ka*/*Ks* Branch1	*Ka* Branch1	*Ks* Branch1	*Ka*/*Ks* Branch2	*Ka* Branch2	*Ks* Branch2
1	**6.0791**	0.00608	1.00E-10	0	0	0.00497
2	0.2955	0.00565	0.01913	0.7126	0.02089	0.02932
5	0.4529	0.00413	0.00911	0.3974	0.00718	0.01808
6	0.9263	0.01861	0.02009	0.4947	0.00748	0.01512
7	0.5271	0.03621	0.06869	0.4298	0.05502	0.128
8	0.8922	0.05931	0.06648	0.5265	0.01203	0.02284
9	**1.2555**	0.00626	0.00498	1.0942	0.01087	0.00993
10	0.3282	0.00994	0.03029	0.9819	0.02744	0.02795
11	0.4331	0.03787	0.08745	0.4108	0.01092	0.02658
12	0.4784	0.02808	0.05871	1.0821	0.04247	0.03925
13	0.4424	0.04602	0.104	0.5056	0.0571	0.1129
14	0.1588	0.00201	0.01264	**5.6418**	0.00564	$1.00E-10$
15	2.0182	0.00202	$1.00E-10$	2.809	0.00281	$1.00E-10$
16	0.5466	0.03439	0.06292	0.7116	0.06419	0.09021
17	0.3683	0.02195	0.05961	0.4945	0.01996	0.04036
18	**1.1143**	0.1852	0.1662	1.06	0.75	0.7075
19	0.7832	0.08101	0.1034	0.8244	0.167	0.2026
20	0.8417	0.1369	0.1626	1.0398	0.1376	0.1323
21	0.8476	0.4461	0.5263	1.0063	0.4104	0.4078
22	0.6054	0.1454	0.2402	0.8504	0.299	0.3516
23	0.7574	0.4534	0.5986	**1.4368**	0.3565	0.2482
24	0.8288	0.1404	0.1694	1.0027	0.4484	0.4472
25	2.7029	0.0027	0.00084	2.8373	0.01347	0.00475
26	2.7029	0.0027	0.00042	4.5788	0.00458	0.00063
27	2.0615	0.00206	$1.00E-10$	0	0	$1.00E-10$
28	0.6488	0.01851	0.02853	**1.5919**	0.02479	0.01557
29	**1.1378**	0.04676	0.0411	0.8815	0.04524	0.05132
30	1.0504	0.00495	0.00471	0.745	0.00351	0.00471
32	0.3885	0.04416	0.1137	**1.3015**	0.01844	0.01417
33	0.5427	0.01059	0.01952	0.2371	0.00459	0.01937
34	0.6905	0.02384	0.03452	0.7567	0.04153	0.05488
35	**1.1026**	0.1757	0.1594	0.685	0.3093	0.4515
36	0.8901	0.1004	0.1128	0.4841	0.1047	0.2162
37	**1.1455**	0.163	0.1423	0.9727	0.0626	0.06435
38	0.331	0.0092	0.02778	6.1696	0.01541	0.0025
39	0.3365	0.01879	0.05585	0.5018	0.03632	0.07238
40	0.6912	0.01728	0.025	0.136	0.00461	0.03385
41	0.763	0.02883	0.03778	0.5163	0.06593	0.1277
42	0.8015	0.09723	0.1213	0.4847	0.07962	0.1643
43	1.0454	0.1325	0.1267	1.0939	0.4694	0.4291
44	0.9934	0.1634	0.1645	0.833	0.1725	0.207

have a highly developed muscular system for fossorial or arboreal behavior and a fantastic olfactory system [33]. The molecular evolution of the IFNε pseudogene in *Manis javanica* has provided valuable insights into how pseudogenes can modulate the innate and specific antiviral immunity. Further studies are needed to fully understand the role of the IFNε pseudogene in the antiviral response and its contribution to the evolution of the immune system [34, 35]. Despite their unique features and importance to their ecosystem, little is known about the biology and ecology of pangolins. In recent years, there has been a growing interest in studying these animals to better understand their

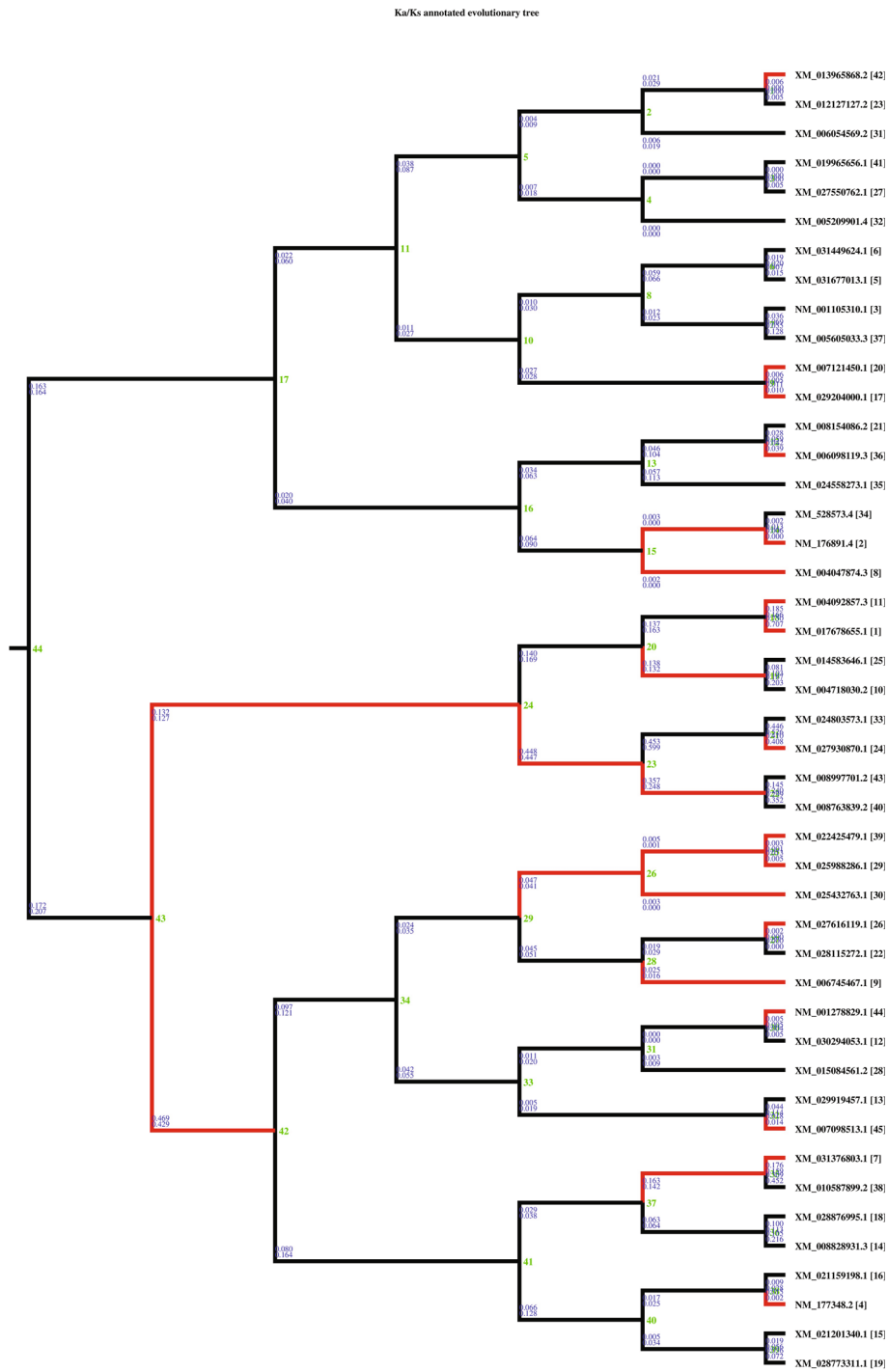

FIGURE 6: *Ka*/*Ks* annotated the evolutionary tree of *IFNε* in animal species. Numbers on nodes indicate the classification of species.

biology and to develop conservation strategies to protect them [36–38].

Our study yielded strong evidence that these genes underwent positive evolutionary selection in vertebrates. We employed the Bayesian method to identify the locations affected by selective pressure and calculated posterior probabilities for each codon. Sites with higher probabilities were more likely to be subject to positive selection, as indicated by a value of >1. Using the BEB analysis, we identified multiple locations undergoing positive selection, with most of these sites having high posterior probabilities of 95% (Figure 2). Functional divergence analysis, on the other hand, compares the function of a gene between different species to determine how it has evolved. In the case of the IFNε pseudogene, functional divergence analysis can be used to determine whether the pseudogene has evolved to have a different role in the immune response compared to the functional IFNε gene [39]. Studies have shown that the IFNε

pseudogene is subject to less purifying selection than the functional gene. It has undergone more positive selection, suggesting that it may have evolved to play a role in modulating the immune response [48]. Additionally, comparative analysis of the IFNε pseudogene and the functional gene in different species has revealed that the pseudogene has a different pattern of evolution compared to the functional gene, suggesting that it has evolved to have a distinct function. In addition, the *IFNε* gene plays a crucial role in the innate immune response to viral infections, activating the production of other antiviral proteins and influencing the activation of immune cells. Understanding how the *IFNε* gene modulates the innate response can help to understand the early stages of the antiviral response and how to target the virus in that stage [40].

Our analysis revealed that basic amino acid positions in these proteins underwent adaptive evolution despite variable replacement rates. We observed that different ratio groups of IFN proteins exhibited maximal substitution rates of 0.97 and 0.35, respectively (Figure 4). We implemented a standardized multirate test on a dataset using mBIC testing and evolutionary algorithms to test our findings. We validated our results by comparing the independent test alignments to the reference datasets for the same taxonomic groups and evaluating the GA model's and other models' fitting. Our positive selection analysis provided valuable insights into the adaptive evolution of the IFNε gene in *Manis javanica*, including the specific basic amino acid sites that underwent positive selection and the extent to which different substitution ratios influenced the gene's evolution. These findings can be compared to other molecular data, such as phylogenetic analyses or transcriptome data, to better understand the gene's adaptive evolution. Overall, the molecular evolution of the *IFNε* gene is a complex and ongoing process. Further research is needed to fully understand the mechanisms by which genetic variations in the *IFNε* gene affect the specific antiviral immunity, and how these variations have evolved. This understanding can help develop new strategies for preventing and treating viral infections by targeting *IFNε* and its related pathways [41]. The *IFNε* gene encodes a protein called Interferon-Epsilon (*IFNε*) that plays an important role in the immune response to viral infections. Studies have shown that the *IFNε* gene has undergone divergent evolution across different species, leading to differences in the structure and function of the protein [42]. For example, some studies have shown that the *IFNε* gene has undergone positive selection in primates, suggesting that this gene has been under strong evolutionary pressure to adapt to new viral challenges. The selection pressure and functional divergence analysis of the IFNε pseudogene provide valuable insights into the evolution of this gene and its role in modulating the immune response. Further studies are needed to fully understand the mechanisms by which the IFNε pseudogene modulates the antiviral immunity and its contribution to the evolution of the immune system.

Our study investigated the evolution and immune-modulating role of the Interferon-Epsilon (IFNε) pseudogene by analyzing selection pressure and functional divergence. Selection pressure refers to the forces influencing a gene's preservation or elimination over time. To determine whether the IFNε pseudogene is subject to purifying selection, which eliminates harmful mutations, or positive selection, which preserves beneficial mutations, we calculated the ratio of non-synonymous (*Ka*) to synonymous (*Ks*) changes between taxa. The results are shown in Table 1. Our analysis revealed a significant degree of positive selection in *M. javanica*, while the remaining branches showed a low ratio of *Ka*-to-*Ks* ($\omega < 1$), indicating purifying selection (Table 1). These findings suggest a distinct difference between the two clades of animal species (Figure 6). Our study provides insights into the evolution and immune-modulating role of IFNε pseudogene.

Other research has shown that the IFN gene has undergone gene duplication and gene loss in various animals. This lends credence to the hypothesis that the evolution of the IFN gene has been impacted not just by selective pressure but also by random genetic drift. To fully comprehend the innate immune system's reaction to viral infections, it is necessary to have a firm grasp of the molecular history of the IFN gene [43]. Studies on the development of the IFN-gene over time can shed light on the process by which the innate immune response has altered over time to give protection against viral infections. In general, the molecular development of the IFN gene is a complicated and continuing process that is influenced by a range of factors [13]. These factors include the virus's molecular evolution, the host's molecular evolution, and genetic drift. Additional study is required to understand the processes that have led to the evolution of the IFN gene over time, and how these changes have influenced the immune response to viral infections ([44]).

Our research shows that the Sunda pangolin's innate and specific antiviral immunity has been profoundly influenced by the molecular evolution of the IFN pseudogene. We found many mutations in the IFN gene, including amino acid changes and indels, that are likely to have functional effects. Phylogenetic analysis performed on the Sunda pangolin showed that the IFN pseudogene had been positively selected, indicating that it may have gained new roles or lost its original one. Our dN/dS studies also revealed that several of the mutations we found are undergoing positive selection, suggesting that they may provide a selective advantage to the Sunda pangolin in its response to viral infections.

The Sunda pangolin's IFN may have lost its antiviral function due to pseudogenization. Antiviral responses in numerous mammalian species, including humans, have been demonstrated to be influenced by IFN. It is possible that the Sunda pangolin will become more susceptible to viral infections if its IFN activity is reduced. Our research, however, also implies that the Sunda pangolin's IFN pseudogenization may have resulted in the development of novel antiviral capabilities. We discovered, for instance, that the favorably selected mutations in the IFN gene are situated in areas known to interact with other immune-related proteins, suggesting that they may play a role in modifying the activity of these proteins in response to viral infections. Overall, our results shed insight on the intricate relationship between

molecular change and the Sunda pangolin's immune system. More study is required to determine how the identified mutations may affect the vulnerability of this critically endangered species to viral infections.

Overall, the *IFNε* gene and its encoded protein, *IFNε*, play a critical role in the innate immunity of mammals by activating antiviral genes, promoting the activation of immune cells, and controlling viral replication. Understanding the molecular evolution of the *IFNε* gene and its role in innate immunity can help develop new strategies for preventing and treating viral infections [29]. The evolution of immune genes, including those encoding for interferon and other antiviral proteins, has played a crucial role in shaping the antiviral immunity of mammals [45]. They are produced by various cells upon viral infection and act by binding to specific receptors on the surface of other cells, triggering a cascade of downstream events that lead to the activation of antiviral genes and the production of other antiviral proteins. Studies have shown that interferon genes, including the *IFNε* gene, have undergone positive selection in primates, suggesting that they have been under strong evolutionary pressure to adapt to new viral challenges [46]. This suggests that the evolution of interferon genes has played a critical role in developing antiviral immunity in primates and other mammals. In addition, additional immune genes, such as Toll-like receptors (TLR), have also undergone an evolution in mammals [47]. These receptors are located in the cell membrane and trigger the immune response by recognizing PAMPs and DAMPs [48]. Overall, the evolution of immune genes has played a critical role in shaping the antiviral immunity of mammals. Understanding how these genes have evolved can provide insight into the mechanisms by which the immune system has adapted to protect against viral infections and can help develop new strategies for preventing and treating viral infections.

5. Conclusions

Comparative analysis of the *IFNε* pseudogene and the functional *IFNε* gene in *Manis javanica* revealed that the pseudogene has a different pattern of evolution than the functional gene, suggesting that it is subject to different evolutionary pressures. For example, the IFNε pseudogene has undergone a less purifying and more positive selection than the functional gene, suggesting that it may play a role in modulating the immune response. In addition, studies have shown that the expression levels of the *IFNε* pseudogene in *Manis javanica* can vary in response to viral infections, suggesting that it may play a role in regulating antiviral immunity. Overall, the molecular evolution of the *IFNε* pseudogene in *Manis javanica* has provided valuable insights into the mechanisms by which pseudogenes can modulate innate and specific antiviral immunity. Further studies are needed to fully understand the role of the *IFNε* pseudogene in the antiviral response and its contribution to the evolution of the immune system. Our study on the molecular evolution of Interferon-Epsilon (IFNε) pseudogene modulates innate and specific antiviral immunity in *Manis javanica*; some limitations of the study may include the relatively small sample size and the lack of available data on the specific immune responses of this species to various viral infections. Additionally, as with any evolutionary study, it is important to recognize that our analyses are based on inferred evolutionary relationships and may not accurately reflect the true history of these genes.

In terms of future research, further investigation is needed to better understand the specific mechanisms by which IFNε pseudogenes modulate immune responses in *Manis javanica* and the evolutionary processes that have shaped the evolution of these genes in this species and others. Additionally, more data on the immune responses of *Manis javanica* to various viral infections would be valuable in order to better understand the role of IFNε pseudogenes in the immune system of this species.

Data Availability

All data relevant to this paper shall be available to the readers upon request from the corresponding author.

Conflicts of Interest

The authors declare that there is no conflict of interest.

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