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EXPERIMENTAL INJECTION OF α-INTERFERON (CHINESE HAMSTER OVARY DERIVED) RECOMBINANT PROTEIN TO RED-CLAW CRAYFISH (*Cherax quadricarinatus*): THE FIRST REPORT

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between both authors. Author DS principal investigator, analyzing data and writing report. Author LO giving assistance in the experimental design, data analyzing and writing. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: Interferon (IFN) was recognized as a natural defense of vertebrates in response to viral infection. In later studies, IFN regulatory factor genes (*IRF*)s within Metazoan Kingdom was identified in Phylum Arthropoda, which are Chinese mitten crab (*Ericheir sinensis*) (*Er*S1, GenBank no. FG360214) and Pacific white shrimp (*Litopenaeus vannamei*) (*PENVA*, GenBank no. AOAOR6M5F1). This study aimed to confirm the existence of an IFN pathway in red claw crayfish (*C. quadricarinatus*), which also belong to Phylum Arthropoda.

Methodology: α-IFN, Chinese hamster ovary derived (CHO-Derived) recombinant protein was subcutaneously injected into adult red-claw crayfish at different dosages per body weight (BW), namely a.100 IU, b. 200IU, and c. 225IU. The right reflecting-time of the red-claw was counted. Polymerase chain reaction (PCR) was conducted using designed primers from Chinese mitten crab (*E. sinensis*).

Results: Basic Local Alignment Search Tool for nucleotides (BLASTn) showed somewhat similar characteristics between our sequences and other crustacean species. The highest matched value was 89% red-claw crayfish (*C. quadricarinatus*) microsatellite cqu.005 (GenBank no. AF156901)) and the lowest was 66% Noble crayfish (*Astacus astacus*) clone Aas8 microsatellite (GenBank no. EU692886). Two of our sequences matched 72 and 75% with a sequence of Chinese mitten crab (*E. sinensis*) microsatellite ES19 (GenBank no. DQ388785).

Conclusion: In conclusion, since the designed primers did not support the idea of an *IRF* as part of an IFN pathway existing in red-claw crayfish, our study tends to confirm that to date, no other *IRF* exists in Arthropoda, except perhaps in the Chinese mitten crab and Pacific white shrimp. This finding supports the rational consideration that *IRFs* within the Metazoan Kingdom, Phylum Arthropoda, might have diverged somewhat or been completely lost during evolution.

Keywords: Interferon; arthropoda; Chinese mitten crab; pacific white shrimp; red-claw crayfish.

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1. INTRODUCTION

Red-claw crayfish *(C. quadricarinatus*) are inherent to the top of the Cape York Peninsula and the river systems flowing into the Gulf of Carpentaria, Arafura Sea and Southern Papua New Guinea. It has become an economically important aquaculture species because of its marked preference for the slower moving reaches of the waterways, lagoons, and creeks. In addition, it can endure a wide range of water quality and climatic environments [1].

There have been many interests in red-claw crayfish aquaculture for the last three decades. Industry developers were rapidly learning the species' benefits, such as grow rapidly, ease of propagation, lack of any free-living larval stages, gregariousness, and robust to poor water quality conditions. That interest encouraged studies that led to the development of optimum aquaculture and best practice techniques. Even so, red-claw farming in Queensland has not lived up to primary predictions and expectations, and production has remained relatively small. There are very few registered red-claw farmers in the Territory [1,2].

Crayfish disease issues have become important with several infections were being described from farms in Australia since the 1990s, following the establishment of the industry. As a result, research studies on virus pathogens of freshwater crayfish have attracted so many interests with a growing number of published reports for the last 10 years. Different viruses, including *C. quadricarinatus* presumptive hepatopancreatic reovirus [3], *Pacifastacus leniusculus* bacilliform virus (PlBV) [4], white spot syndrome virus (WSSV) or white spot syndrome associated baculovirus (WSBV) [5], Infectious pancreatic necrosis virus (IPNV) [6], Infectious hypodermal and hemotopoietic necrosis virus (IHHNV) [7], *Chequa iflavirus* [8], and Athabvirus [9], to name but view.

Interferons (IFN)s were discovered fifty years ago and are family members of the cytokines produced by leucocytes [10]. Interferon was recognized as a natural defense in response to viral infection for several reasons [11]. First, there is a strong correlation between the production of IFN and natural recovery in many viral infections. Second, the severity of infection is in line with the inhibition of IFN production or action. Third, IFN treatment eliminates viral infection [11].

Even though it was believed that IFN regulatory factor genes (*IRF*)s only existed in vertebrates, the study of their origins has still to be confirmed [12,13,14]. For example, previous studies [15,16,17] detected IRF-like genes in genomic and expressed sequence tags (EST)s and genomic databases of Metazoa and Protostomia. In fact, a comprehensive search of *IRF* sequences in available databases and an analysis of different genes and proteins of *IRF*s in metazoans [18] confirmed the incidence of IRF family members within metazoans. For example, two (*IRF*)s existed in the genome of Porifera, Placozoa, and possibly Ctenophora. Since all the sequences belong to any single species cluster together in these groups, partial diversity of *IRF* sequences was obvious.

On the other hand, there were up to five *IRF* family members per genome in Cnidaria. The diversification of genes increased because the genes formed two separate clusters. In the Protostomian group of Lophotrochozoa, the number of genes differed significantly with Mollusca and Platyhelminthes representing two extremes. The *IRF* family in Mollusca diversified up to seven per genome and their sequences formed four clusters. In contrast, just one *IRF* was found in some genomes of Platyhelminthes. Finally, a reduction of the IRF family complexity was suspected to occur in the Protostomian group of Ecdyzoa. There was no sign of *IRF* DBD sequences in the genomes and EST sequences of Ecdyzoans such as Nematoda and Hexapoda. However, several *IRF*s were found in other Arthropoda such as Chelicerata and Crustacea. For example, two *IRF*s per genome were found in Chelicerata (*Ixodes scapularis*). Interestingly, even though extensive sequences existed in crustaceans within Arthropoda, *IRFs* were detected in Chinese mitten crab (*E. sinensis*) which was *ErS1*. The original sequence of *ErS1* was deposited (GenBank no. FG360214) [19]. Another *IRF* of crustacean was identified in Pacific white shrimp (*L. vannamei)* which was *PENVA* (GenBank no. A0A0R6M5F1) [20]. Therefore, it suggested that the *IRF* family was either eliminated or diverged at least twice during the evolution of Metazoan Kingdom [18].

This study aimed to obtain preliminary information on the tolerance of the challenged crayfish (*C. quadricarinatus*) by comparing the effect of different dosages of α-IFN (CHO-DERIVED) recombinant protein injected into the crayfish and to confirm the existence of an *IRF* in red-claw crayfish using PCR. The study will contribute to an overview of the existence of an IFN pathway in red-claw crayfish (*C. quadricarinatus*).

2. MATERIALS AND METHODS

2.1 Source of Experimental Animals

Adult red-claw crayfish (*C. quadricarinatus*) were obtained from the breeding facility at James Cook University (JCU), Townsville Australia and

transferred into separate tanks for the study. The experiment was set in randomly designed blocks of two recirculation tank systems (A and B) as the experimental tanks for three weeks. Red-claw crayfish (the average BW was 26.17± 2.26 g) were randomly distributed within the two tank systems. Small baskets were placed inside the aquaria (each of 28 cm in wide, 51 cm in length, and 26 cm high) and used to house the red-claw crayfish with the initial stocking densities of 2 or 3 animals per aquarium. The red-claw were given mixed food, including vegetables (chopped potatoes, carrots, broccoli, and lettuce), chicken and prawn pellets *ad libitum*.

2.2 Challenge Tests

Prior to injection of interferon, a 20 ml stock of the drug was made by diluting $5x10^5$ IU of highly active IFN or α-IFN (CHO-Derived) recombinant protein (GenWay Biotech Inc., Australia) into a mixed solution of 1 ml distilled water and 19 ml Phosphate buffer saline (PBS), according to the manufacturer's protocol (25 IU is equal to 1 µl drug).The stock was refrigerated at 4°C until required.

Crayfish were subcutaneously injected with interferon during the first week to assess their tolerance to the drug at three different dosage levels per gram body weight (BW), namely a.100 IU, b. 200 IU, c. 75 IU inoculated three times, and one group of crayfish was used as a control (without injection). Each treatment had two replicates of five animals. The crayfish were placed immediately into the rearing aquaria following injection. The experimental period began on the first day of injection and concluded on day 21.

All crayfish were tested daily to find out their righting reflex time by reversing their bodies to dorsal recumbency and counting the actual time of reversal back to the normal position. The average righting reflex time was the ratio between the average righting reflex time and the number of days alive whereas last righting reflex time was the righting reflex time on the last day alive.

2.3 Statistical Analyses

All statistical analyses were performed using a Statistical Package for the Social Sciences (SPSS) version 18 (PASW®Advanced Statistics 18) following the instructions. The effect of treatment (different dosages of interferon), tank allocation and the righting reflex test were determined by univariate analysis of variance (ANOVA). Data were assessed for normality using Q-Q plots and failed. Therefore, it was transformed using log10. When the ANOVA test showed a significant difference, comparisons between significant means for both analyses were performed using least significant difference (LSD).

2.4 DNA Extraction

Total DNA was extracted from approximately 15-30 mg of pleopod tissues of five male red-claw crayfish using High Pure PCR Template Preparation Kit (Roche Diagnostics) following the manufacturer's protocol. However, DNA was eluted in 100 µl prewarmed elution buffer. Total DNA was quantified by spectrophotometry using a NannoPhotometer™ (Implen, Germany). Two wavelengths (260 nm (A_{260}) and 280 nm (A_{280})) were recorded against an elution buffer (blank). Estimation for the purity of the nucleic acid in a DNA sample was confirmed by the ratio between the two different wavelength readings (A_{260} and A_{280}). A ratio between 1.8 and 2.0 indicated pure DNA preparations.

2.5 Polymerase Chain Reaction (PCR)

Two sets of primers, namely 370F/R and 376F/R (Sigma-Aldrich. Co.) that were designed from a deposited sequence of Jiang et al. 2009 (GenBank no. FG360214) using Oligo7 software (Table 1) was used for amplification. The PCR reaction mixture contained 1 U PCR Master Mix 2x (Promega Australia), 0.2 µM each primer, and a 50 ng eluted DNA template. The PCR reaction volume in all amplifications was adjusted with nuclease-free water to reach a final volume of 25 µl.

A negative control containing no DNA template was applied in both PCR amplification using decapod and designed primers whereas no positive control was applied due to the lack of availability. Amplification was conducted in a thermocycler (Eppendorf, Germany). The amplification profiles began with an initial denaturation at 94°C for 4 min and 35 cycles of denaturation at 94°C for 30 s, annealing was 43°C, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. Amplified products were run on a high-speed gel electrophoresis system (Biokeyston Co., USA) and visualized on 1.2% agarose gels containing GelRed (Biotium, USA) at a concentration of 0.05 µl ml^{-1}. In addition, an ultraviolet transillumination box (Syngene, USA) that was linked with Gensnap software was used to preview and photograph the amplified gels.

Fragments of DNA with a range of product lengths between 200 and 1000 bp were chosen and excised using a clean scalpel blade and transferred into 1.5 ml microcentrifuge tubes and were directly used for DNA cloning to obtain plasmid DNA which was then sequenced.

Table 1. Designed primers used in the present study

Primer pairs	Primer sequences (5'-3')	Expected products (bp)	T_A (°C)	Reference
370 F/R	ACATGTCCCCTTCCGTCACACC TTGGCCCAGTCTGTGAACCT	370	43	[19]
376 F/R	CGCCGCCACATCACCCGTTC GTCCCCGTCGGTGGAGCGTCT	376	43	

T_A: Annealing temperatures

2.6 DNA Cloning and Sequencing

The PCR products were initially purified by centrifugation with the Wizard®SV Gel and PCR clean-up system (Promega Australia Part#TB308) to eliminate primer-dimers or unexpected reaction products, and to enhance efficient ligation. At the next stage, the amplicons were cloned into 50 µl JM109 High-Efficiency Competent Cells (Promega Australia), using the pGEM®-T and pGEM®-T Easy Vector systems (Promega Australia Part# TM042). At least two blue-white colonies were screened for isolation of the recombinant plasmids using the Wizard Plus®SV minipreps DNA purification system (Promega Australia Part# TB225) following the manufacturer's protocol. Two forward and two reverse reactions were performed for each clone.

Sequencing was performed by Macrogen Inc. using universal primers M13pCU (Forward: 5'GTTTTCCCAGTCACGAC 3'/Reverse: 5'CAGGAAACAAGCTATGAC 3'). A series of contiguous, overlapping, cloned DNA fragments (contigs) was performed using Sequencher® version 4.10.1 (Gene Codes Corporation). Nucleotide sequences were submitted into a basic local alignment search tool (BLAST) for comparison against known nucleotide sequences submitted to the GenBank databases (National Centre for Biotechnology Information, NCBI) (http://www.ncbi.nlm.nih.gov).

3. RESULTS AND DISCUSSION

3.1 Challenge Test

There was no significant difference for the mean of righting reflex time (p>.05) and the last righting reflex time (p>.05) across the four treatments and the two systems. All treatment groups showed the mean of righting reflex time and the last righting reflex time longer than that of control groups. It suggests that the dosage level of interferon that was used in our study was toxic to crayfish. Therefore, lower dosages should be applied in future studies.

3.2 Polymerase Chain Reaction (PCR) and Sequence Analyses

The analysis of some of our obtained nucleotide sequences against known nucleotides through BLASTn showed somewhat similar characteristics to other crustacean species (Table 2). The highest matched value was 89% (Red-claw crayfish (*C. quadricarinatus*) microsatellite cqu.005 (GenBank no. AF156901)) and the lowest was 66% (Noble crayfish (*Astacus astacus*) clone Aas8 microsatellite (GenBank no.EU692886)). Two of our sequences matched 72 and 75% with a sequence of Chinese mitten crab (*E. sinensis*) microsatellite ES19 (GenBank no. DQ388785) (Table 2).

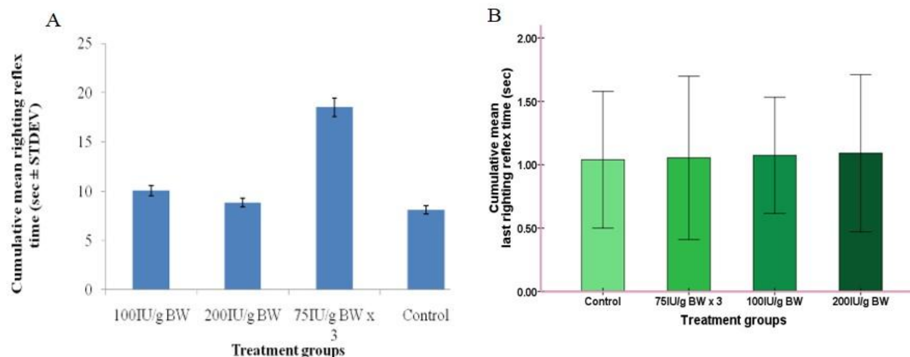

Fig. 1. A. Cumulative mean righting reflex time (sec ± STDEV); B. Cumulative mean last righting reflex time (sec ± STDEV) across the four treatment groups of red-claw crayfish (*C. quadricarinatus*)

Table 2. Basic local alignment search tool (BLASTn) results

Contigs*	Matched species		Accession no.	E-value	Query coverage	Identitities	Length (bp)	Decription of sequences
	Common names	Scientific names						
$A7_1$	Red-claw crayfish	*C. quadricarinatus*	GQ286098	4.00E-34	50%	180/242	467	clone GB_8B mRNA
$A7_2$	Noble crayfish	*Astacus astacus*	EU313798	3.00E-17	53%	164/237	337	microsatellite Aas790
	Noble crayfish	*Astacus astacus*	EU692886	2.00E-13	62%	184/274	707	clone Aas8 microsatellite
A6	Noble crayfish	*Astacus astacus*	EU313798	3.00E-17	53%	164/237	337	microsatellite Aas790
	Noble crayfish	*Astacus astacus*	EU692886	7.00E-12	62%	183/274	707	clone Aas8 microsatellite
$A10_1$	Red-swamp crayfish	*Procambarus clarkii*	EF564124	2.00E-06	16%	57/72	447	clone PCL25 microsatellite
	Chinese mitten crab	*Eriocheir sinensis*	DQ388785	3.00E-04	17%	59/78	860	microsatellite ES19
	Noble crayfish	*Astacus astacus*	EU692886	3.00E-23	60%	188/269	707	clone Aas8 microsatellite
	Noble crayfish	*Astacus astacus*	EU313798	6.00E-25	47%	152/208	337	microsatellite Aas790
$B4_2$	Noble crayfish	*Astacus astacus*	EU692886	1.00E-32	37%	194/267	707	clone Aas8 microsatellite
	Noble crayfish	*Astacus astacus*	EU313798	3.00E-27	23%	156/211	337	microsatellite Aas790
	Red-swamp crayfish	*Procambarus clarkii*	EF564124	4.00E-25	28%	183/257	447	clone PCL25 microsatellite
	Red-claw Crayfish	*C. quadricarinatus*	DQ847885	6.00E-10	10%	74/96	436	clone h4_D5 mRNA
	Chinese mitten crab	*Eriocheir sinensis*	DQ388785	5.00E-05	13%	86/119	860	microsatellite ES19
$C3_2$	Noble crayfish	*Astacus astacus*	EU692886	2.00E-15	29%	180/264	707	clone Aas8 microsatellite
	Noble crayfish	*Astacus astacus*	EU313798	2.00E-15	23%	149/214	337	microsatellite Aas790
	Red-claw crayfish	*C. quadricarinatus*	AF156901	5.00E-05	6%	49/60	218	microsatellite cqu.005
$C5_1$	Noble crayfish	*Astacus astacus*	EU313798	4.00E-19	24%	173/246	337	microsatellite Aas790
	Noble crayfish	*Astacus astacus*	EU692886	2.00E-17	42%	288/435	707	clone Aas8 microsatellite
	Red-claw crayfish	*C. quadricarinatus*	AF156901	2.00E-11	5%	53/59	218	microsatellite cqu.005
	Red-swamp crayfish	*Procambarus clarkii*	EF564124	1.00E-07	7%	60/74	447	clone PCL25 microsatellite
$E42_1$	Noble crayfish	*Astacus astacus*	EU692886	1.00E-31	81%	194/269	707	clone Aas8 microsatellite
	Noble crayfish	*Astacus astacus*	EU313798	6.00E-24	455	117/151	337	microsatellite Aas790

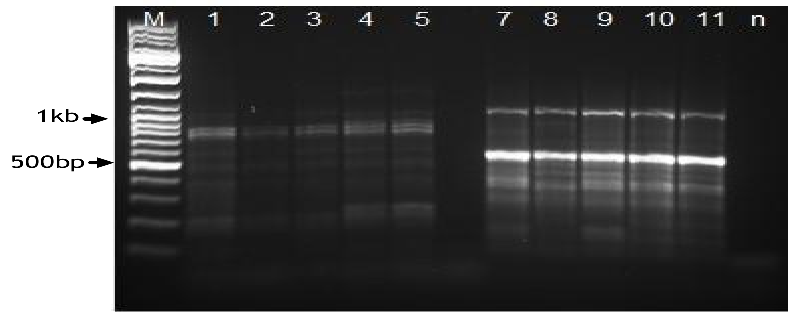

Fig. 2. Amplification of five pleiopods of red-claw crayfish (*C. quadricarinatus*) using primers 370 F/R (lane 1-5); 376 F/R (lane 7-11); n: negative controls; M:10kb marker GeneRuler^{TM} (Fermentas, Australia)

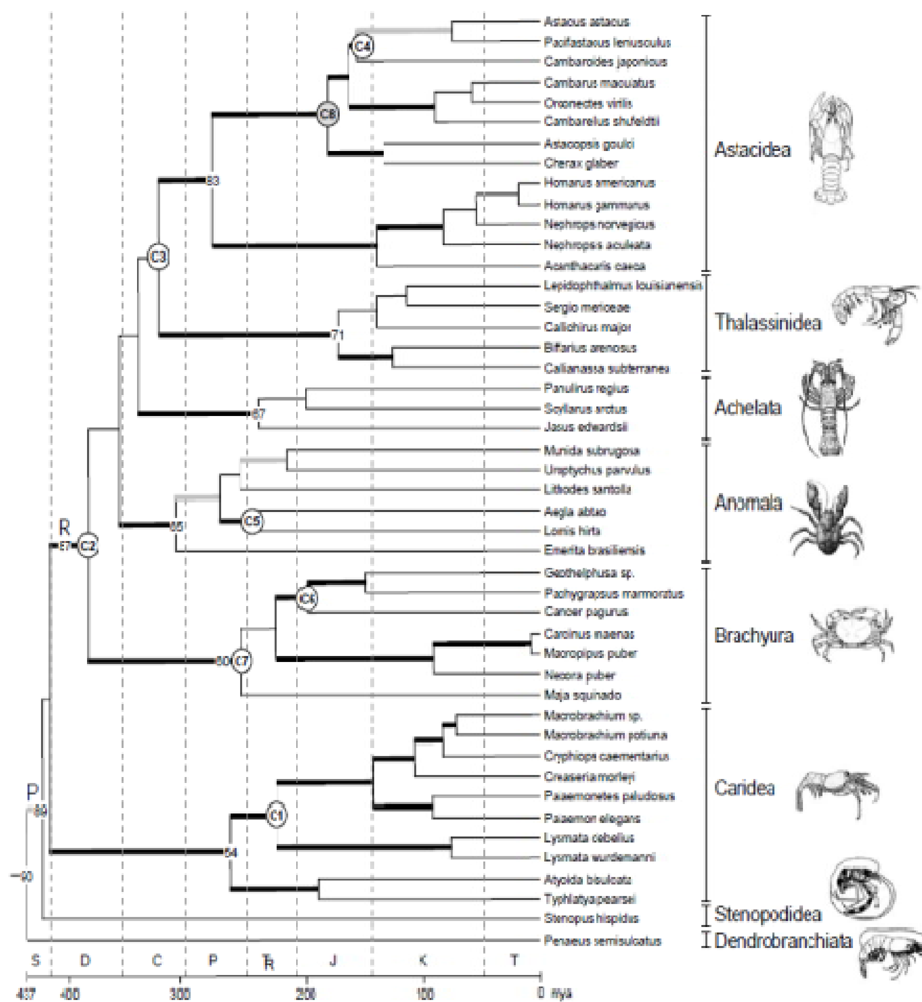

Fig. 3. Chronogram of Order decapoda showing estimated divergence time using maximum likelihood (ML) tree with the focus of suborder Pleocymata (P) and Reptantia (R) (modified from Porter et al. 2005)

The successful PCR amplification using designed primers from Chinese mitten crab (*E. sinensis*) (Fig 2). indicated that the primers effectively worked during the amplification of our red-claw crayfish DNA. However, since the description of matched sequences were microsatellites (see in Table 2) which

differed from our targeted genes (*IRF*)s, it suggested that the evidence for the interferon (IFN) pathway in red-claw crayfish (*C. quadricarinatus*) was negative.

There are different views expressed in different papers. Some argued that interferon-like protein (IntlP) existed in crustaceans [21], whilst others claimed that IFN and/or IntlP did not exist in crustaceans [22]. Taking into account these differences and coupled with the fact that no one has either studied nor deposited *IRF* sequences of crayfish (*C. quadricarinatus*) in GenBank, our results tend to support the discovery of IRF genes (*IRF*)s by the incidence of *IRF* family members in the kingdom Metazoa (in which decapods belong to), using extensive phylogenetic analyses of *IRF* family [17]. Furthermore, our result was consistent with the previous molecular phylogenetic study of Order Decapoda [23].

The divergence between the three Infraorder within Metazoan Kingdom (Phylum Arthropoda, Subphylum Crustacea, Order Decapoda), including Infraorder Brachyura (such as Chinese mitten crab, in which *Er*S1 was detected), Infraorder Dendrobranchiata (such as Pacific white shrimp in which *PENVA* was detected) and Infraorder Astacidea (such as red-claw crayfish in our study) occurred more than 350 million years ago or since the Devonian geological period (Fig. 3). Against these background studies, the lack of signs of IFN pathways (or an IFN pathway) which was found in our study, tends to confirm that to date, no other *IRF* exists in crustaceans except perhaps in the Chinese mitten crab (*E. sinensis*) [19] and in Pacific white shrimp (*L. vannamei*) [20]. It is also rational to consider that interferon regulatory factor genes IRFs as factors of an interferon (IFN) pathway within crustacean species including crayfish (*C. quadricarinatus*) were either eliminated or diverged during evolution.

4. CONCLUSION

This study is the first attempt to examine the existence of an interferon pathway in crayfish (*C. quadricarinatus*) using an experimental injection of an α-IFN (CHO-derived) recombinant protein accompanied by a molecular study by PCR using designed primers from deposited IRF sequences (GenBank no. FG360214). Even though the signs of an IFN pathway in the red-claw crayfish was not strong, or tend to be negative, several crucial notes should be considered. Firstly, since the level of interferon dosages that were used was toxic to the red-claw crayfish, lower dosages should be applied. Secondly, the designed primers do not support the idea of *IRF*s as part of an IFN pathway existing in red-claw crayfish. Thirdly, our study supports the rational consideration that *IRFs* within Metazoan Kingdom including crustaceans might have diverged somewhat or been completely lost during evolution. Therefore, opportunity to reveal immune-based genes in crayfish using PCR analyses with newly designed primers still exists.

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COMPETING INTERESTS

The authors declare no conflict of interest in the collection, analyses, or interpretation of data, in the writing of the manuscript, and in the decision to publish the results.

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