MOLECULAR CLONING, CHARACTERISATION AND FUNCTIONAL ANALYSIS OF GROWTH ARREST SPECIFIC GENE 2 IN PEARLSPOT (*Etroplus suratensis*)

by,

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(2014-09-117)

THESIS

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DECLARATION

I, hereby declare that the thesis entitled "**MOLECULAR CLONING, CHARACTERISATION AND FUNCTIONAL ANALYSIS OF GROWTH ARREST SPECIFIC GENE 2 IN PEARLSPOT (*Etroplus suratensis*)**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Place: Vellayani
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CERTIFICATE

This is to certify that this thesis entitled **"Molecular cloning, characterisation and functional analysis of growth arrest specific gene 2 in Pearlspot (*Etroplus suratensis*)"** is a record of research work done by **Ms. Amiya Thalakkattu (2014-09-117)** under my guidance and supervision and that this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Amiya Thalakkattu

Dedicated to my beloved parents

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LIST OF ABBREVIATIONS

%	Percentage
A260	Absorbance at 260 nm wavelength
A280	Absorbance at 280 nm wavelength
bp	Base pair
cm	Centimeter
RNA	Ribonucleic Acid
DNA	Deoxy ribonucleic Acid
EDTA	Ethylene diamine tetra-acetic acid
et al.	et alia
EtBr	Ethidium bromide
F	Forward primer
R	Reverse primer
GAS1	Growth Arrest Specific Gene 1
GAS2	Growth Arrest Specific Gene 2
GAS3	Growth Arrest Specific Gene 3
GAS5	Growth Arrest Specific Gene 5
GAS6	Growth Arrest Specific Gene 6
ICAR- CMFRI	Indian Council of Agricultural Research – Central Marine Fisheries Research Institute
KAU	Kerala Agricultural University
PCR	Polymerase Chain Reaction
C DNA	Complimentary DNA
FCS	Fetal Calf Serum
TAM	Tyro3, Axl, MerTK
GLA	Gamma Carboxy Glutamic
DEPC	Di-ethyl Pyro Carbonate
RT- PCR	Real Time Polymerase Chain Reaction
d NTP	Deoxy Nucleotide Tri phosphate
$CaCl_2$	Calcium chloride
kb	Kilobases
M	Molar
mg	Milligram

min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
ng	Nanogram
nm	Nanometer
°C	Degree Celsius
OD	Optical Density
RNase	Ribonuclease
s	Second
TBE	Tris- borate EDTA
TE	Tris EDTA
TM	Trademark
Tm	Melting temperature
Tris HCl	Tris (Hydroxy methyl) aminomethane hydrochloride
UV	Ultra violet
V	Volt
µg	Microgram
µl	Microliter
µM	Micromolar

INTRODUCTION

1. INTRODUCTION

Aquaculture plays a major role in ensuring a consistent supply of aquatic species for human consumption. Fish food which is rich in omega - 3 fatty acids and proteins is one of the most healthy and recommended thing to be included in human diet.

The family Cichlidae comprises over 700 species of fishes that occur in freshwater as well as brackish water habitats (Keenlyside, 1991).Pearl spot (*Etroplus suratensis*), commonly known as "Karimeen", is a species of cichlid fish inhabiting both freshwater and brackish water in southern India and Srilanka.

In 2010, *E. suratensis* was named the official state fish of Kerala. It is essentially a brackish water fish that has become naturally acclimatized to freshwaters. It is an economically important food fish and is a delicacy that fetches very high price. The fish breeds naturally in confined conditions and is ideally compatible for polyculture with both freshwater and brackish water fishes and prawn species. It is monogamous and identification of sexes is possible only during breeding season. *E. suratensis*, the largest among the three indigenous cichlids, is a native of peninsular India, occurring primarily in Kerala and Southern Karnataka; the other species being *E. maculatus* and *E. canarensis*. *E. maculatus* occur in backwaters of Kerala while *E. canarensis* (Bloch) is restricted to the coastal wetlands of Karnataka.

Aquaculture in the present scenario of climate change is facing many adverse environmental conditions which will affect the growth and development of fish. One of the most ambient temperature affecting the growth of fish is water temperature. Growth arrest specific gene 2 is a component of microfilament system that plays a major role in cell cycle, regulation of microfilaments and cell morphology during apoptotic processes (Brancolini *et al.*, 1992). It was reported to be expressed at temperature stress, cell contact inhibition and serum starvation. It was originally identified in murine fibroblasts under growth arrest conditions. Serum stimulation of

quiescent, non-dividing cells leads to the down regulation of growth arrest specific gene 2 and results in the re-entry into the cell cycle (Brancolini *et al.*, 1997)

Growth arrest specific genes are concerned with growth arrest, cell contact inhibition, micro filament activity and apoptosis. The growth arrest specific genes mostly studied are *GAS*1, *GAS2, GAS3, GAS5, GAS6* (Polyak *et al.*, 1996).

The protein coded by the *GAS2* gene is a caspase-3 substrate that plays a role in regulating microfilament activity and cell shape during apoptosis. Little information is available on fish growth arrest specific gene 2. Since growth and reproduction in fish is closely related to water temperature, light, gas pressure, and other climacteric factors, (Yang *et al.*, 2017) the tolerance to wide range of temperatures and the biochemical changes caused by these factors are attributed to modulations in gene expression patterns (Geralch *et al.*, 1990).

The objectives of the study are:-

- Molecular cloning, characterization and functional analysis of growth arrest specific gene2 *(GAS2).*
- Quantitative expression analysis of *GAS2* at different water temperatures.

GAS2 is studied in tilapia and is reported to be expressed at low water temperatures. The expression pattern may vary from species and this difference will affect the overall growth and development of fish. Studying the expression of the gene at different temperatures and their effect on the growth and development will help us understand the *GAS2* function more deeply and will enable us to suggest an ideal rearing water temperature for the farmers in Pearlspot aquaculture. The use of real time PCR to compare the expression of the gene will help to observe the minute changes in expression pattern. This will enable us to understand the expression pattern of the gene at different water temperatures and thus allow to come up with the condition in which *GAS2* is least expressed.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 ETROPLUS SURATENSIS

Etroplus suratensis is a fish belonging to family Cichlidae which is most abundantly seen in estuaries and inland water of India and Srilanka. The family Cichlidae contains more than 700 species of fishes which live in brackish water as well as fresh water habitats. Cichlid fishes inhabit still or slow moving water and their natural preferred habitat is one with many hiding places such as plant roots and plant thickets. They appear to be conservative structurally, but studies have shown that they occupy a wide range of ecological niches and their speciation is miraculous in tropical lakes (Keenleyside, 1991). Human relationship with cichlids has been reported to be quite long since cichlid specimens have been recognized from ancient frescoes (Keenleyside, 1991). The native fisheries in tropics mainly rely on the stock of cichlids. Till the Second World War, Cichlids were not considered popular among aquarists because of their habit of destroying the vegetation of their habitat and also because of the fight among individuals in the species and other fished in the habitat. These habits shown by cichlids are said to be due to their prolific production, high breeding in confined conditions and parent care which demands lots of free space. The reproductive behavior varies widely among family from monogamous biparental care of fries and eggs to mouth brooding by females as observed in tilapias. Cichlids are broadly divide into two groups the mouth brooders and the substrate brooders(De silva *et al.,* 1984) Among the various species of cichlid, *E. suratensis* is the only one endemic to India. Within the species it has low fecundity rate with about 500 eggs laid during a single spawning (Jayaprakas *et al.*, 1990). Eggs get attached to submerged logs, rocks and also to aquatic weeds. The eggs will hatch in 4 days after laying (Dhas *et al.*, 2010)

2.1.1 **Morphological features**

E. suratensis which is a euryhaline cichlid is an indigenous species seen in India and southern coast of Srilanka (Munro, 1955). They have spiny rays and are distinguished primarily by only one nostril at each side of their head. The body

shape may vary from pike like to disc form with genus. Most species appear deep with large eyes and head, large fairy scales, and a strong jutting jaw with well-developed lips. Dorsal fin of the fish is single and long based, The front part is spiny and is larger than the after part which is softer.

In almost all cichlids the dorsal anal fin is rounded in females and are pointed among males. Its lateral line appear as two parts. The first extending up to the soft rayed part of dorsal from the gill cover whereas the next parts to be discontinuous. The body is light greenish in color and has eight yellowish bands, the first band passes through the occipital part of head and the last crosses the base of the caudal. They have a pearly spot on the scales above the lateral line. It attains maturity at the end of the first year. It show sexual dimorphism which is identifiable only during the breeding season (Padmakumar *et al.*, 2004). Pearlspot exhibits a low fecundity rate which ranges from 500 to 7550 (Kumari *et al.*, 1981). Egg laying and fertilizations usually occur several times and these eggs get cemented in a manner in which they don't touch each other. Spawning in pearl spot is rapid and is completed in 45 to 50 minutes. The eggs hatch in 72 hours and *E. suratensis* exhibit a long period of parental care and the parents take care of the young till they attain a size of 30 to 40 mm (Padmakumar *et al.*, 2012).

2.1.2 **Habitat**

E. suratensis is a most abundantly seen in estuaries and inland waters of India and Srilanka. It is the largest among the three indigenous cichlids. Other species of Etroplus are *E. maculatus and E. canarensis. E. maculatus* is widely distributed in the backwaters of Kerala while *E. canarensis* is seen restricted to coastal wetlands in Karnataka. The family cichlidae contains more than 700 species of fishes which live in brackish water as well as freshwater habitats

E. suratensis is widely distributed along the coastal tracts of south Canara to Malabar to Chilka lake in the east coast (Hora *et al.*,1962; Jhingran *et al.*, 1969). Popularly known as Karimeen in kerala, it is identified as the state fish of Kerala.. The backwaters of Kerala is a potential source of its seed. It is a delicacy that fetches

a very high price in tourist sector .The quality and taste of the fish is highly dependent on the habitat in which it is grown.

It is one the most suitable fish in aquaculture due to its good palatability, herbivorous feeding habit and non predacious nature. They are also considered as ornamental fishes due to its rare colour and remarkable pattern. Since the fish breeds naturally in confined conditions, it can be grown in polyculture along with other fresh water and brackish water fishes. The fish was reported to constitute almost 10 percent of total fish landings from the backwaters of Kerala during the sixties and total landing was indicated to vary from 14000 to 17000 tons (George and Sebastian, 1970). The Vembanad lake in Kerala (Lat. 09°31′ and 09°41′ N and Long. 76°21′ and 76°26′ E),is considered as a heritage reservoir of Pearl spot supports a rich natural fishery for this species since long, although the annual landings have reduced to 200 tons (Padmakumar *et al.*, 2009) from 1252 tons reported earlier

2.2 RESPONSES OF ORGANISMS TO TEMPERATURE STRESS

Most of the species of animals are able to survive a temperature beyond the zone of tolerance , it is indicated as the zone of resistance (Rajaguru, S. and Ramachandran, S., 2001). Biochemical, physiological and life history activities of fish is largely influenced by temperature. The molecular mechanisms behind these temperature acclimatization behaviors remain largely unknown. The variations in environmental temperature affect many physiological characteristics such as functional characters of cells and biomolecules (Somero, 1995). These include properties such as folding assembly, rigidity and structure of proteins and lipids (Pan *et al.*, 2008; Simon and McIntosh, 1995) the permeability of cell membrane and its fluidity (Ueda *et al.*, 1976). Since various structure and cellular components are ubiquitously dependent on temperature, even small changes can affect the homeostasis and attenuate physiological performance (Long *et al.*, 2012). Water temperature is supposed to be an abiotic master factor which controls and limits all the physiological and biochemical characteristics since the body temperature of most fishes gets equilibrated very fast with ambient temperature (Donaldson *et al.*,

2008; Beitinger *et al.*, 2000). Fishes experience many sources of temperature fluctuations from sources such as thermocline temperature variations, solar heat changes, abnormal water movements, changes in seasonal temperature and also sudden rapid precipitation events (Beitinger *et al.*, 2000; Bret J. R. 1956). To compact the effects due temperature changes, fishes have evolved different mechanisms to maintain body homeostasis under different harsh temperature environments ranging from -2°C in the polar oceans to over 45°C in hot springs (Cossins and Crawford, 2005). Some fishes belonging to eurythermal habitat even adapt seasonal variations in temperature ranging from near freezing to greater than 36°C (Ju *et al.* 2002) and can withstand a daily temperature cycle up to 20°C (Podrabsky and Somero, 2004).

The observation that fishes get adapted to changing environmental conditions have led to more studies related to this acclimatization phenomenon. It is well studied that the biochemical changes caused by temperature stress are attributed to modulations of gene expression (Geralch *et al.*, 1990; Tine M, 2017). In a study which monitored the transcriptomics of larval Zebra fish under different temperatures, a total of 2680 genes were found to be specifically regulated by temperature stress. Protein folding was found to be overrepresented in most of the heat stress response genes (Langheinrich *et al.*, 2002; Johnson and Weston, 1995.).

Experiments were conducted to study the response of fruit fly to heat stress. Heat shock lead to the appearance of expanded chromosomal puffs which is an indicative of locally enhanced transcription (Ritossa, 1962; Martinez *et al.*, 2016)). In eukaryotes the main effect caused by heat stress is the damage to cytoskeleton (Richter *et al.*, 2010). Minor stresses leads to the changes in actin and microfilament cytoskeltons whereas heavy stress leads to the aggregation of filament forming proteins like vimentin and finally leading to cell collapse (Toivola *et al.*, 2010; Welch and Suhan, 1985)

Temperature tolerance capability of estuarine fishes *E. suratensis* (Bloch), *Therapon jarbua* (Forsskal), *Ambassis commersoni* (Cuvier) sampled from Vellar estuary, South India in 1986–87 was determined in laboratory.

More tolerance was obtained in *E. suratensis* and *T. jarbua*. *E. suratensis* exhibited tolerance at a range of 16.5 to 41.5°C . It was in the range 13.5–40.6°C for *T. jarbua*. *A. commersoni* was found to tolerate from 15.5 to 38.5°C. The tolerance area of 512 units was found in *E. suratensis*, 629 units in *T. jarbua* and 442 units in *A. commersoni*. Among the fishes tested *T. jarbua* had a high tolerance area (629 units) than other fishes tested. It is evident from the results that 15°C increase in acclimation temperature cause a shift of 4.02°C (*E. suratensis)* and 3.05°C (*T. jarbua* and *A. commersoni*) in upper incipient lethal temperature.(Rajguru S, 2002). Boltana *et al.* 2017, in his study of the influences of thermal environment in fish growth in Atlantic salmon, suggested that the thermal heterogeneity showed substantial impacts in the growth trajectories and muscle structure of the fish. All the growth trajectories including morphological/histological parameters, gene expression regulation, muscle structure showed significant difference in the control(restricted temperature range) and experimental individuals(wide temperature range)(Kinne O, 1963) The size trajectory of RTR individuals displayed similar growth patterns to those observed in other fish species conditioned to restricted temperatures (Johnston, 2003; Valente *et al.*, 1999).

2.3 GROWTH ARREST SPECIFIC GENES

In multicellular organisms cell growth and cell death must be regulated and monitored continuously in order to maintain tissue homeostasis. Observations in studies suggests that this is maintained partially by coupling the process of cell cycle progression and cell death by using many controlled set of factors. The cell cycle is suggested as a process in which a cell divides all its material and become two separate identical cells. Cell cycle progresses through 4 main steps Gap 1 (G1), Synthesis phase(S), Gap 2 (G2) and mitosis (King and Cidlowski, 1995). Cell proliferation is determined by the dynamic equilibrium maintained between some positive and negative elements signaling cells to stay in or out of the cell cycle. Both of these factors are important because they help in governing the kinetics of cellular equilibrium (Brancolini *et al.*, 1991). Vast number of elements in the positive circuit was identified among oncogenes. Nuclear oncogenes remain tightly

coupled with the cytoplasmic and membrane oncogenes through a very complex signal transduction cascade (Aaronson, 1991; Schedl *et al.,* 1984). Communication within these positive circuits is usually mediated by the level of phosphorylation (Ser-Thr/Tyr) of the individual elements, modulating their signaling activity (Hanks *et al.,* 1988; Cantley *et al.,* 1991).

Growth arrest specific genes are mainly involved in cell growth inhibition, microfilament activity and apoptosis. There are a wide range of growth arrest specific genes involved in the maintenance of cellular integrity and homeostasis. These genes are expressed during cell contact inhibition, cold stress, serum starvation etc. The genes usually expressed in these conditions are growth arrest specific and growth arrest and DNA age specific genes. (Rees *et al.*, 1999). Among the different growth arrest specific genes, the mostly studied ones are GAS1,GAS2,GAS3,GAS4,GAS5,GAS6. p53, p21, ATM, ATK, cyclin dependent kinase inhibitors etc are the other genes involved in growth arrest (Polyak *et al.*, 1996).

GAS1 belongs to a category of genes earlier recognized as particular growth arrest since their expression is down-regulated following growth induction of arrested NIH 3T3 cells (Schneider *et al.*, 1988). In vitro transcription-translation studies using the pCITE-1 vector scheme suggests that the *GAS1* is an integral membrane protein. The impact on DNA synthesis of ectopic *gas 1* expression was explored. Automated micro injection was used to give GAS 1 cDNA to the recipient of NIH 3T3 cells, cloned in pGDSV7 vector. The expressed cells were immunostained with an antibody or anti-hu-tr-specific monoclonal antibody purified with affinity (Del Sal *et al.*, 1992). At the appropriate time, the expression of *GAS1* in microinjected cells was found to be significantly higher than the endogenous level. The product of the *GAS1* gene induces growth arrest by means of a mechanism dependent on p53 (Caelles *et al.*, 1994). To explore whether *GAS1* is a tumor suppressor gene, *GAS1*-negative human tumor cells were transfected with *GAS1* plasmids and stable transfectants were evaluated for growth features. Results indicate that *GAS1* suppresses the development and tumorigenicity of

human tumor cells and inhibits the *GAS1*-mediated growth-suppressing pathway by overexpression of MDM2 or p53 gene (Evdokiou *et al.*, 1998).

Growth arrest specific gene 3 expression was studied from the mRNA isolated from serum starved NIH 3T3 cells and at different times after 20 % FCS addition. At growth arrest (48 h in 0.5 percent FCS), the RNA identified by the *GAS3* probe (about 1.8 kilobases) was abundantly displayed and reached the smallest amount of expression after 6 hours of serum addition (Manfioletti *et al.*, 1990).

Growth arrest specific gene 5 was identified in growth arrested cells in connection with its interaction with glucocorticoid receptor (Smith and Steitz, 1998). The availability of nutritional elements affect the cell growth and transcriptional activity(Chang *et al.*, 2016). Gene transcription is also influenced by availability of glucocorticoids. *GAS5* was mainly identified in cells whose growth arrest occurred due to starvation or lack of growth factors. This suggest that the expression of *GAS5* in such tissues sensitize cell to apoptosis by hindering the glucocorticoid mediated responsive genes such as that involved in coding cellular apoptosis inhibitors (Liu *et al.*, 2018) *GAS5* act as a riborepressor of glucocorticoid receptor by competing with DNA glucocorticoid receptors (Kino et al., 2010; Mourtada et al., 2008).Growth arrest specific gene 6 was identified in connection with its structural similarity to the natural anticoagulant protein S which has over 40 % sequence identity with *GAS6* (De Frutos *et al.*, 2007; Fernandez *et al.*, 2008). *GAS6* is noticed due to its distinctive interaction with receptor thyrosine kinases of TAM family of receptors. This include the Tyro 3, Axl and MerTK .(Godowski *et al.*, 1995; Lemke and Rothlin, 2008). Recent studies also points that *GAS6* has significant roles in inflammation, homeostasis and cancer *GAS6* employs a unique mechanism of action, inter-acting through its vitamin K-dependent GLA (γ-carboxyglutamic acid) module with phosphatidylserine- containing membranes and through its carboxy-terminal LamG domains with the TA membrane receptors (Takahashi *et al.*, 2014; Laurance *et al.*,2012). It belong to the plakin family of proteins (Leung *et al.*, 2002). Thus the coagulation cascade as vitamin K dependent

element and the innate immunity has been found to be connected (Tjwa *et al.*, 2008; Kulman *et al.*,2006; Manfioletti *et al.*, 1993)

2.4 GROWTH ARREST SPECIFIC GENE 2

Growth arrest specific two *(GAS2)* sequence is a part of the microfilament system that plays a serious role within the cell cycle, regulation of microfilaments, and cell morphology throughout apoptotic processes (Yang *et al.*, 2017). It had been originally known in murine fibroblasts under growth arrest conditions. Serum fluid stimulation of quiescent, non-dividing cells results in the down regulation of *GAS2* and ends up in re- entry into the cell cycle (Pucci *et al.*, 2000).

GAS2 is a protein that in humans is encoded by *GAS2* gene. It is a caspase 3 substrate that plays a role in microfilament dynamics and also have a crucial role in preventing cell morphology changed due to apoptosis(Brancolini *et al.*, 1992). It can also modulate cells response to necrobiosis or apoptosis by regulating the calpain activity. GAS2 is reported to be a caspase-3 substrate, cleaved by caspase3 and regulate the morphological changes occurring during apoptosis. (Lee *et al.*, 1999; Benetti *et al.*, 2001). Another study showed that the over expression of this gene does not directly lead the cells to undergo apoptosis, but make them sensitive to apoptotic signals (Benetti *et al.*, 2001).

homology (CH) near to the N terminus and a *GAS2* domain which bind tubulin near the C terminus. Between the CH and Gas2 domain there are 2 low complexity regions which give the protein high structural flexibility due to the presence of two proline- seriene repeats (PS repeats) in the second region of the low complexity region.

2.4.1 **Protein activity**

Cytoskeletal dynamics regulate many fundamental cellular processes like would healing, cell division and cell motility (Heng *et al.*, 2010). Actin and microtubules undergo dramatic rearrangements at the time of cell division for processes like changing morphology of cells, chromosome segregation and

cytokinesis. The cytoskeleton's capacity to adapt to continuing physiological modifications is mediated by actin and microtubule binding proteins. Many of these proteins have been recognized, but their functions and regulatory mechanisms stay uncertain(Rodriguez *et al.*, 2003). The *GAS2* protein is one such cytoskeleton interacting protein.

The *GAS2* protein belongs to the protein family of growth-arrest and is commonly expressed in human tissues (Collavin *et al.*, 1998). Although *GAS2* has a putative N-terminal actin-binding calponin homology (CH) domain(Brancolini *et al.*, 1992) and a C-terminal tubulin-binding *GAS2* domain, there has been no direct proof of *GAS2* interactions with cytoskeleton. Studies of immunofluorescence, however, have shown that the full length *GAS2* co-locates with filamentous actin (F-actin) in the cell cortex and in stress fibers in growth-arrested NIH 3T3 fibroblasts (Sun *et al.* 2001) and the *GAS2* domain co-locate in COS-7 cells with microtubules (Brancolini *et al.*, 1992). Although most of an organism's cells are quiescent, they can re-enter the cycle of the cells and proliferate after stimulation. Several lines of evidence favor *GAS2*'s role in the development of the cell cycle. *GAS2* protein has been shown to co-locate in mammalian interface cells with actin and microtubules that help rearrange cytoskeletons during cell cycle and cell division.

GAS2 protein belongs to GAS2 family of proteins, They play a major role in mediating the interaction between filamentous actin and microtubules. It also regulate cell cycle, apoptosis and calpain activities. (Lee *et al.*, 1999; Zhou *et al.*, 2014) .It was found to be up-regulated in chronic myeloid leukemia cells and was found required for their growth

Most of the actin binding proteins comprise a 250 amino acid stretch called actin binding domain. This domain is assumed to have arisen by duplicating a domain found in single copy in number of other proteins like calponin or the vav protooncogene and is called calponin homology CH) domain (Stradal *et al.*, 1998; Keep *et al.*, 1999).

A detailed analysis of The CH domain-containing proteins has shown that they can be divided in three groups (Stradal *et al.,* 1998)

- The fimbrin family of monomeric actin cross-linking molecules containing two ABDs
- Dimeric cross-linking proteins (alpha-actinin, beta-spectrin, filamin, etc.) and monomeric F-actin binding proteins (dystrophin, utrophin) each containing one ABD
- Proteins containing only a single amino terminal CH domain.

Each single ABD, comprising two CH domains, is able to bind one actin monomer in the filament. The amino terminal CH domain has the intrinsic ability to bind actin, with lower affinity than the complete ABD, whereas the carboxy terminal CH bind actin extremely weakly or not at all. Nevertheless both CH domains are required for a fully functional ABD; the C-terminal CH domain contributing to the overall stability of the complete ABD through inter-domain helix-helix interactions (Stradal *et al.,* 1998) Some of the proteins containing a single CH domain also bind to actin, although this has not been shown to be via the single CH domain alone. In addition, the CH domain occurs also in a number of proteins not known to bind actin, a notable example being the vav protooncogene. The resolution of the 3D structure of various CH domains has shown that the conserved core consist of four major alpha-helices (Keep *et al.*, 1999; Marchler-Bauer *et al.,* 2016).

GAS2 is specifically cleaved by an unidentified ICE like protease at its C terminus during apoptosis, and the processed protein induces drastic rearrangements in the cytoskeleton when over-expressed in several kinds of cells. During apoptosis it is cleaved and the cleaved form induces drastic rearrangements of the cytoskeleton actin and powerful changes in the shape of impacted cells (Brancolini *et al.,* 1992)

2.4.2 **Major functions**

The molecular functions of *GAS2* include microtubule and actin binding. *GAS2* is the cellular component of cytoskeleton, cytosol, actin filament, membranes and cytoplasm. The biological processes in which this plays a role are cell cycle, regulation of cell shape, growth arrest, microtubule cytoskeletal organization, actin cytoskeletal organization.

The role of *GAS2* in cell division is studied extensively. Evidences suggest the role of tis gene in cell cycle progression. This gene was identified originally from culture of murine fibroblasts which were maintained under growth arrest conditions (Schneider *et al.,* 1988). It was also found to be down regulated when stimulated wit serum or growth factor (Brancolini *et al*, 1992). In addition to this, the protein coded by this gene, *GAS2* was found to be phosphorylated on a seriene residue at the G0 to G1 transition which allowed the quiescent cells in G0 phase to re-enter the cell cycle. (Brancolini *et al.,* 1992).But there is no direct evidence about the direct involvement of *GAS2* in cell division or its cytoskeleton binding properties

2.4.3 **GAS2 in Tilapia**

There is little data on fish GAS2. The tilapia (*Oreochromis niloticus*) *GAS2* gene was first cloned and described in a study (Yang *et al.,* 2017). The open reading frame was 1020 bp, with 340 amino acids encoded; the 5′-untranslated area (UTR) was 140 bp and the 3′-UTR was 70 bp, with a poly (A) tail. In the regulatory region, the greatest promoter activity happened (–3000 to –2400 bp). The fusion protein Gas2-GFP has been allocated in the cytoplasm (Yang *et al.*, 2015)Quantitative reverse transcription-polymerase chain reaction and Western blot analysis disclosed that low temperature stress obviously influenced the concentrations of *GAS2* gene expression in the liver, muscle, and brain. Results from *GAS2* RNAi showed reduced *GAS2* and P53 gene expression. These findings indicate that apoptosis induced by stress at low temperature may involve the tilapia *GAS2* gene.

2.4.4 *GAS2* in human

Growth arrest specific gene 2 is expressed in most human tissues undergoing growth arrest, the expression being the highest level in lung, liver and kidney. It was observed to be strongly expressing in embryos in regions which undergo extensive apoptosis such as cartilage of bones, craniofacial mesenchyma and intervertebrate tissues. The overexpression of *GAS2* increases the susceptibility of cell to apoptosis following the exposure to DNA damaging agents such as etoposide, methyl methonate sulphate treatments and the effect of these depend on the stability of p53 and transcription stability (Benneti *et al.*, 2001). Gnathodiaphyseal diysplasia is a disease associated with *GAS2*. *GAS2 L3* is an important paralog of this gene.

MATERIALS ANDMETHODS

3.MATERIALS AND METHODS

The study entitled "Molecular cloning, characterization and functional analysis of Growth arrest specific gene 2 in Pearlspot (*Etroplus suaratensis)*" was carried out at the Marine Biotechnology division, ICAR – Central Marine Fisheries Research Institute (ICAR- CMFRI), Kochi during 2018-2019. Details regarding the experiments and methods used in the study are elaborated in this chapter. It describes the collection of samples, storage, RNA isolation protocol, cDNA synthesis with the steps and thermal cycling conditions. The oligonucleotide gene specific primers used for amplification of the gene along with steps and cycling conditions, protocol for preparation of competent cells, cloning , sequence analysis softwares, the house keeping genes and gene specific primers used for real time PCR are also described .

3.1 COLLECTION OF FISH SAMPLE

Juvenile *Etroplus suratensis* were procured from a private hatchery at Chovara, Ernakulam district and was maintained at a water temperature of 26°C in the mariculture experimental facility at CMFRI, Kochi. The fishes were allowed to acclimatize for a period of 15days. The fishes were then separated into 3 groups with 15 fishes in each group for exposure to temperature stress. The control group was maintained at a water temperature of 26°C and the temperature stressed groups were maintained at water temperatures (using a thermostat) viz, 30 °C, 32 °C for a period of 3 days and the 3 fishes were sampled at 1 hour, 12 hour, 72 hour period. The liver, muscle and brain tissue from each sample was collected and stored in RNA later solution at -80°C deep freezer.

3.2 TOTAL RNA ISOLATION

Total RNA was isolated from each sample by using TRIzol™ Reagent (Invitrogen) using manufacturers protocol.

3.2.1 Preparation of glasswares and reagents

All glass wares used for RNA isolation was made RNase free by treating with 0.1% Diethyl Pyro Carbonate (DEPC) in milli Q water overnight and autoclaved . Sterile disposable RNase free plastic wares were used for the isolation procedure. The procedure was carried out in a RNase free bench using disposable gloves.

3.2.2 Reagents

All the reagents were prepared using 0.1 % DEPC treated milli Q water. The autoclavable reagents were sterilized at 121°c at 15 LBS PSI.

3.2.3 Protocol

25 mg of the tissue samples were weighed and homogenized in a DEPC treated autoclaved mortar pestle using liquid nitrogen. The homogenized samples were mixed in 1ml of TRIzol™ Reagent (Invitrogen) and transferred into a RNase free eppendorf tube. The sample was incubated at room temperature for 5 minutes for the complete dissociation of nucleic acids. In to the tube, 0.2 ml of chloroform was added for each ml of TRIzol™ reagent and mixed thoroughly. The sample was incubated at room temperature for 2-3 sec for phase separation. It is then centrifuged in a pre-cooled centrifuge for 15 minutes at 12000 rpm at 4°C. The sample will get separated into three phases. The upper aqueous phase has the RNA. The aqueous phase is carefully transferred into a fresh RNase free tube. 0.5 ml of isopropanol was added for each 1 ml of TRIzol™ added. The tube was incubated at 4°C for 10 minutes for precipitating the RNA. It is then centrifuged at 12000 rpm for 10 minutes. The total RNA forms a pellet at the bottom of the tube. The supernatant was discarded. The wash step was carried out using 70% ethanol at 8500 rpm for 5 minutes. The ethanol was discarded and the pellet was air dried in a laminar air hood for 5- 10 minutes. The air dried pellet is dissolved in sterile RNase free water and stored at -80°C till use.

3.2.4 Quantification of RNA

3.2.4.1Biophotometry

The total RNA isolated was quantified spectrophotometrically using Eppendorf Biophotometer plus by measuring the optical density ratio of A260 nm to A 280 nm. Quality of the isolated total RNA was determined using 1 % agarose gel electrophoresis.

3.2.4.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to quantify the RNA isolated. A 1% agarose gel was prepared using 1X TBE buffer and ethidium bromide was added into it for staining. After solidification 2 µl of isolated RNA mixed with tracking dye is loaded into the wells and separated by electrophoresis. The gel was imaged in a gel documentation system (BIORAD)

3.3 OLIGONUCLEOTIDE PRIMERS FOR c DNA SYNTHESIS

All the primers were custom synthesized from Sigma Aldrich India. All primers were designed using Primer 3 software

3.4 cDNA SYNTHESIS

cDNA synthesis involves the synthesis of first strand cDNA from RNA using reverse transcriptase enzyme.

3.4.1 Synthesis of first strand cDNA

After quantifying the quality and purity of RNA by agarose gel electrophoresis and spectrophotometry 1 µg of total RNA was reverse transcribed to first strand cDNA using reverse transcriptase. The total RNA up to 1µg was mixed with 1 µl of dNTP, 2µl of reverse primer, 1 µl RiboLock RNase Inhibitor (Thermo Fisher Scientific). And the reaction is made up to 10 µl. It was then denatured at 65°C for 5 minutes. 2 µl of

M- MuLv buffer and 1 µl reverse transcriptase was added and the total reaction was made up to 20 µl using 7 µl of sterile water. cDNA synthesis was carried out by incubating the mix at 42°C for one hour. Inactivation was carried out by heating the mix at 65°C for 20 minutes. The cDNA thus prepared was stored at -20°C until further use for carrying out the amplification of *GAS2* using gene specific primers.

3.5 AMPLIFICATION OF GAS2 SEQUENCE USING SPECIFIC PRIMERS

3.5.1 Primer selection and design

The oligonucleotide primers were designed based on the conserved regions in *GAS2* gene sequences for fish species available in NCBI database.

Table 1 Primers used for amplification of *GAS2* gene

Name of the primer	Primer sequences	Primer source
GAS2 _AF	AACCAGCATCTCACAGGACGAG	*Oreochromis niloticus*
GAS2-R	CACTTTTTGCTGCGGTAGTGCG	*Oreochromis niloticus*

3.5.2 Amplification of *GAS2* using specific primers

Table 2 Reaction mixture for PCR amplification of *GAS2* gene.

Q5 reaction buffer	5µl
10 mM d NTP	0.5 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
cDNA	2 µl
Q5 DNA polymerase	0.25 µl
ddH2O	Up to 25 µl

Table 3 The thermal cycling conditions for PCR amplification

Initial denaturation	98°c	30 seconds	
Denaturation	98°c	15 seconds	30 cycles
Annealing	65°c	15 seconds	
Elongation	72°c	32 seconds	
Final elongation	72°c	10 minutes	
Hold	4°c	Infinity	

3.5.3 Agarose gel electrophoresis, purification and quantification of the amplified PCR products

The products obtained from PCR reactions were analyzed on a 1.5% agarose gel in 1x TBE buffer with ethidium bromide (µg/ml). Samples were mixed with sample dye were loaded. The electrophoresis was carried out in a sub marine electrophoresis unit (Hoefer Inc., USA) with a constant voltage of 90 V. The gel was documented in Gel Doc-Gel documentation system (Bio Rad, Hercules, CA, USA). The amplified products were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) system as described by the manufacturer.

3.5.4 PCR Purification procedure

• Five volumes of buffer PB was measured and added to 1 volume of the PCR reaction and mixed well.

• The column provided in the kit was placed in the 2 ml collection tube.

• Sample was applied to the column and centrifuged for 1 min at 10000 rpm.

• Flow-through was discarded and column was placed back into the same collection tube.

• 750 µl Buffer PE was added to the column to wash away unwanted molecules and centrifuged for 1 min at 10000 rpm.

• Flow-through was discarded and the column was placed back in the same collection tube.

• The column was centrifuged for 1 min at maximum speed.

• The column was placed in a sterile 1.5 ml microcentrifuge tube

The purified sample was eluted from the column by adding 15µl of DNase free water.

The eluted PCR products were quantified using Eppendorf biophotometer and the concentration and the absorption ratio at 260/280 nm was recorded.

purification carried out using QIAquick PCR purification kit in a precooled micro centrifuge(Hermle) All the centrifugation steps were carried out at 10000 rpm .

3.6 MOLECULAR CLONING OF PURIFIED PCR PRODUCTS

3.6.1 Bacterial strain used for preparing competent cells

Invitrogen USA Top 10 bacterial host cells were made chemically competent by $CaCl_2$ method and used for transformation with recombinant p JET plasmid.

3.6.2 Preparation of competent cells

E. coli Top10 cells (Invitrogen USA) were used for the preparation of chemically competent host cells by using $CaCl_2$ method (Sambrook and Russel, 2001). The culture of Top 10 bacterial cell was streaked on a LB agar plate and incubated overnight. A single colony was picked using a sterile toothpick and was inoculated into 10 ml LB broth in a test tube and was grown overnight in a 37°c shaker incubator at 200 rpm, 5ml of the overnight culture was inoculated into 50 ml LB broth and incubated at 37°C for 2 hours in a shaking incubator at 120 rpm. The cells are harvested at the log

phase of growth by centrifugation. The supernatant was decanted and the cell pellet was dissolved in 10 mM $CaCl_2$ ($1/4^{th}$ original cell culture) by gentle mixing. The tube was incubated in ice for 30 minutes with intermittent mixing. The cell suspension was finally centrifuged at 6000 rpm for 10 minutes at 4°C. The supernatant decanted and the cells were dissolved in 1 ml of 0.1 M $CaCl_2$

3.6.3 pJET 1.2 blunt end cloning vector

pJET blunt end cloning vector (CloneJET PCR Cloning Kit, Thermo Fisher scientific) was used for cloning. It can take inserts in the range of 6 bp to 10 kb. All blunt ended PCR products can be directly ligated to pJET 1.2 cloning vector. The selection of transformed colonies does not need blue white screening since only transformed colonies appear on the plate. It has a size of 2974 bp, ampicillin resistance as selectable marker and a range of restriction sites.

3.6.4 Ligation of *GAS2* fragment to p JET 1.2 vector.

The ligation reaction of the PCR amplified product to the cloning vector is done as per the protocol advised by the manufacturer.(CloneJET PCR Cloning Kit, ThermoFisher scientific)

Table 4 Ligation reaction mixture for molecular cloning of *GAS2*.

2X reaction buffer	5µl
PCR product	1 µl
pJET blunt end cloning vector (20ng/µl)	0.5 µl
Milli Q water	3 µl
T4 DNA ligase	0.5 µl
Total Volume	10 µl

After mixing thoroughly by spinning ,the reaction mix was incubated at 22°c for 30 minutes in a thermal cycler(ABI Veriti 96 well thermal cycler, Applied Biosystems) .This ligation is directly used for transformation.

3.6.5 Transformation protocol

- Competent cell aliquot(100µl) stored at -80°c was kept in ice for 5 minutes prior to transformation
- 5 µl of the ligation mixture is added to the competent cell and mixed by gentle flicking
- It is then incubated in ice for 30 minutes.
- The mixture is given a heat shock at 42°c for 90 seconds in a preset water bath.
- The cells are immediately placed in ice for 2 minutes to avoid damage of cells.
- 100 µl of SOC medium is added to the tube and incubated at 37°c in a shaker incubator for 1 hour.
- 75 µl of the transformed cells are plated on an ampicillin positive LB agar plate (100µg/ml) and incubated at 37°c overnight in an incubator shaker (MaxQ6000, thermofischer scientific)

3.6.6 Confirmation of cloned *GAS2* genes

The colonies obtained on LB agar plates were screened for recombinants by colony PCR.The primers used were vector specific. pJET1.2F (5`-CGA CTC ACT ATA GGG AGA GCG GC-3`) and pJET1.2R (5`-AAG AAC ATC GAT TTT CCA TGG CAG-3`).

- A small portion of the selected colonies were picked up using sterile tooth picks and dispensed into 5µl sterile water in PCR tubes and labeled.
- 5 µl of master mix consisting of 2µl 2X reaction buffer,0.2 µl pJET 1,2 forward primer, pJET 1.2 reverse primer, 0.2 µl dNTP, taq polymerase enzyme, and water is added to each tube containing the cells.

- This is mixed thoroughly and amplified in a thermal cycler

Table 5 Thermal cycling conditions for colony PCR

Initial denaturation	95°c	5 minutes	
Denaturation	95°c	30 seconds	35cycles
Annealing	55°c	30 seconds	
Elongation	65°c	1 minute	
Final elongation	65°c	5 minutes	
Hold	4°c	Infinity	

- Electrophoresis was done on 1% agarose gel stained with ethidium bromide using 1X TAE buffer and the colonies transformed with the required fragment is identified.
- The identified colonies are inoculated in 5 ml of LB broth with ampicillin (100µg/ml)
- The culture is incubated overnight in a shaking incubator at 37°c.
- The obtained culture is used for isolating plasmid DNA for sequencing.

3.7 PLASMID ISOLATION

Plasmid isolation was carried out using a column based plasmid isolation kit. (GeneJET plasmid miniprep kit, Thermoscientific).

- 4 ml of the overnight culture is pelleted by centrifugation at 8000 rpm for 5 minutes.
- The pellet is resuspended in 250µl of resuspension buffer by vortexing and pipetting.
- 250 µl of lysis solution provided is added to the above mixture and mixed by inverting 5-6 times. The solution becomes slightly clear and viscous.
- The mixture is neutralized by adding neutralization solution.

- The cell debris is sedimented by centrifugation at 12000 rpm for 5 minutes.
- The supernatant with the DNA is loaded on to a column provided with the kit.
- It is centrifuged at 12000 rpm for 1 minute and the collective is discarded and the column is placed back in the same collection tube.
- 500µl of wash solution provided is added to the column and centrifuged at 12000 rpm for 1 minute
- It is discarded and the wash step is repeated
- An additional centrifugation step is carried out to remove all the residual ethanol from the column.
- The plasmid DNA is eluted from the column into a clean Eppendorf tube by adding 20 µl sterile water and centrifuging at 12000 rpm for 1 minute.
- The quality and quantity of plasmid DNA is ensured by electrophoresis and spectrophotometry.

3.7.1 Sequencing of the positive plasmid DNA

The isolated plasmid DNA from the recombinant colonies was sent to Agrigenome, Kochi for sequencing.

3.7.2 Storage of recombinant clones and plasmids

750 µl of the recombinant colonies culture in LB broth was added to 750 µl of sterile glycerol in cryo vials and stored at -80°C

3.8 CONSTRUCTION OF PHYLOGENETIC TREE

The sequence availed from Agrigenome, DNA sequencing facility was compared with the gene sequence in similar and dissimilar organisms. FASTA sequences of these gene sequences were collected and used for the construction of a phylogenetic tree to study the evolutionary history of the gene. MEGA X software was used for the phylogenetic tree construction.

3.9 SEQUENCE ANALYSIS OF GAS2 USING BIOINFORMATICS TOOLS

The ORF sequence was identified from the sequenced data using NCBI ORF finder. This ORF sequence was used in ExPASy translate tool to obtain the amino acid sequence of GAS2. The nucleotide and amino acid sequences were compared using NCBI BLAST and the sequence distances, comparing GAS2 sequences from other organisms were calculated using Clustal W. The phylogenetic tree was constructed using MEGAX software using GAS2 sequences from related and distant species. The GAS2 protein parameters were assessed using Protparam tool and its 3D structure was predicted using ExPAsy Swiss modelling software. The conserved domains were identified using NCBI conserved domain search tool and ExPASt *Superfamily* server. The domains were shaded on multiple aligned GAS2 amino acid sequences using Jalview software.The protein was checked for the presence of signal peptides and disulphide bonds.

3.9 EXPRESSION ANALYSIS OF GAS2 AT DIFFERENT TEMPERATURES

Real time PCR analysis was performed to compare the expression of *GAS2* in muscle, liver and brain tissues collected from *Etroplus suratensis* exposed to water temperature of 30 and 32 °C for different time intervals (1 hour, 12 hour and72 hour) as compared to control maintained at 26°C.

- The juvenile fish was exposed to 30 and 32 °C for a time period of 3 days.
- The fish was sampled in triplicates in 1 hour, 12 hour and 72 hour after the exposure.
- The muscle, liver and brain tissue from each fish was collected and stored in RNAlater® (Invitrogen) properly labeled and stored at -80°C.
- The total RNA from each tissue was isolated.
- DNase treatment was employed to remove DNA contamination
- cDNA preparation was done using iScript cDNA Synthesis Kit (Biorad).

- The cDNA prepared was used as template in qPCR

3.9.1RNA isolation for realtime PCR

RNA was isolated from the tissues of biological triplicate fishes exposed to the same temperature. Thus total RNA from all the collected tissues were isolated.

3.9.2 cDNA preparation

The RNA of equal concentration from all samples 1µg was used for c DNA synthesis. cDNA synthesis was done using iScript cDNA synthesis kit.The prepared c DNA was diluted to different concentrations for standardization of real time PCR.

3.9.3 Primer Designing

Specific primers for both GAS2 amplification and housekeeping genes were designed using Beacon Designer™ software. The primer and cDNA specificity was validated by preparing standard curves using different concentration and volume of cDNA and primers.

Table 6 Sequence data of Realtime PCR primers

GAS2_3F	CTTTGCACGGGACAACACAG
GAS2_7R	CGGCCCAGCTCTAAAAGACA
Beta - actin F	ACAGGTCCTTACGGATGTCG,
Beta - actin R	CTCTTCCAGCCTTCCTTCCT

Beta actin specific primers (Tine. M. 2017)

3.9.4 Real Time PCR

Real time PCR assay was carried out using LightCycler® 96 Instrument (Roche life science) in a 96 well plate using Biorad iTaq™ Universal SYBR® Green Supermix. Three positive control samples and 3 non template controls were maintained for each assay. The house keeping gene used was β actin.

Table 7.The standardized reaction mixture for real time PCR

Components	Volume
iTaq™ Universal SYBR® Green Supermix	10µl
Forward primer(400nM)	2 µl
Reverse primer(400 nM)	2 µl
c DNA (500ng/ µl)	5 µl
dd H2O	Upto 20 µl

Table 8. Thermal cycling conditions for realtime PCR analysis.

Realtime PCR system	Setting/ Mode	Polymerase Activation and DNA denaturation at 95°c	Amplification		
			Denaturation at 95°c	Annealing/ extension+ plate read at 60°c	Cycles
Roche light cycler 96	Standard	30 sec	15 sec	60 sec	50

3.9.5 Preparation of standard curve for optimization of qPCR conditions

Different concentration of cDNA and primers were trialed for optimizing the constant expression pattern of GAS2 and housekeeping genes. A standard curve was plotted for each tissue samples and the ideal PCR conditions were determined. A concentration was selected when the slope and r2 value reached standard levels.

The slope obtained from the standard curve was used to determine the efficiency of the reaction. The efficiency values were calculated from the slopes obtained from lightcycler 96 software.

Efficiency of real time PCR = $10^{(-1/\text{slope})}$

The relative expression ratio (R)of a target gene is calculated based on efficiency value € an CP value of our unknown sample versus that of the control, in reference to a reference gene. CP (Crossing Point) value is a term referred to the point at which the fluorescence produced by a gene reaches appreciably higher level than background fluorescence.

$$\text{Relative expression ratio (R)} = \frac{(\text{ Efficiency of target })^{\Delta CP\ \text{target}}}{(\text{Efficiency of reference })^{\Delta CP\ \text{control}}}$$

ΔCP target = CP elevation of control- CP elevation of sample of target gene.

Note: When the CP of the chosen reference gene is the same in the control and the sample (Pfaffl and M.W., 2001)

ΔCP control = 0

$$\text{Then } R = \frac{(\text{ Efficiency of target })^{\Delta CP\ \text{target}}}{(\text{Efficiency of reference })^{0}}$$

$$\mathbf{R = (\ Efficiency\ of\ target\ )^{\Delta CP\ target}}$$

RESULTS

4. RESULTS

The results of the study entitled "Molecular cloning characterisation and functional analysis of Growth Arrest Specific Gene 2 in Pearlspot (*Etroplus suratensis*) conducted at ICAR- Central Marine Fisheries Research Institute, Kochi during 2018-19 are depicted in this chapter.

4.1 SAMPLING OF TEMPERATURE STRESSED FISH SAMPLES

E. suratensis juveniles acclimatised at mariculture experimental facility and exposed to temperature 26°C, 30°C, 32°C were collected at 1 hour, 12 hour and 72 hour after experiment. The muscle, brain and liver tissues from each fish were collected and stored in RNAlater (Invitrogen) at -80 °C till use.

4.2 ISOLATION OF NUCLEIC ACIDS

4.2.1 Fish RNA isolation

The total RNA was isolated from each collected sample using TRIzol™ reagent. The quantity and quality of the isolated RNA was estimated using Eppendorf Biophotometer and Agarose gel electrophoresis. The gel image showed good quality of RNA obtained as bright bands and readings of the biophotometer are shown in fig.2 and table 9 respectively.

4.3 cDNA PREPARATION

Synthesis of first strand cDNA was done using M-MuLv reverse transcriptase enzyme using reverse primer specific to the gene of interest. The primers were custom synthesised from Sigma Aldrich.

4.4 AMPLIFICATION OF GENE OF INTEREST

4.4.1 Amplification of GAS2 using specific primers

PCR was employed to amplify GAS2 from the cDNA prepared from *E. suratensis* RNA using specific primers. A non-template reaction was kept as the negative control. A positive band representing the *GAS2* amplicon was observed

by agarose gel electrophoresis (Fig 3). There was no band in the negative control which indicated that there is no cross contamination.

Table 9 Biophotometer values of RNA isolated.

Concentration of RNA(ng/µl)	260/280 ratio
226	1.96

Fig. 1 *Etroplus suratensis*

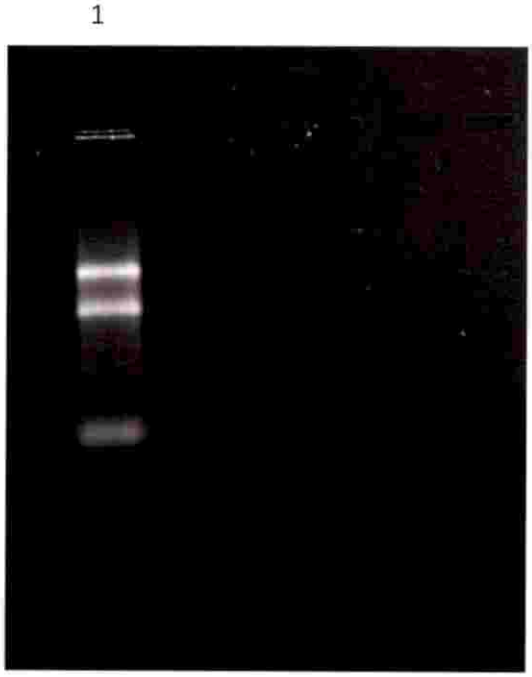


Fig. 2. Lane 1- RNA isolated from *E. suratensis* visualised on agarose gel (1%).

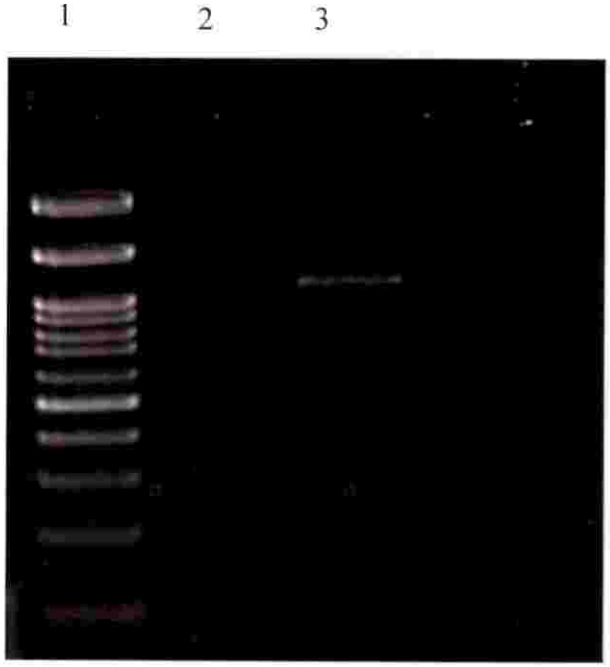


Fig 3. Gel image showing positive amplicons of GAS2 obtained by PCR using specific primers.

1 - 100 bp ladder(100bp to 3kb)
2- negative control
3- *GAS2* PCR product (1080bp)

The PCR amplicon obtained was purified using QIAquick PCR purification kit (QIAgen) to remove contaminants which may negatively affect downstream processing. The purified PCR product was analysed using agarose gel electrophoresis and biophotometer.

4.5 MOLECULAR CLONING OF GAS2

Molecular cloning was done using pJET 1.2 blunt end cloning vector

4.5.1 Ligation of GAS2 amplicon on pJET vector

Ligation of the gene of interest was done using T_4 DNA ligase enzyme in a thermal cycler by incubating the reaction mix including buffer, insert , vector and enzyme at 22°c for 30 minutes.

4.5.2 Transformation

Top 10 competent cell colonies transformed with the vector ligated product was obtained on Amp+ LB agar plates. Around 10 distinct colonies were obtained. Fig.4 shows the transformed colonies on LB ampicillin agar plate

4.5.3 Confirmation of recombinant clones using colony PCR

The colony PCR products with expected size were confirmed by agarose gel electrophoresis. The agarose gel image is shown in fig.5. A control plate was also maintained to confirm that no contaminant colonies appear.

4.5.4 Isolation of plasmid from the selected clones

Plasmid DNA was isolated from the selected positive colonies after culturing them overnight in Amp+ LB broth. The plasmid DNA obtained was checked for quality and quantity using agarose gel electrophoresis and spectrophotometer and was sent to Agrigenome DNA sequencing facility, Kochi for sequencing along with pJET specific primers. The sequencing result electropherograms were obtained.

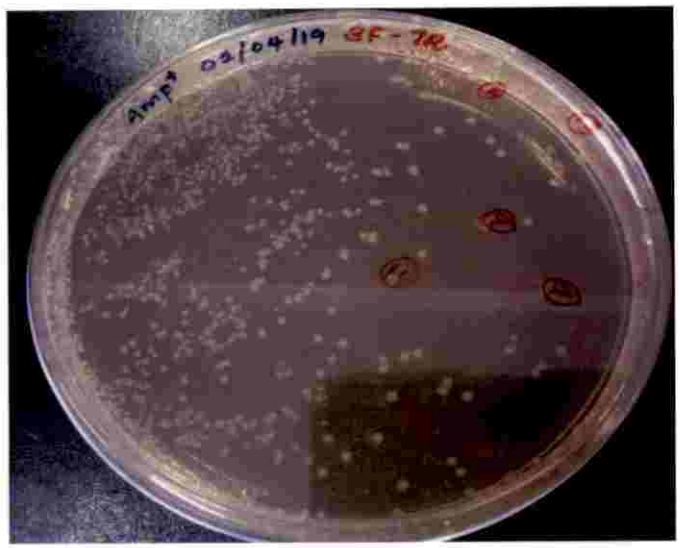


Fig.4 Transformed colonies on LB Ampicillin plate.

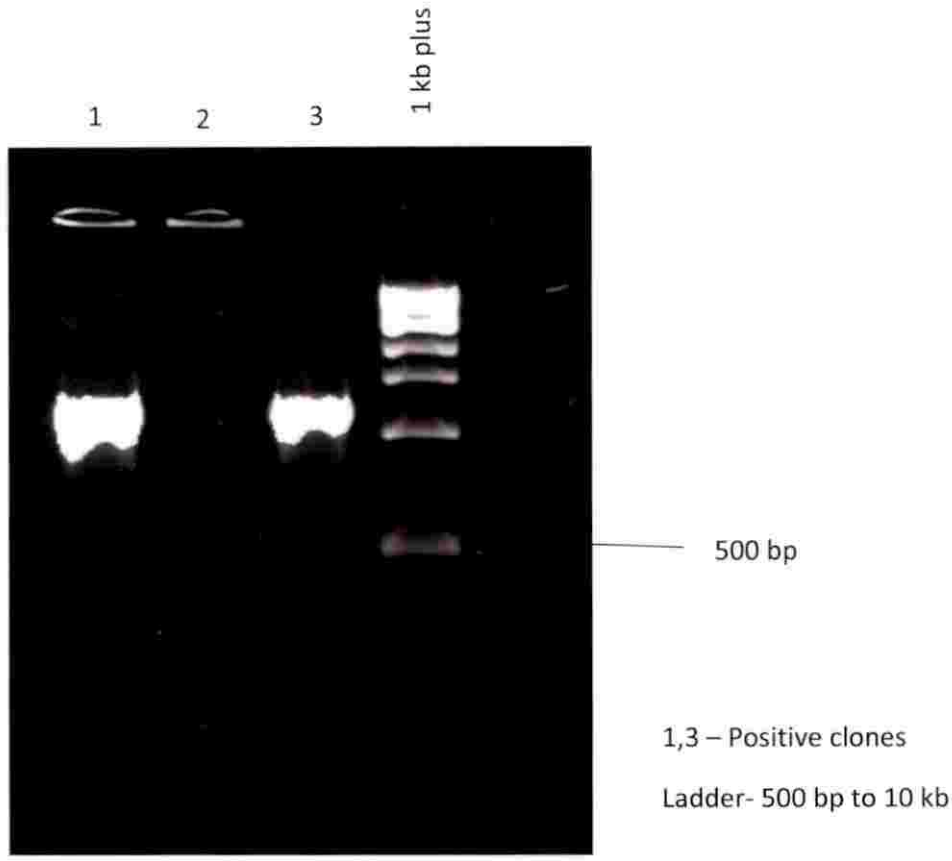


Fig. 5 Gel image showing the colony PCR products

4.6 SEQUENCE ANALYSIS OF GAS2 USING BIOINFORMATICS TOOLS

Plasmid DNA isolated from the positive colonies were sequenced. A 1081 bp sequence was obtained:

AACCAGCATCTCACAGGACGAGTGAGAGGAAGGTGCGCACCAACGAG
CTGACCCTCAGGGGAACATGTACAGCTCTTTGAGCCCCAAACAGCCCT
GCGGCCCCGGCCTGACAGACATGCAGCAATACCACCAGTGGCTTTCCA
GTCGACATGAAGCCAGCCTGCTGCCCATGAAGGAAGACCTGGCCCTGT
GGCTCACAAATATCCTTGGTCTGGAAATCACTGCTGAGAGCTTCATGG
ACCGCTTAGACAATGGATTCCTGTTGTGCCAGCTGGCTGAGACACTAC
AGGAGAAGTTCAGGCAAAGTAATGGAGATCTGCCTACCCTTGGCAAC
AGCAAGAGAATTCCTGATCGGAGAATACCATGTCGGCGGAGTGCTCCC
TCTGGTTCCTTCTTTGCACGGGACAACACAGCCAACTTCCTGGCCTGGT
GTCGTGAGGTTGGAGTTGGAGAAACATGTCTGTTTGAGTCAGAGGGTT
TAGTTCTTCATAAGCAGCCACGAGAAGTGTGCCTCTGTCTTTTAGAGCT
GGGCCGGATAGCATCACGGTACAATGTGGAGCCTCCCGGCCTTATAAA
ACTGGAGAAAGAGATTGAACAAGAAGAGAAGGCACCTCTACCTCCTC
CGTCACCCATGTCTCCAGCTCAGCCTCTCCCTCCATCTCCCTCTTCATCT
CCTTCTCCATCTCCTTCCCCATCCCCAACTCCAACTCCTACCAAAGTGA
CTTCTACCAAAAAAAGTACAGGGAAGCTGTTGGATGATGCCGTAAGAC
ATATTGCTGATGATCCACCTTGCAGATGTGTGAATAAGTTCTGTGTAG
AGAGGCAGTCGCAGGGTCGATATCGTGTTGGGGAGAAGATGCTCTTTA
TACGGATGCTGCACAACAAGCATGTGATGGTGCGAGTCGGTGGAGGCT
GGGAGACGTTTGAGAGCTACCTGCTGAAACATGACCCATGCCGCATGC
TCCAAATTTCAAGAGTAGAGGGCAAGATATCCCCAATCAGCAGCAAGT
CCCCGAACATCAAGGACCTCAGCCCTGACAGCTACCTGGTGGTGGCAG
CGCACTACCGCAGCAAAAAGTGA

4.6.1 NCBI ORF finder

The sequence was analysed in NCBI ORF finder,the open reading frame was a 1017 bp sequence.

ATGTACAGCTCTTTGAGCCCCAAACAGCCCTGCGGCCCCGGCCTGACA
GACATGCAGCAATACCACCAGTGGCTTTCCAGTCGACATGAAGCCAGC
CTGCTGCCCATGAAGGAAGACCTGGCCCTGTGGCTCACAAATATCCTT
GGTCTGGAAATCACTGCTGAGAGCTTCATGGACCGCTTAGACAATGGA
TTCCTGTTGTGCCAGCTGGCTGAGACACTACAGGAGAAGTTCAGGCAA
AGTAATGGAGATCTGCCTACCCTTGGCAACAGCAAGAGAATTCCTGAT
CGGAGAATACCATGTCGGCGGAGTGCTCCCTCTGGTTCCTTCTTTGCAC
GGGACAACACAGCCAACTTCCTGGCCTGGTGTCGTGAGGTTGGAGTTG
GAGAAACATGTCTGTTTGAGTCAGAGGGTTTAGTTCTTCATAAGCAGC
CACGAGAAGTGTGCCTCTGTCTTTTAGAGCTGGGCCGGATAGCATCAC
GGTACAATGTGGAGCCTCCCGGCCTTATAAAACTGGAGAAAGAGATTG
AACAAGAAGAGAAGGCACCTCTACCTCCTCCGTCACCCATGTCTCCAG
CTCAGCCTCTCCCTCCATCTCCCTCTTCATCTCCTTCTCCATCTCCTTCC
CCATCCCCAACTCCAACTCCTACCAAAGTGACTTCTACCAAAAAAAGT
ACAGGGAAGCTGTTGGATGATGCCGTAAGACATATTGCTGATGATCCA
CCTTGCAGATGTGTGAATAAGTTCTGTGTAGAGAGGCAGTCGCAGGGT
CGATATCGTGTTGGGGAGAAGATGCTCTTTATACGGATGCTGCACAAC
AAGCATGTGATGGTGCGAGTCGGTGGAGGCTGGGAGACGTTTGAGAG
CTACCTGCTGAAACATGACCCATGCCGCATGCTCCAAATTTCAAGAGT
AGAGGGCAAGATATCCCCAATCAGCAGCAAGTCCCCGAACATCAAGG
ACCTCAGCCCTGACAGCTACCTGGTGGTGGCAGCGCACTACCGCAGCA
AAAAGTGA

4.6.2 Translation of GAS2 nucleotide sequence

The *GAS2* nucleotide sequence was translated into amino acid sequence using ExPASy ProtParam tool. The result obtained was a 338 amino acid sequence. The molecular weight of the sequence was predicted to be 37.8 k. Da.

AMINO ACID SEQUENCE OF GAS2 PROTEIN

MYSSLSPKQPCGPGLTDMQQYHQWLSSRHEASLLPMKEDLALWLTNILG
LEITAESFMDRLDNGFLLCQLAETLQEKFRQSNGDLPTLGNSKRIPDRRIPC

ATGTACAGCTCTTTGAGCCCCAAACAGCCCTGCGGCCCCGGCCTGACA
GACATGCAGCAATACCACCAGTGGCTTTCCAGTCGACATGAAGCCAGC
CTGCTGCCCATGAAGGAAGACCTGGCCCTGTGGCTCACAAATATCCTT
GGTCTGGAAATCACTGCTGAGAGCTTCATGGACCGCTTAGACAATGGA
TTCCTGTTGTGCCAGCTGGCTGAGACACTACAGGAGAAGTTCAGGCAA
AGTAATGGAGATCTGCCTACCCTTGGCAACAGCAAGAGAATTCCTGAT
CGGAGAATACCATGTCGGCGGAGTGCTCCCTCTGGTTCCTTCTTTGCAC
GGGACAACACAGCCAACTTCCTGGCCTGGTGTCGTGAGGTTGGAGTTG
GAGAAACATGTCTGTTTGAGTCAGAGGGTTTAGTTCTTCATAAGCAGC
CACGAGAAGTGTGCCTCTGTCTTTTAGAGCTGGGCCGGATAGCATCAC
GGTACAATGTGGAGCCTCCCGGCCTTATAAAACTGGAGAAAGAGATTG
AACAAGAAGAGAAGGCACCTCTACCTCCTCCGTCACCCATGTCTCCAG
CTCAGCCTCTCCCTCCATCTCCCTCTTCATCTCCTTCTCCATCTCCTTCC
CCATCCCCAACTCCAACTCCTACCAAAGTGACTTCTACCAAAAAAAGT
ACAGGGAAGCTGTTGGATGATGCCGTAAGACATATTGCTGATGATCCA
CCTTGCAGATGTGTGAATAAGTTCTGTGTAGAGAGGCAGTCGCAGGGT
CGATATCGTGTTGGGGAGAAGATGCTCTTTATACGGATGCTGCACAAC
AAGCATGTGATGGTGCGAGTCGGTGGAGGCTGGGAGACGTTTGAGAG
CTACCTGCTGAAACATGACCCATGCCGCATGCTCCAAATTTCAAGAGT
AGAGGGCAAGATATCCCCAATCAGCAGCAAGTCCCCGAACATCAAGG
ACCTCAGCCCTGACAGCTACCTGGTGGTGGCAGCGCACTACCGCAGCA
AAAAGTGA

4.6.2 Translation of GAS2 nucleotide sequence

The *GAS2* nucleotide sequence was translated into amino acid sequence using ExPASy ProtParam tool. The result obtained was a 338 amino acid sequence. The molecular weight of the sequence was predicted to be 37.8 k. Da.

AMINO ACID SEQUENCE OF GAS2 PROTEIN

MYSSLSPKQPCGPGLTDMQQYHQWLSSRHEASLLPMKEDLALWLTNILG
LEITAESFMDRLDNGFLLCQLAETLQEKFRQSNGDLPTLGNSKRIPDRRIPC

RRSAPSGSFFARDNTANFLAWCREVGVGETCLFESEGLVLHKQPREVCLC
LLELGRIASRYNVEPPGLIKLEKEIEQEEKAPLPPPSPMSPAQPLPPSPSSSPS
PSPSPSPTPTPTKVTSTKKSTGKLLDDAVRHIADDPPCRCVNKFCVERQSQ
GRYRVGEKMLFIRMLHNKHVMVRVGGGWETFESYLLKHDPCRMLQISR
VEGKISPISSKSPNIKDLSPDSYLVVAAHYRSKK

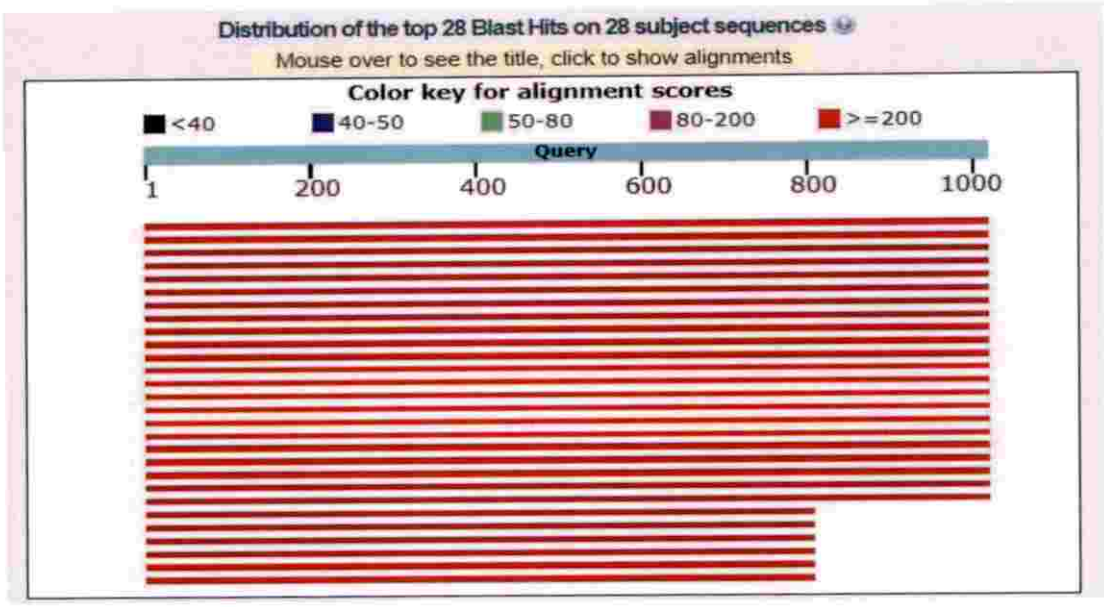
4.6.3 NCBI BLAST analysis

4.6.3.1 *NCBI BLAST analysis of E. suratensis GAS2 nucleotide sequence*

The sequence obtained was used for BLAST search in NCBI database. The *E. suratensis* GAS2 nucleotide sequence was found to have 93.81 % sequence similiarity to *Neolambricus brichardi GAS2* transcript variant 3.The results of BLAST search are shown in fig.6

4.6.3.2 NCBI BLAST analysis of E. suratensis GAS2 amino acid sequence

The amino acid sequence of GAS2 obtained from ExPASy translate was used for BLAST search in NCBI database. GAS2 protein sequence of *Oreochromis niloticus* showed the highest sequence homology with a percentage identity of 96.47 %. The NCBI – BLAST output is represented in fig.7.



Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
PREDICTED: Neolamprologus brichardi growth arrest-specific protein 2-like (LOC102797154), transcript variant X3, mRNA	1524	1524	100%	0.0	93.81%	XM_006789184.1
PREDICTED: Neolamprologus brichardi growth arrest-specific protein 2-like (LOC102797154), transcript variant X2, mRNA	1524	1524	100%	0.0	93.81%	XM_006789183.1
PREDICTED: Neolamprologus brichardi growth arrest-specific protein 2-like (LOC102797154), transcript variant X1, mRNA	1524	1524	100%	0.0	93.81%	XM_006789182.1
PREDICTED: Oreochromis niloticus growth arrest specific 2 (gas2), transcript variant X5, mRNA	1519	1519	100%	0.0	93.55%	XM_005450832.4
PREDICTED: Oreochromis niloticus growth arrest specific 2 (gas2), transcript variant X4, mRNA	1519	1519	100%	0.0	93.55%	XM_005450831.4
PREDICTED: Haplochromis burtoni growth arrest-specific 2 (gas2), transcript variant X4, mRNA	1519	1519	100%	0.0	93.71%	XM_014341137.1
PREDICTED: Astatotilapia calliptera growth arrest specific 2 (gas2), transcript variant X4, mRNA	1513	1513	100%	0.0	93.61%	XM_026175688.1
PREDICTED: Maylandia zebra growth arrest specific 2 (gas2), transcript variant X2, mRNA	1513	1513	100%	0.0	93.61%	XM_004575110.3
PREDICTED: Pundamilia nyererei growth arrest-specific 2 (gas2), transcript variant X3, mRNA	1513	1513	100%	0.0	93.61%	XM_005727970.1
PREDICTED: Pundamilia nyererei growth arrest-specific 2 (gas2), transcript variant X2, mRNA	1513	1513	100%	0.0	93.61%	XM_005727969.1

Fig.6 NCBI- BLAST output for *E.suratensis GAS2* nucleotide sequence.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
growth arrest-specific protein 2 isoform X2 [Oreochromis niloticus]	669	669	100%	0.0	96.47%	XP_005450888.1
growth arrest-specific protein 2 isoform X2 [Maylandia zebra]	667	667	100%	0.0	96.75%	XP_004575167.1
growth arrest-specific protein 2 isoform X1 [Oreochromis niloticus]	663	663	100%	0.0	94.52%	XP_005450886.1
growth arrest-specific protein 2 isoform X1 [Maylandia zebra]	660	660	100%	0.0	94.78%	XP_004575166.1
putative growth arrest-specific protein 2 [Scophthalmus maximus]	604	604	100%	0.0	92.01%	AWP08319.1
PREDICTED: growth arrest-specific protein 2 [Lates calcarifer]	603	603	100%	0.0	92.04%	XP_018521931.1
growth arrest-specific protein 2 [Acanthochromis polyacanthus]	600	600	100%	0.0	90.53%	XP_022048578.1
growth arrest specific 2 [Oreochromis niloticus]	598	598	100%	0.0	95.00%	ASN66810.1
growth arrest-specific protein 2 isoform X2 [Seriola lalandi dorsalis]	596	596	100%	0.0	92.01%	XP_023278174.1
growth arrest-specific protein 2 [Seriola dumerili]	592	592	100%	0.0	91.74%	XP_022611933.1
growth arrest-specific protein 2 [Perca flavescens]	589	589	100%	0.0	90.83%	XP_028440569.1
growth arrest-specific protein 2 isoform X2 [Monopterus albus]	588	588	100%	0.0	90.83%	XP_020478715.1
growth arrest-specific protein 2 [Echeneis naucrates]	587	587	100%	0.0	92.92%	XP_029379481.1
growth arrest-specific protein 2 isoform X1 [Monopterus albus]	585	585	100%	0.0	90.56%	XP_020478704.1
growth arrest-specific protein 2 [Fundulus heteroclitus]	582	582	100%	0.0	92.42%	XP_021163098.1
growth arrest-specific protein 2 isoform X2 [Anabas testudineus]	581	581	100%	0.0	88.17%	XP_026221141.1
growth arrest-specific protein 2-like [Parambassis ranga]	579	579	100%	0.0	92.92%	XP_028285847.1

Fig.7 NCBI- BLAST output for *E.suratensis* GAS2 amino acid sequence.

4.6.4 Analysis of GAS2 sequence using multiple alignment tools

4.6.4.1 Multiple alignment analysis of GAS2 nucleotide sequence

GAS2 nucleotide sequence was aligned and compared with the GAS2 nucleotide sequences from other species. The highest homology of GAS2 gene sequence was found with *Neolambrichus brichardi* (93.8%) (XM_006789184) (fig.8)

4.6.4.2 Multiple alignment analysis of GAS2 *amino acid sequence*

The GAS2 amino acid sequences of different species obtained by BLASTP search were aligned and compared. The GAS2 protein sequence of *Maylandia zebra*showed the highest homology of 96.4 %. (fig.9)

4.6.5 Phylogenetic analysis

A phylogenetic tree was constructed using the nucleotide sequence of growth arrest specific gene 2 from different organisms and the evolutionary pattern was analysed.

The *E. suratensis GAS2* sequence was observed as a separate lineage along with other fish families and represented a separate branch with common branch points with *O. niloticus* and *N. brichardi.* (Fig.10)

Table 10. Details of the GAS2 nucleotide sequences used for multiple alignment and phylogenetic analysis.

Organism Name	NCBI accession number
Neolamprologus brichardi	XM_006789184
Perca flavescens	XM_028584770
Hippocampus comes	XM_019857026
Homo sapiens	XR_001747829
Canis lupus	XM_022407941
Mus musculus	XM_011250796
Mus caroli	XM_029479606
Felis catus	XM_019812192
Capra hircus	XM_018043608
Macaca mulatta	XM_028832581
Bos Taurus	XM_005226733
Myotis davidii	XM_015566620
Crocodylus porosus	XM_019552024
Oryctolagus cuniculus	XM_008268897
Aquila chrysaetos canadensis	XM_011575557
Equus caballus	XM_023646179
Lipotes vexillifer	XM_007457640

Table 11. Details of the GAS2 amino acid sequences used for multiple alignment and phylogenetic analysis.

Organism	NCBI Accession number
Oreochromis niloticus GAS2	XP_005450888
Seriola lalandi dorsalis	XP_023278174
Monopterus albus	XP_020478715
Maylandia zebra	XP_004575167
Anabas testudineus	XP_026221141
Amphiprion ocellaris	XP_023128597
Homo sapiens	XP_016873021
Felis catus	XP_003993122
Bos taurus	NP_001098471 XP_871577
Cervus elaphus	ABB99418
Camelus dromedarius	KAB1269916

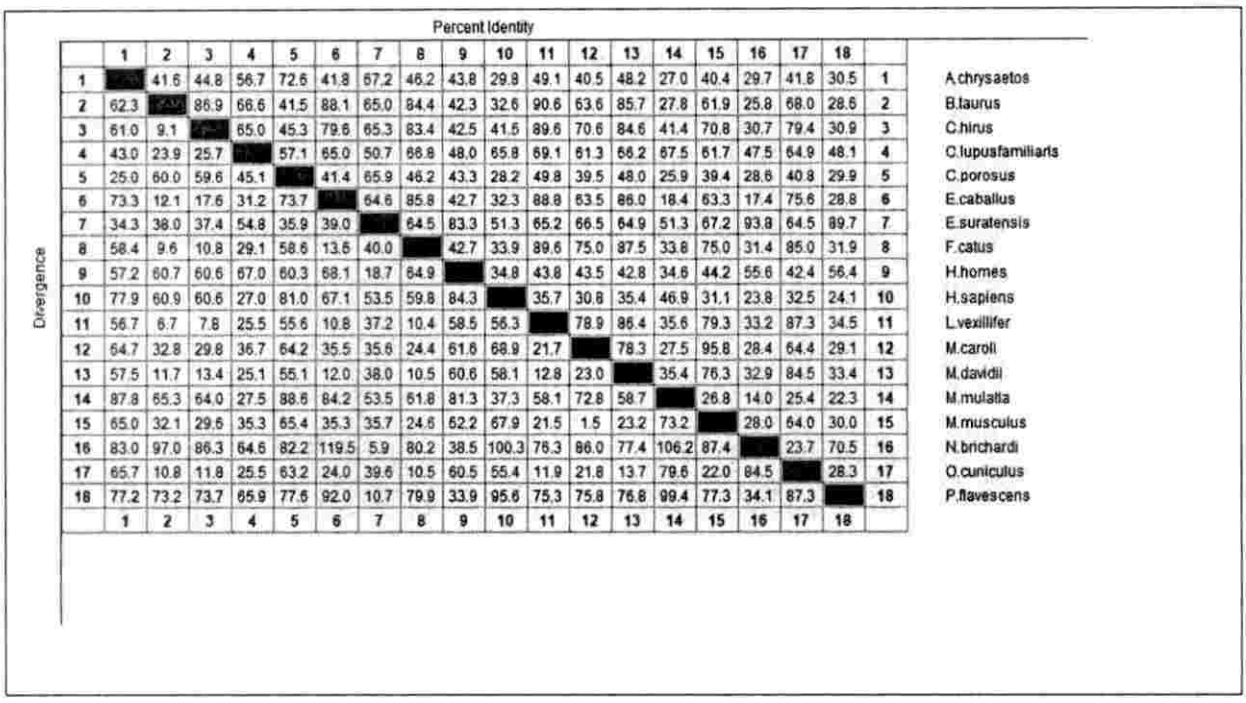


Fig.8 Picture showing the sequence distance of various *GAS2* gene sequences

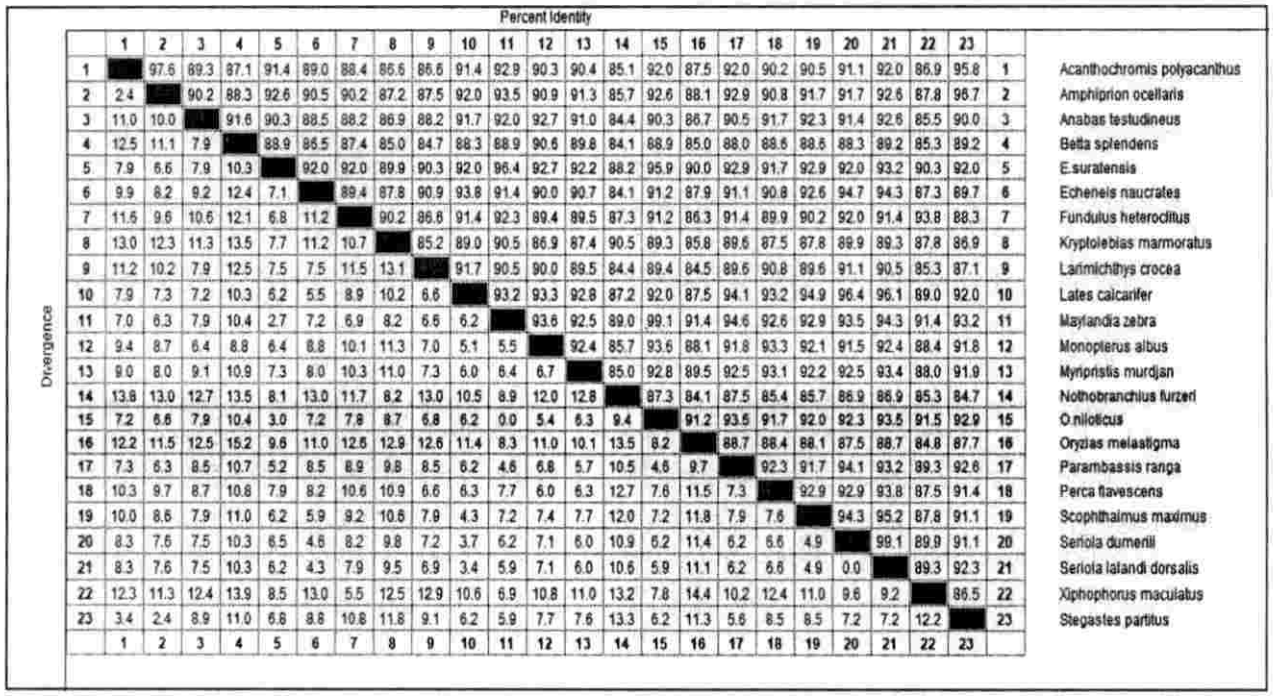


Fig.9 Picture showing the sequence distance of various GAS2 amino acid sequences

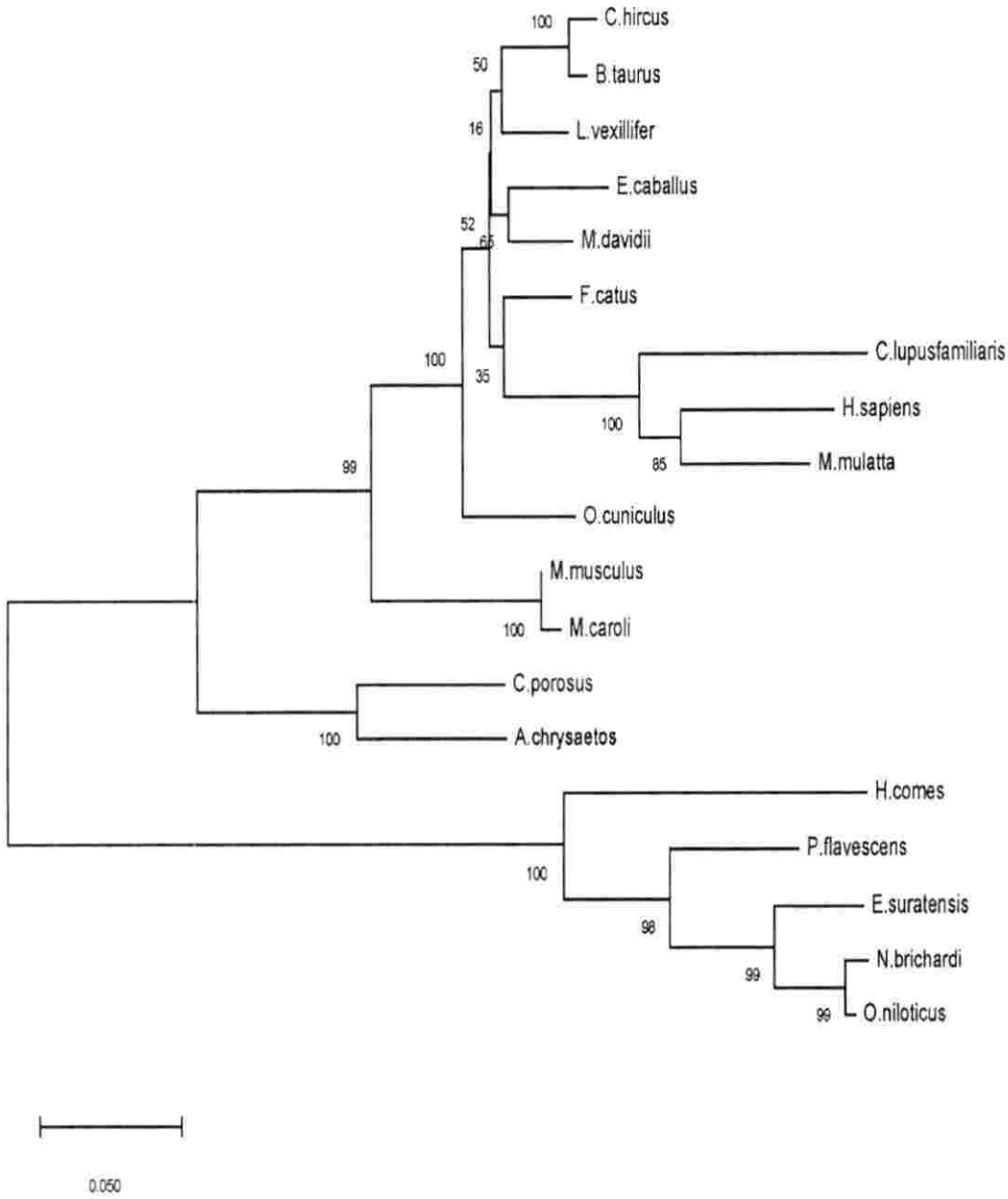


Fig.10 Phylogenetic tree showing the evolutionary relationship of *E.suratensis GAS2* with other GAS2 sequences

4.6.6 GAS2 protein characterisation

The aminoacid sequence of *E. suratensis* GAS2 was analysed using ExPASy ProtParam tool. The molecular weight of GAS2 protein was predicted to be 37.8 k. Da. with 338 amino acids and an isoelectric point of 8.497. The total number of negatively charged residues (Asp+ Glu) are 37 and the positively charged residue (Arg +Lys) are 44.

The predicted amino acid composition and atomic composition of GAS2 protein is represented in table 10 and table 11.

4.6.6.1 Conserved domains in GAS2

The conserved domains in GAS2 protein was identified using NCBI conserved domain search. GAS2 was found to have a putative actin binding surface with Calponin homology (CH) domain and GAS2 domain as conserved domains (fig. 11, 12, 13,14).

GAS 2 contains a CH domain at the 30- 179 amino acid region and a GAS2 domain in the 229 to 308 amino acid region. Calponin homology domain is an actin-binding domain which may be present as a single copy or in tandem repeats (which increases binding affinity). The CH domain is found in cytoskeletal and signal transduction proteins, including actin-binding proteins like spectrin, alpha-actinin, dystrophin, utrophin, and fimbrin, proteins essential for regulation of cell shape (cortexillins), and signaling proteins (Vav).

The GAR (Gas2-related) domain is commonly observed in plakin family members and Gas2 family members. It comprises about 57 amino acids and has been shown to bind to microtubules (Applewhite et al., 2010; Sun et al., 2001). This domain has an alpha-beta (5)-alpha fold with two layers (alpha/beta) consisting of meander antiparallel sheet.

The 3D structure of Gas2 protein was predicted using ExPASy Swiss modelling software. The result obtained is shown in fig.15

Table 12 Amino acid composition of GAS2 protein

Ala (A)	15	4.4%
Arg (R)	23	6.8%
Asn (N)	10	3.0%
Asp (D)	14	4.1%
Cys (C)	11	3.3%
Gln (Q)	13	3.8%
Glu (E)	23	6.8 %
Gly (G)	19	5.6%
His (H)	8	2.4%
Ile (I)	13	3.8%
Leu (L)	38	11.2%
Lys (K)	21	6.2%
Met (M)	9	2.7%
Phe (F)	10	3.0%
Pro (P)	34	10.1%
Ser (S)	36	10.7%
Thr (T)	14	4.1%
Trp (W)	4	1.2%
Tyr (Y)	7	2.1%
Val (V)	16	4.7%
Pyl (O)	0	0.0%
Sec (U)	0	0.0%

Table 13 Atomic composition of GAS2 protein

Carbon	C	1666
Hydrogen	H	2663
Nitrogen	N	471
Oxygen	O	493
Sulphur	S	20

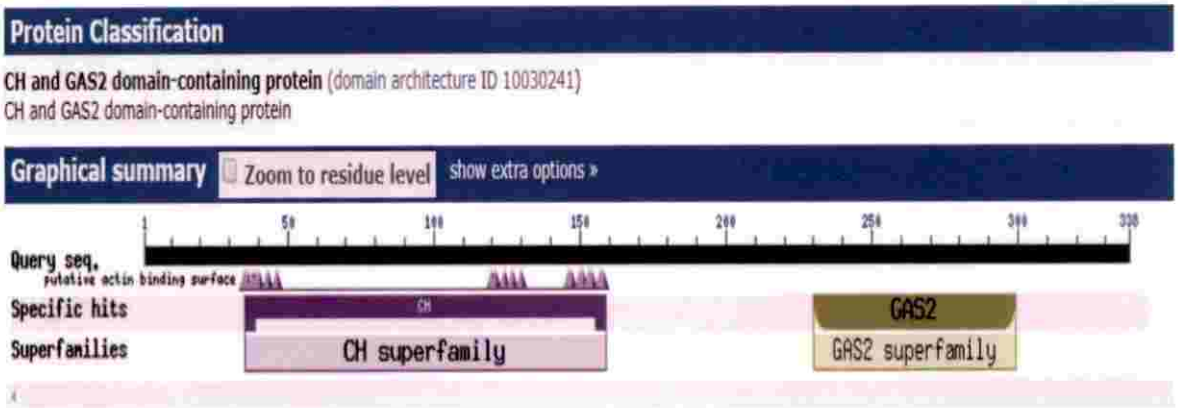


Fig.11. NCBI conserved domain search tool output

Sequence:	GAS2	
Domain Number 1	Region: 30-179	
Classification Level	Classification	E-value
Superfamily	Calponin-homology domain, CH-domain	1.45e-39
Family	SCOP hierarchy in SUPERFAMILY	0.069
Further Details:	Family Details Alignments Genome Assignments Domain Combinations	

Sequence:	GAS2	
Domain Number 2	Region: 229-308	
Classification Level	Classification	E-value
Superfamily	GAS2 domain-like	3.66e-30
Family	GAS2 domain	9.15e-06
Further Details:	Family Details Alignments Genome Assignments Domain Combinations	

Sequence:	GAS2	
Domain Number -	Region: 183-222	
Classification Level	Classification	E-value
Superfamily	PDB	1.31e-04
Family	SCOP hierarchy in SUPERFAMILY	0.040
Further Details:	Family Details Alignments Genome Assignments Domain Combinations	

Fig.12 ExPASy Superfamily server output

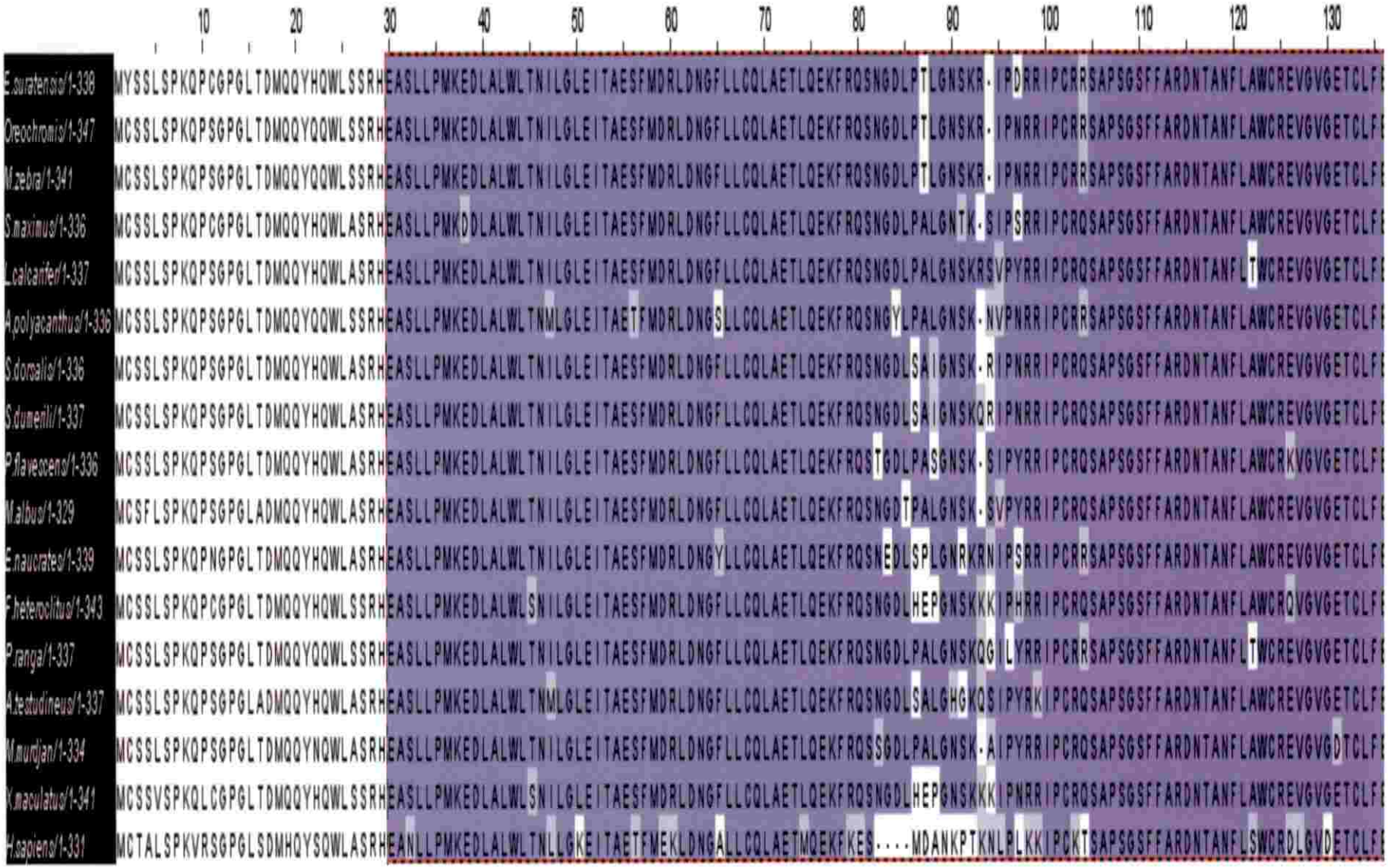


Fig. 13 Calponin homology domain in GAS2 protein.

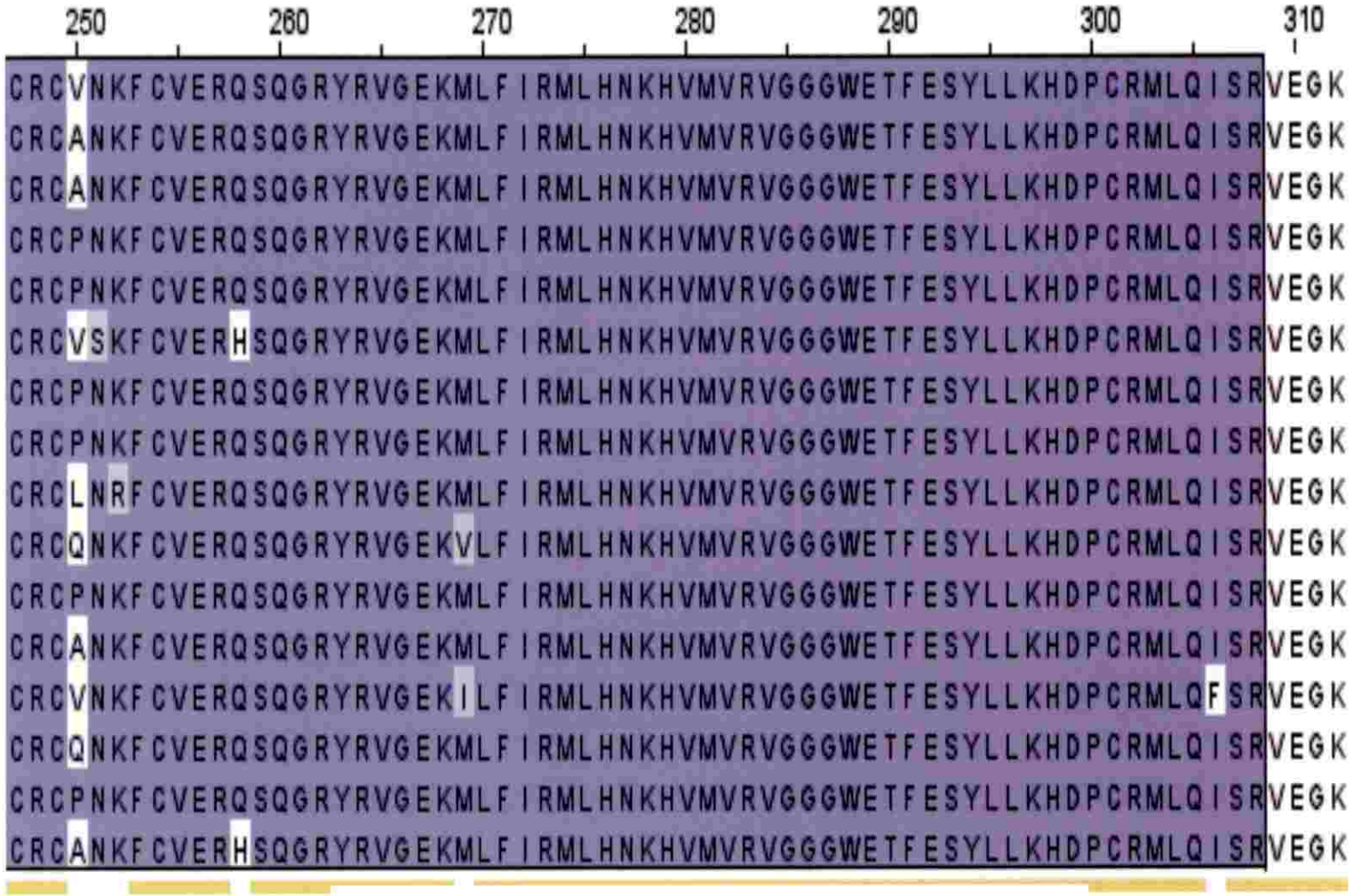


Fig. 14 GAS2 conserved domain

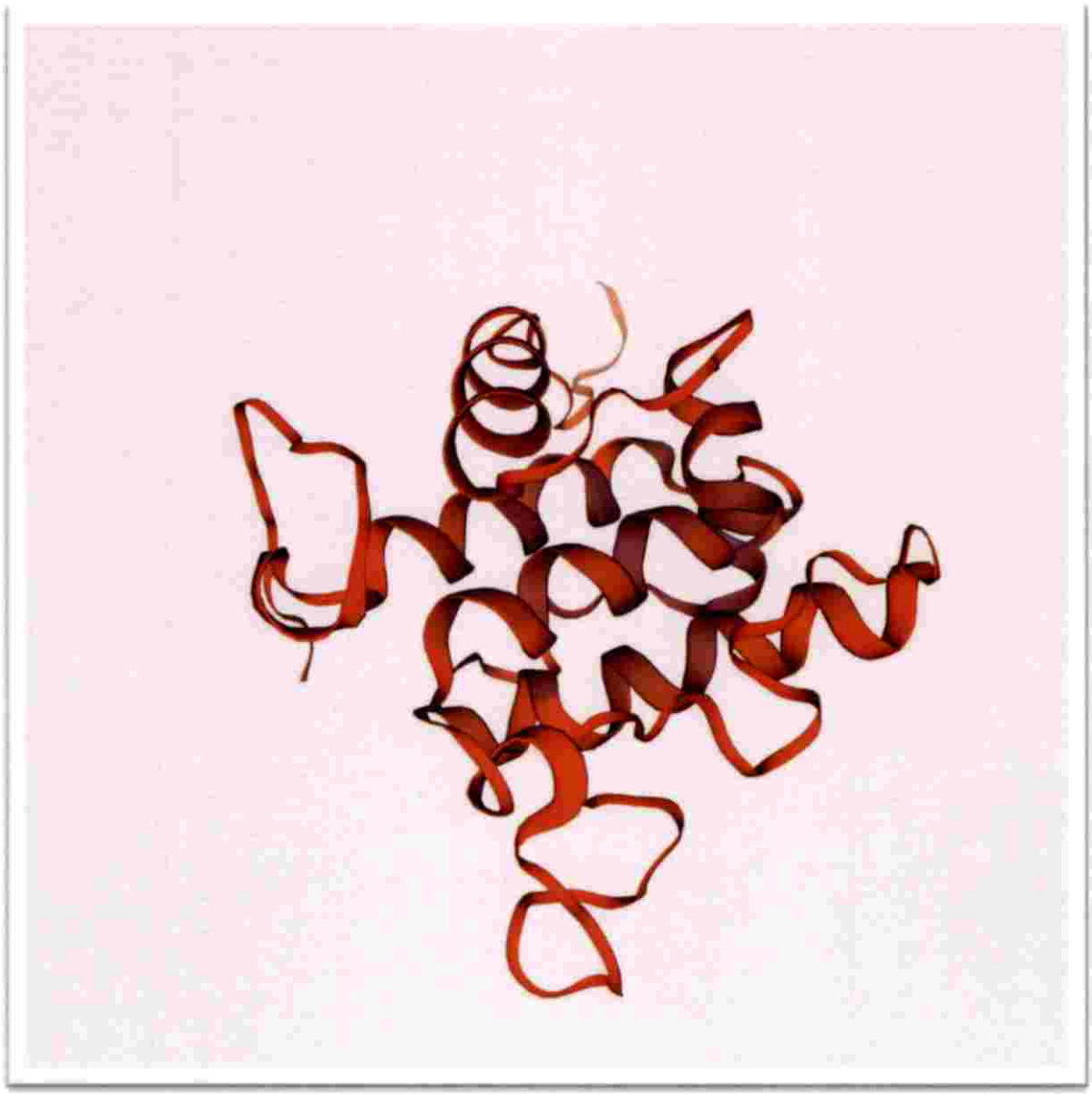


Fig. 15 3D structure of GAS2

4.7 COMPARITIVE STUDY OF EXPRESSION OF *GAS2* AT DIFFERENT TEMPERATURES

4.7.1 Standard curve plot

Standard curve was prepared using using different known concentration of cDNA. Standard curve was plotted using Roche light cycler 96 application software by plotting the CP value of samples against the concentration used. The slope and r^2 obtained was used to optimise the conditions for real time analysis.

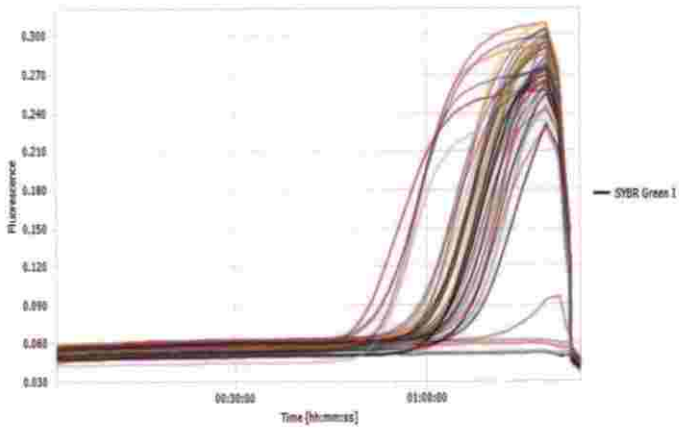
4.7.2 Real time PCR analysis of *GAS2*

Real time PCR was carried out with muscle, brain and liver samples separately with a positive and non-template control. The control used was the samples collected from *E suratensis* maintained at 26°C. The light cycler software was used to analyse the data obtained. The Ct values and Ct curves obtained are shown in fig. 16.

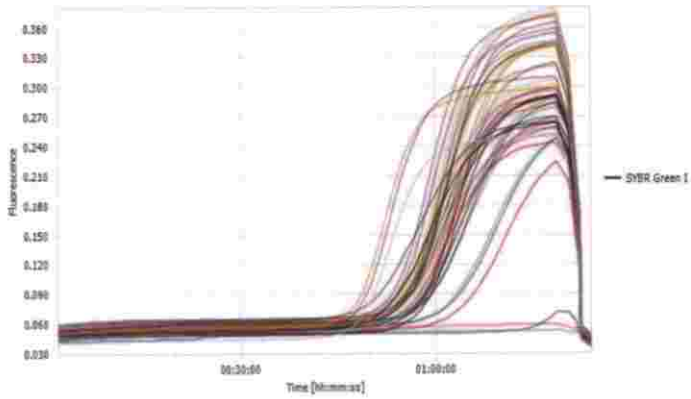
4.7.3 Comparative analysis of *GAS2* expression at different temperatures

The data obtained from real time PCR was used to plot graphs showing the expression of *GAS2* at different temperatures at different exposure times. The graphs were plotted using Microsoft excel programme and used for further comparison.

MUSCLE	CT CYBR
Control	45.3966
30 1hr	44.01333
30 12 hr	44.95333
30 72 hr	45.50666
32 1 hr	43.92
32 12 hr	43.19
32 72 hr	44.66666



BRAIN	Ct SYBR
control	44.19
30 1hr	44.536667
30 12 hr	44.696667
30 72 hr	45.68
32 1 hr	45.623333
32 12 hr	44.536667
32 72 hr	44.303333



LIVER	Ct SYBR
control	44.566667
30 1hr	45.526667
30 12 hr	46.756667
30 72 hr	47.163333
32 1 hr	46.266667
32 12 hr	46.22
32 72 hr	45.94

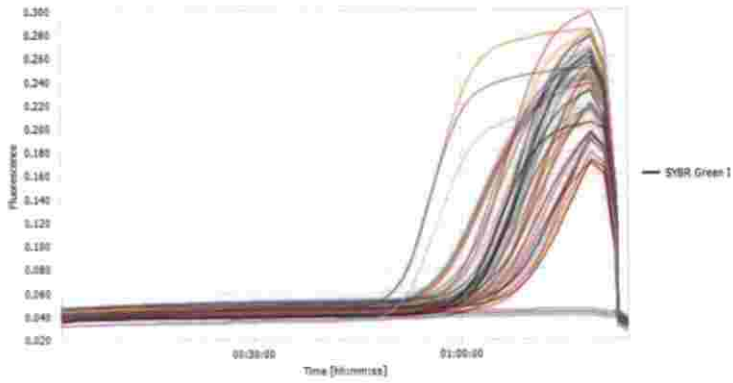


Fig. 16 Real time data obtained from Roche 96 light cycler

4.7.3.1Muscle tissue

The comparative expression of *GAS2* in muscle tissues exposed to different temperature for different time duration was calculated by taking the values obtained from 26°C exposed fish muscle samples as control.

$$R = (\text{ Efficiency of target })^{\Delta Cp\ target}$$

The real time data showed that the expression of *GAS2* in muscle decreases with time in fish exposed to 30°C. The expression ratio value was observed as 2.608704 at one hour of exposure, which reduced to 1.359742 and 0.926588 at 12 hour and 72 hour of exposure respectively. The results are presented in table no. 12 and fig.17.

In contrast the relative expression ratio value of muscle tissue of fish exposed to 32°C for the same time period of 1 hour to 72 hour was found to increase with time. The relative expression ratio in comparison to the muscle tissue of fish maintained in the control temperature of 26°C was found to increase at 32°C. The R value was observed as 1.658639 at the first hour sampling, which increased to 1.874709 at 12 hour and 2.78305 at the 72 hour sample. The relative expression ratio values and graph showing the relative expression are shown in table 13 and fig.18

4.7.3,2 Brain tissue

The relative expression ratio value obtained from real time PCR of brain tissue samples suggest that the expression of GAS2 decreases when the fish is exposed to 30°C and continues to decrease at 72 hours of exposure. The relative expression value of GAS2 compared to control brain tissue are 0.786399 afterl hour of exposure, 0.703847 after 12 hour and decreases to 0.356013 (Table 14, fig.19).

The relative expression of GAS2 at 32 increases in reference with 26°C. The relative expression value at 32°C were 0.370274 after 1 hour, 0.786399 after 12 hour, 0.92445 after 72 hours. (table 15) (fig. 20)

4.7.3.3 Liver tissue

The real time data showed that the expression of GAS2 in liver tissue of fish exposed to 30°C decrease in time compared to the expression of GAS2 in liver of the control. . The relative expression values observed were 0.514057 at 1hour, 0.219151 at 12 hour and 0.16532 at 72 hour. The results are presented in table 16 and fig. 21.

In contrast the relative expression ratio value of liver tissue of fish exposed to 32°C for the same time period of 1 hour to 72 hour was found to increase with time. The relative expression ratio in comparison to the liver tissue of fish maintained in the control temperature of 26°C was found to increase at 32°C. The R value was observed as 0.307786 at the first hour sampling, which increased to 0.317905 at 12 hour and 0.385998 at the 72 hour sampling. The relative expression ratio values and graph showing the relative expression are shown in table 17and fig.22

4.7.3.4 Relative expression in muscle, brain and liver tissues

The expression was compared in muscle, brain and liver tissues. The expression was compared in tissues at 30°C and 32°C. The highest expression was observed in muscle tissue at 30°C and 32°C at every point of sampling. The least expression was observed in liver at every sampling during the experiment. (fig. 23 & 24)

Table 14. Relative expression ratio values of *GAS2* in muscle tissue of fish exposed to 30°C

MUSCLE	Relative expression ratio (R)
30°C 1 HOUR	2.608704
30°C 12 HOUR	1.359742
30°C 72 HOUR	0.926588

Table 15. Relative expression ratio values of *GAS2* in muscle tissue of fish exposed to 32°C

MUSCLE	Relative expression ratio(R)
32 °C 1 HOUR	1.658639
32 °C 12 HOUR	1.874709
32 °C 72 HOUR	2.78305

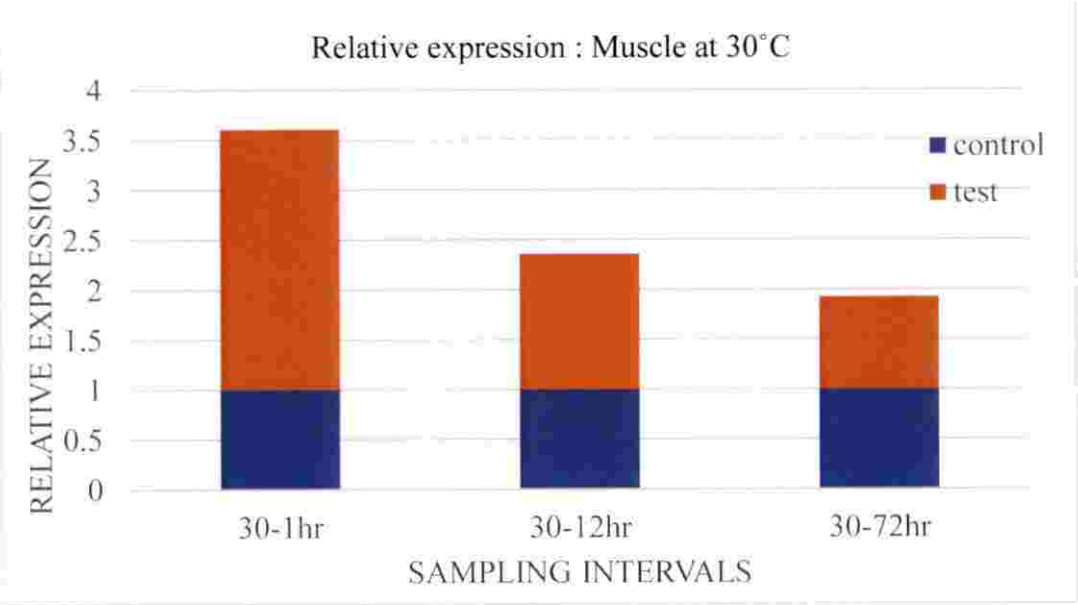


Fig. 17: Graph showing the relative expression of GAS2 in muscle tissue of fish exposed to 30˚C.

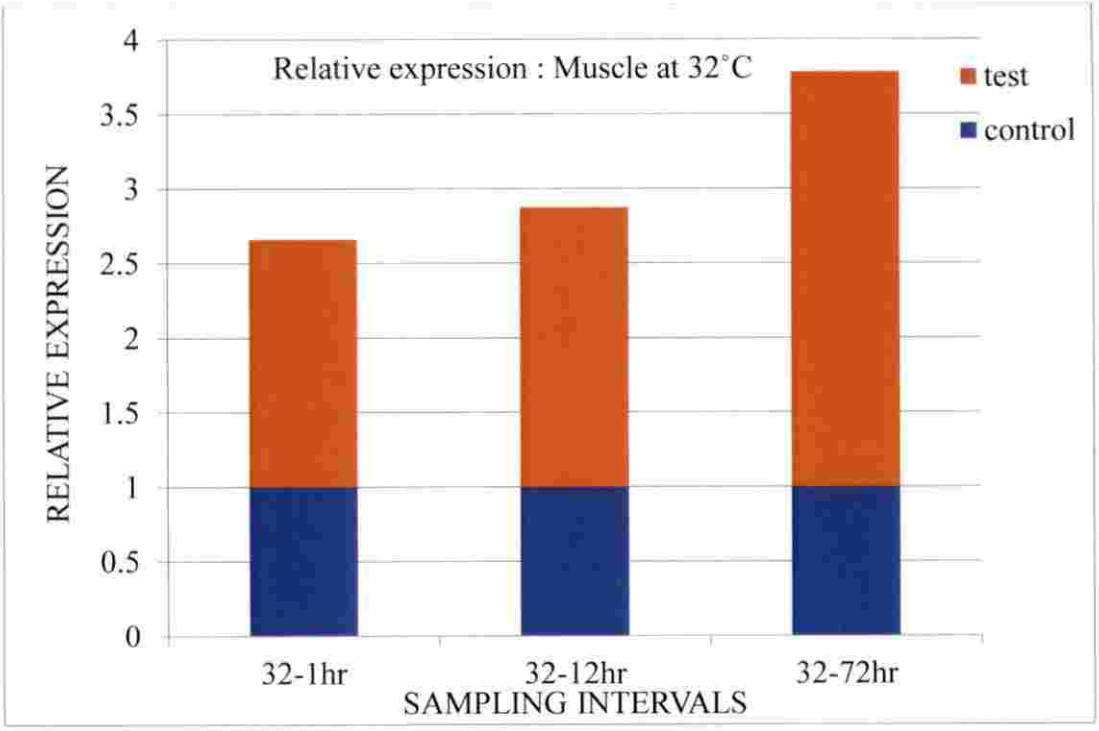


Fig. 18: Graph showing the relative expression of GAS2 in muscle tissue of fish exposed to 32 ˚C

Table 16. Relative expression ratio value of GAS2 in brain tissue of fish exposed to 30°C

BRAIN	Relative expression ratio (R)
30°C 1 HOUR	0.786399
30°C 12 HOUR	0.703847
30°C 72 HOUR	0.35601

Table 17. Relative expression ratio value of GAS2 in brain tissue of fish exposed to 32°C

BRAIN	Relative Expression Ratio (R)
32 °C 1 HOUR	0.370274
32 °C 12 HOUR	0.786399
32 °C 72 HOUR	0.92445

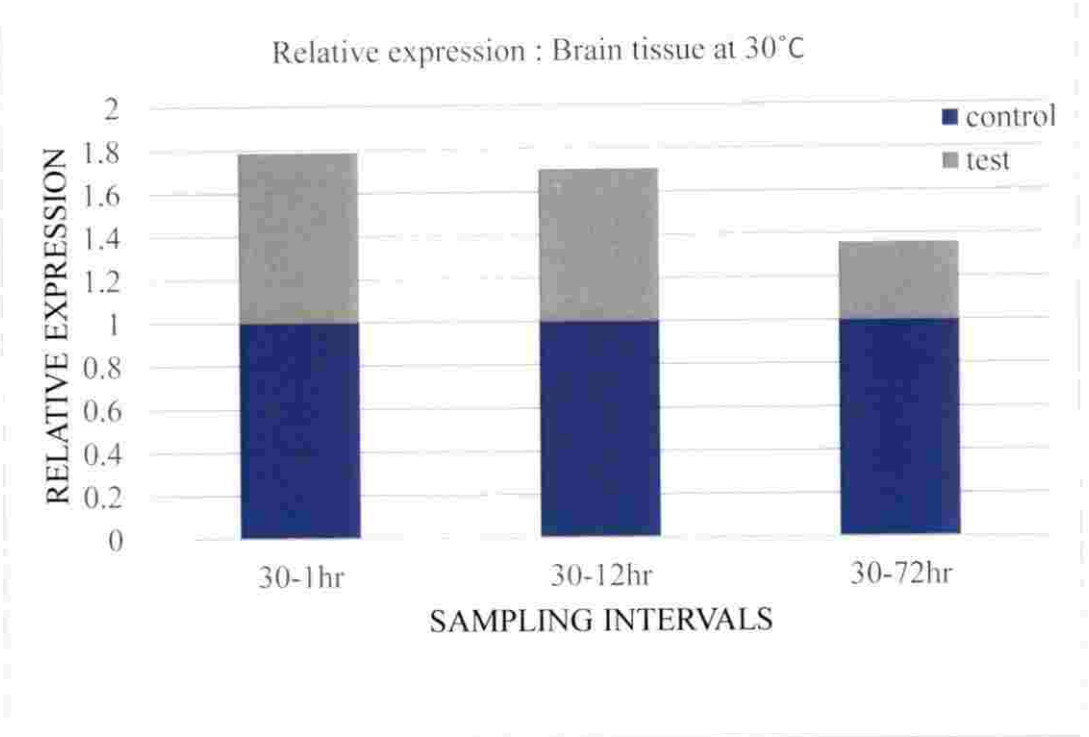


Fig 19. Graph showing the relative expression of GAS2 in brain tissue of fish exposed to 30 °C.

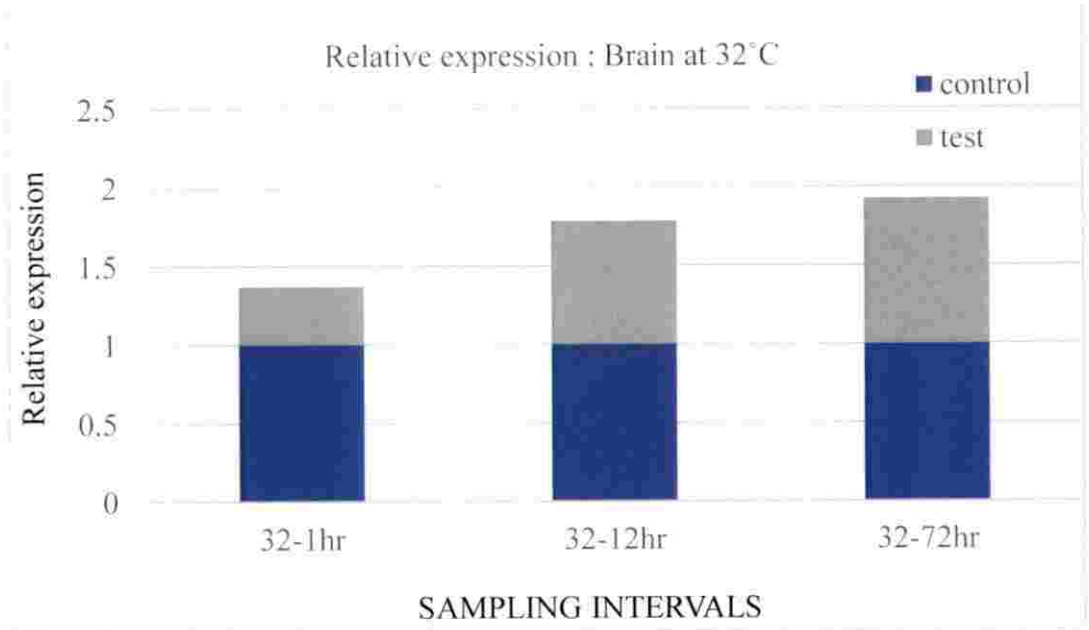


Fig 20. Graph showing the relative expression ratio of GAS2 in brain tissue of fish exposed to 32°c for a period of 1 to 72 hours

Table 18. Relative expression ratio value of GAS2 in liver tissue of fish exposed to 30°C

LIVER	Relative expression ratio (R)
30°C 1 HOUR	0.514057
30°C 12 HOUR	0.219151
30°C 72 HOUR	0.16532

Table 19. Relative expression ratio value of GAS2 in liver tissue of fish exposed to 32°C

LIVER	Relative expression ratio (R)
32 °C 1 HOUR	0.307786
32 °C 12 HOUR	0.317905
32 °C 72 HOUR	0.385998

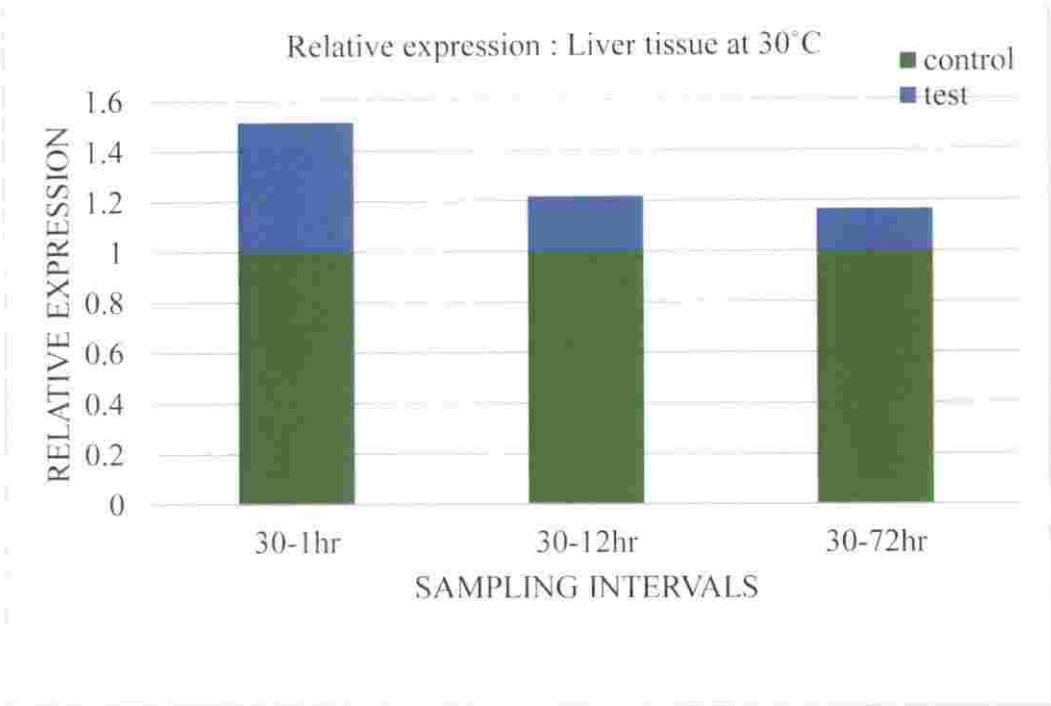


Fig 21. Graph showing the relative expression ratio of GAS2 in liver tissue of fish exposed to 30˚C.

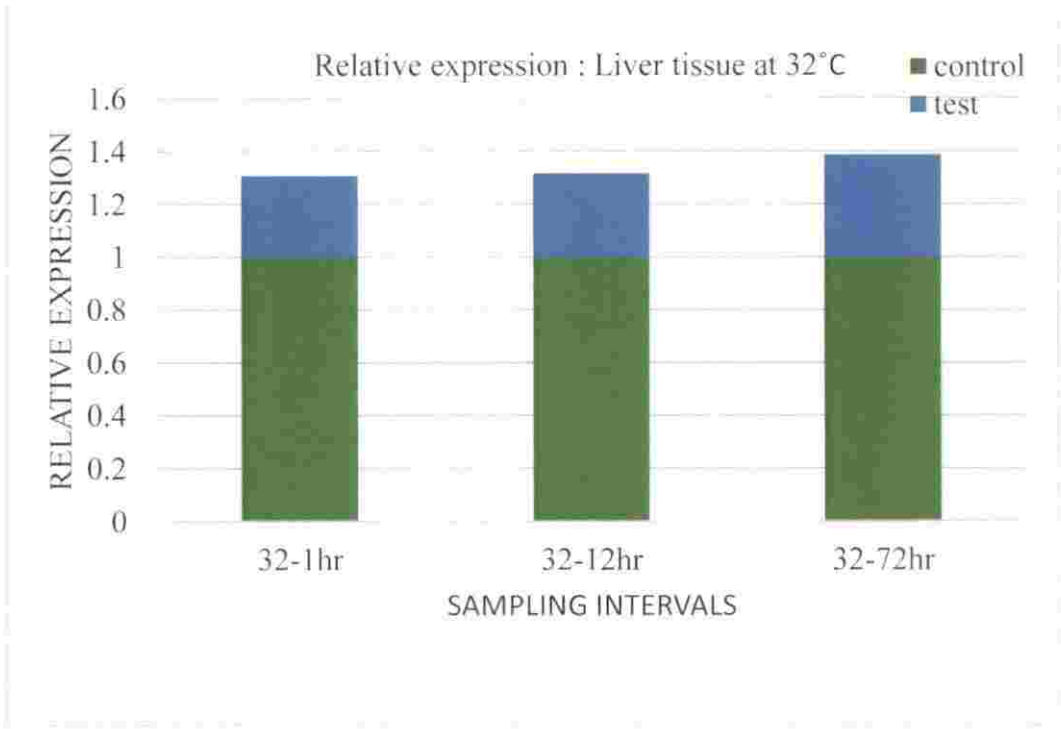


Fig 22. Graph showing the relative expression ratio of GAS2 in liver tissue of fish exposed to 32˚C.

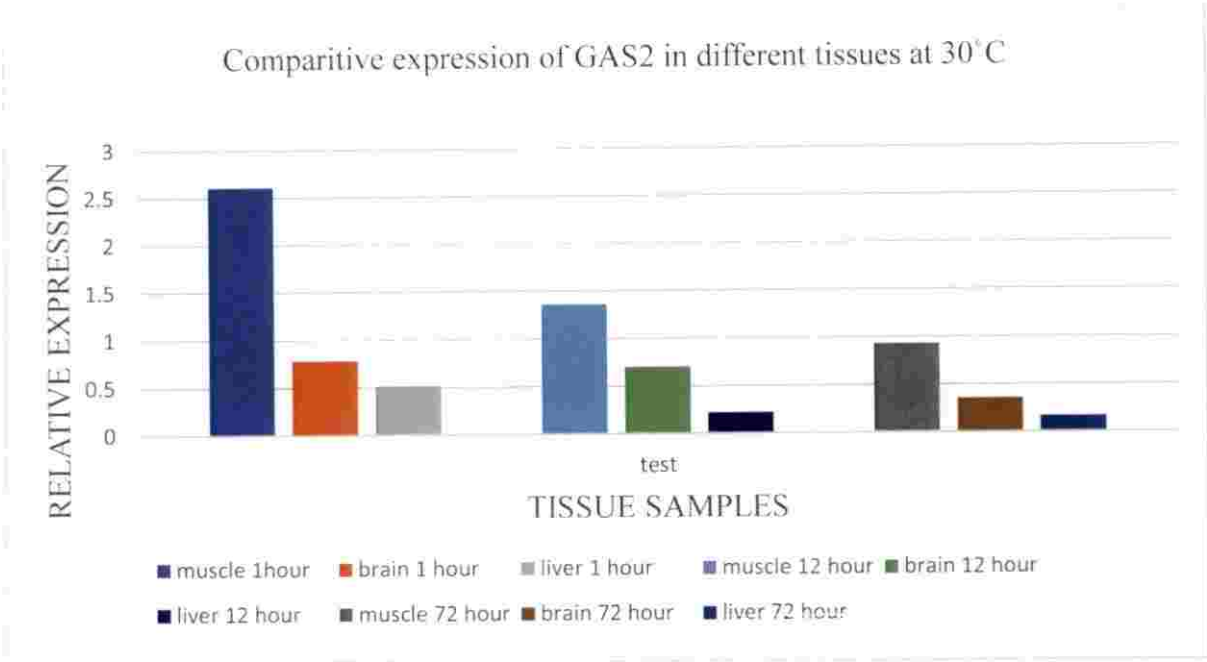


Fig.23 Graph showing the comparative expression of *GAS2* in 30˚C in muscle, brain, liver at different sampling intervals.

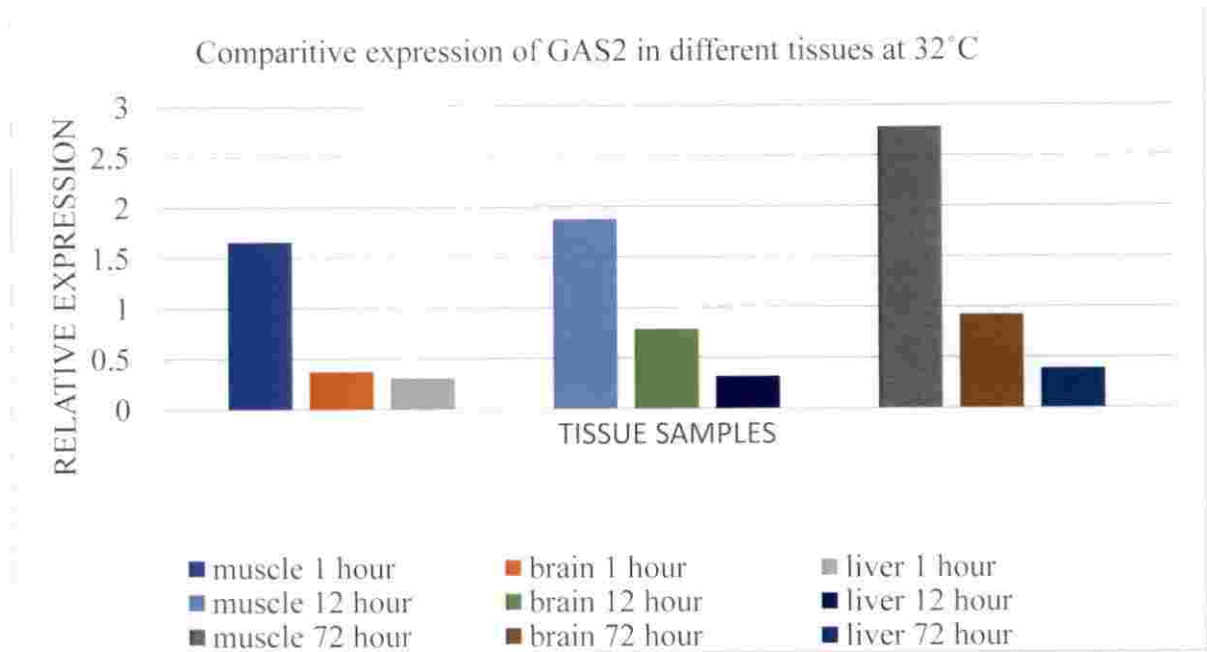


Fig. 24 Graph showing the comparative expression of *GAS2* at 32 ˚C in muscle, brain and liver tissues at different temperatures at different sampling intervals.

DISCUSSION

5. DISCUSSION

E. suratensis is a food fish belonging to the family *Cichlidae*. It is one of the most important fish delicacy in Kerala and is recognised as the state fish of Kerala. The fish has tolerance to a wide range of water temperatures ranging from 16.5°C to 41.5 °C (Rajguru.S, 2002). This temperature tolerance allows the breeding of Pearlspot in a wide variety of habitats. Changes in the environmental conditions and climacteric factors adversely affect the growth and development of fish. Hence understanding the expression of stress associated genes which hinder the normal development of the fish is important. Growth arrest specific gene 2 is a component of the microfilament system which plays a major role in cell death and apoptosis (Brancolini *et al.*, 1992). GAS genes are studied to be upregulated during invitro serum starvation and cell contact inhibition (Manfioletti *et al.*, 1990; Schnieder *et al.*,1988). It was found to be upregulated in tilapia under low temperature stress (Yang *et al.*, 2017)

Real time PCR is a powerful tool to analyse the expression of genes because of its high sensitivity. qPCR detect even the minute levels of expression making the study more reliable and accurate. It is more convenient to study the protein expression patterns through real time PCR than doing conventional protein expression experiments.

The cDNA prepared from total RNA was amplified by specific primers to obtain the sequence of GAS2 gene. The predicted nucleotide sequence obtained after sequencing was searched against NCBI database. This showed significant sequence similarity to GAS2 nucleotide sequence of *N.brichardi*. The amino acid sequence of GAS2 obtained using ExPASy translate tool was also used for BLAST search and it was found to be the most homologous to GAS2 protein sequence of *Oreochromis niloticus*. The ORF was found to be a 1017 bp sequence.

The protein parameter studied using ExPASy ProtParam tool suggested that GAS2 consist of 338 aminoacids and have a molecular weight of 37.8 k. Da. The conserved domains in the sequence found were calponin homology (CH) domain

and GAS2 related (GAR) domain. The GAS2 binds CH domain in the 30- 179 amino acid region and a GAS2 domain in the 229 to 308 amino acid region.

The CH domain was first identified as a protein molecule of about 100 residues at the N terminus of calponin, which is an active actin binding protein involved in muscle contraction. (Korenbaum *et al.*,2002).

GAS2 protein belongs to GAS2 family of proteins, They play a major role in mediating the interaction between filamentous actin and microtubules. It also regulate cell cycle, apoptosis and calpain activities. (Lee *et al.*, 1999;Zhou *et al.*, 2014) .It was found to be up-regulated in chronic myeloid leukaemia cells and was found required for their growth.

Most of the actin binding proteins comprise a 250 amino acid stretch called actin binding domain (ABD). This domain is assumed to have arisen by duplicating a domain found in single copy in number of other proteins like calponin or the vav protooncogene and is called calponin homology CH) domain (Stradal *et al.*, 1998; Keep *et al.*, 1999).

Each single ABD, comprising two CH domains, is able to bind one actin monomer in the filament. The amino terminal CH domain has the intrinsic ability to bind actin, with lower affinity than the complete ABD, whereas the carboxy terminal CH bind actin extremely weakly or not at all. Nevertheless both CH domains are required for a fully functional ABD. The C-terminal CH domain contribute to the overall stability of the complete ABD through inter-domain helix-helix interactions (Stradal *et al.*, 1998). Some of the proteins containing a single CH domain can also bind to actin, but it was studied that this interaction was not only by the single CH domain. (Keep *et al.*, 1999). CH domain is present in other proteins not involved in actin binding such as protooncogene .GAR domain bind to microtubules and comprises about 57 amino acids (Sun *et al.*, 2001). Transcription factors which bind to these sites are mainly involved in growth and development, regulation of cell cycle, immunoregulation and cell apoptosis. One of the transcription factor involved is Fork head box (FoxO) which has a major role in

stress tolerance. (Yang *et al.*, 2017; Bramham *et al.*, 2002). Its molecular functions include microtubule binding.

Heat stress can trigger the expression of series of gene activation and physiological responses, which include cell proliferation mechanisms, cell cycle changes and cell apoptosis (Sonna *et al.*, 2002). Yao *et al.* 2012 had shown that mussels show the same pattern of low and high temperature stress response mechanisms and is dependent on caspase 3. They also suggested a link between DNA damage at high and low temperatures and subsequent stress reaction such as induced cell apoptosis (Allanson and Noble, 1962)

The study conducted at mariculture facility CMFRI, Kochi was also aimed to study the expression of *GAS2* at higher temperatures. Pearlspot belongs to the cichlid family of fishes and is one of the most valued food fish in Kerala. Since it is of high demand regardless of its high price, it is an important candidate in aquaculture. Since, temperature is one of the most influential factor affecting fish growth, it is necessary to identify a temperature at which *GAS2* gene, which hinder growth is down regulated. The experiments which were conducted at controlled conditions suggested results with significant differences in *GAS2* expression based on the temperatures at which the fish were held. The expression pattern of the gene significantly differed from the control and this suggested that this variation in expression is not resulted from any other natural driving forces. In a study by Yang et al. 2017 *Tilapia* (which belong to the same family) showed high expression of *GAS2* at low temperature stress and expression was found to increase further when the temperature was decreased. Their experimental temperature ranged from 25°C to 10°C. The highest expression was reported in liver tissues of fish exposed to 10°C (Yang *et al.*, 2017). This suggested that GAS2 is a temperature stress associated gene.

In our experiment, the expression of *GAS2* was found to decrease with time when exposed to 30°C. But in the experimental fishes at 32°C, the expression was found to increase in time and reached the highest value towards 72 hour. These inferences were made by comparing the experimental data to the expression value

obtained in control fishes maintained at 26°C. The relative expression value of GAS2 in muscle tissue of fish juveniles maintained at 30°C at one hour of exposure was 2.608704 which reduced to 1.359742 after 12 hour and 0.926588 after 72 hour of exposure. The trend was found to be opposite in the fish juveniles maintained at 32°C. The relative expression ratios were 1.6586391 after one hour, which increased to 1.874709 at 12 hour and 2.78305 at 72 hour of exposure.

In brain tissue of fish juveniles exposed to 30°C, the relative expression value in the 1 hour sample was found to be 0.786399, the expression decreased as time elapsed. In the brain tissue collected after 12 hour, the expression ratio was found to have decreased to 0.703847 which came down to 0.35601 after 72 hours. In fish juveniles exposed to 32°C, the expression was found to increase in brain tissues when compared to the control. The expression ratio was found to be 0.370274 at 1 hour which increased to 0.786399 after 12 hour and reached 0.92445 after 72 hour.

In the liver tissue the expression was very low and was found to decrease further on 30°C exposure. At the one hour sampling, the ratio was 0.51407 which decreased to 0.219151 after 12 hour and 0.16532 after 72 hour. Even if the expression in liver tissue was feeble, at 32°C it showed an increase of 0.307786, 0.317905 and 0.385998 at one hour, 12 hour and 72 hour respectively

The relative expression in the three tissues studied were compared. At every point of sampling the expression was found to be the highest in muscle tissue and the lowest expression was found in the liver tissue. This is in contrast to the low expression of *GAS2* in muscle tissues of Tilapia exposed to low temperatures (10 °C) (Yang et al., 2017). In their study at low temperature, the highest expression was found in the liver tissue. Thus we found that the *GAS2* expression pattern results obtained from two fishes belonging to the same family are relatable.

The protein GAS2 is a caspase-3 substrate which was found to be cleaved by caspase 3 and regulate the morphological changes occurring during apoptosis. (Lee *et al.*, 1999; Benetti *et al.*, 2001). Another study has shown that the over

expression of this gene does not directly lead the cells to undergo apoptosis, but make them sensitive to apoptotic signals (Benetti *et al.*, 2001).

After comparing the tissue distribution, results suggested that the gene was expressing ubiquitously, with the highest level of expression in muscle tissues constantly, This indicates that muscle may be one of the most important location where GAS2 carryout physiological function at high temperatures.

From the observations described, we came to an inference that a temperature above 26˚C and below 32˚C is an ideal temperature range where the expression of *GAS2* is comparatively lower in Pearlspot. So adopting a water temperature in this range for Pearlspot breeding may help to avoid *GAS2* modulated growth arrest. *GAS2* is a gene which is studied to be expressed at low serum levels, cell contact inhibition and many other conditions. So we cannot conclude that the expression pattern we observed is only dependent on the temperature stress and it needs further study.

SUMMARY

6. SUMMARY

Etroplus suratensis is a fish belonging to family Cichlidae. It was recognized as the state fish of Kerala in 2010 and is a fish delicacy which fetches a very high price. Karimeen is so popular among the people due to its exceptional taste. It is a brackish water fish which can easily get acclimatised to marine and fresh water habitats. The family Cichlidae contains more than 700 species of fishes which live in brackish water and fresh water habitats. Various environmental factors affecting the growth and development of fish are studied. Growth arrest specific gene is reported to be expressed at temperature stress conditions, cell contact inhibition and serum starvation.

Growth arrest specific gene 2(*GAS2*) is a component of the microfilament system which plays a major role in growth arrest and apoptosis. *GAS2* is a gene expressed at low temperature stress in fish. The study was aimed at amplifying the gene from *Etroplus suratensis* and studying the expression pattern of the gene at different water temperatures (26˚C – 32˚C).

Total RNA was isolated from *Etroplus suratensis* using TRIzol method. The cDNA was prepared and *GAS2* was amplified using specific primers. The PCR product was cloned in pJET blunt end cloning vector and the sequence was obtained. Pearl spot juveniles were purchased and acclimatised at the mariculture facility at CMFRI, Kochi. The fish juveniles were exposed to 26˚C, 30˚C and 32˚C using a thermostat for 72 hour. The muscle, brain and liver tissues were collected from triplicate fishes held at each temperature at 1 hour, 12 hour and 72 hour intervals. The RNA was isolated from the tissues and cDNA was prepared and stored at -80 ˚C. The real time PCR analysis was done for muscle, brain and liver tissues with β actin as reference gene.

The ORF region was found to be a 1017 bp sequence. It was translated into a 338 amino peptide sequence with a predicted molecular weight of 37.8 k.Da. Two protein conserved domains were identified, a GAS2 related (GAR) domain and calponin homology(CH) domain. The Gas2 domain is usually observed in GAS2

family of proteins and plakin family proteins. GAS2 related domain (GAR) domain comprises about 57 amino acids and is studied to bind to microtubules. Calponin homology domain is an essential actin binding domain and is found in cytoskeletal proteins and in proteins involved in signal transduction.

The expression pattern of *GAS2* was found to vary under high temperature stress. The control tissues was collected from fish juveniles exposed to 26˚C. In tissue samples collected from fishes exposed to 30˚C, the expression of *GAS2* was found to decrease when compared to the expression observed at the control fish in muscle, brain and liver tissues. The expression of the same was found to increase with time in real time PCR of tissues exposed to 32˚C when compared to the control. This variation in expression pattern suggested that *GAS2* is a temperature stress associated gene.

The present study at higher temperature and previous studies in fishes belonging to same family at low temperatures showed relatable results. *GAS2* was found to be a temperature stress associated gene. Since environmental and climacteric changes in the present scenario is one of the most important factor affecting aquaculture, this study is very relevant and studying the expression pattern of the gene at different temperatures and finding an ideal temperature with the least expression of the gene will be a big milestone to the Pearlspot breeding industry. From our results, we came to a conclusion that a temperature above 26˚C, in a range of 27 ˚C to 30˚C will be ideal to grow Pearlspot since it will be effective to hinder the increased expression of growth arrest specific gene 2.

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APPENDICES

APPENDIX I

70% Ethanol

100% ethanol	70 ml
Distilled water	30 ml

APPENDIX II

TE buffer

Tris HCl (pH=8)	10m M
EDTA	1 mM

APPENDIX III

Gel loading dye

TBE buffer (10 X)	700µl
Glycerol	200 µl
Dye	100 µl

APPENDIX IV

TBE buffer(10 X)

Tris HCl	108 g
Boric acid	55g
EDTA	7.44

APPENDIX V

Agarose gel for AGE(1%)

TBE buffer (1X)	40 ml

Agarose	0.4 g

APPENDIX VI

LB Broth

LB broth	2.5 g
Distilled water	100 ml

APPENDIX VII

LB Agar

LB broth	2.5 g
Agar powder	1.5 g
Distilled water	100 ml

APPENDIX VIII

Ampicillin (100 mg/ ml)

Ampicillin (100mg)	100 mg
Double distilled water	1 ml

APPENDIX IX

Kanamycin (50 mg/ ml)

Kanamycin	50 mg
Double distilled water	1 ml

ABSTRACT

MOLECULAR CLONING, CHARACTERISATION AND FUNCTIONAL ANALYSIS OF GROWTH ARREST SPECIFIC GENE 2 IN PEARLSPOT (*Etroplus suratensis*)

by,

AMIYA THALAKKATTU

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Abstract of the thesis submitted in partial fulfillment of the requirement of the degree of

B. Sc. – M. Sc. (INTEGRATED) BIOTECHNOLOGY

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ABSTRACT

Etroplus suratensis is the State fish of Kerala which is a delicacy which has very high economic value. Water temperature is a very important factor which affect the growth of Pearlspot. There are many genes expressed at water temperature stress. The objective of the study was "Molecular cloning, characterization and functional analysis of Growth Arrest Specific gene 2 in Pearlspot *(Etroplus suratensis)*". The study was carried out at the maricuture facility, CMFRI, Kochi. Growth arrest specific gene 2 is a component of the microfilament system which plays a major role in cell death and apoptosis. This was studied to be highly expressed at cell contact inhibition, serum starvation and temperature stress The growth arrest specific gene was amplified using specific primers. The *GAS2* ORF was found to be a 1017 bp sequence which was translated into a 388 amino acid sequence. The predicted molecular weight of GAS2 was found to be 38.7 k.Da. and was found to have calponin homology domain and GAS2 as conserved domains. In the study, fish juveniles were exposed to 3 water temperatures 26˚C, 30˚C and 32˚C for 72 hours. The muscle, brain and liver samples were collected at 1 hour, 12 hour and 72 hour. The expression was studied in each tissue sample by real time PCR assay. The expression patterns at each temperature and each duration was compared with the expression observed in 26˚C samples maintained as controls.

The GAS2 expression was found to decrease proportional to the duration of exposure in fishes exposed to 30˚C when compared to that in the control sample maintained at 26˚C. The trend was found to be reverse in fish exposed to 32˚C, the expression increased at a constant level compared to the 26˚C control. In both experiments the highest expression was observed in muscle tissue and the lowest expression was found in liver tissue. *GAS2* was found to be a stress related gene which show variable expression in different water temperatures with least expression in a range of above 26˚C and below 32 ˚C.