Indian Journal of Animal Sciences **78** (12): 1426–1430, December 2008

Sequence analysis and tissue-specific expression of the ß$_2$–microglobulin gene in *Aeromonas hydrophila* infected *Cirrhinus mrigala*

B R MOHANTY[1] and P K SAHOO[2]

Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneshwar Orissa 751 002 India

Received: 23 October 2007; Accepted: 26 June 2008

ABSTRACT

Partial complementary DNAs of ß$_2$–microglobulin genes were cloned from the livers of five species in four genera of Indian carps (Indian major carp species: *Labeo rohita*, *Catla catla*, *Cirrhinus mrigala*; medium carp: *Puntius sarana*; minor carp: *Labeo bata*) subsequent to polymerase chain reaction amplification with self-designed primers derived from conserved regions of ß$_2$–microglobulin cDNA sequences. The partial ß$_2$–microglobulin cDNAs of the five species of carps had sizes from 175 to 206 bp and encoded open reading frames consisting of 58–68 amino acids. The alignment of carp cDNA sequences showed 90.2 to 98.8% homology and that of the deduced amino acid sequences showed 82.7 to 96.5% homology. The relationship among the four genera of Indian carps is well correlated with that derived from classic morphologic analyses as showed by a phylogenetic tree constructed of amino acid sequences of ß$_2$–microglobulin cDNAs from carps. The tissue-specific expression of the ß$_2$–microglobulin gene was studied in various tissues (liver, kidney, spleen, brain, muscle, testis, heart, intestine, gill and fin) from apparently healthy (control), moribund and survived *Cirrhinus mrigala*, experimentally infected with *Aeromonas hydrophila*. ß$_2$–microglobulin mRNA was detected in all the tissues analyzed irrespective of the bacterial infection.

Key words: *Aeromonas hydrophila*, ß$_2$–microglobulin, Carp, *Cirrhinus mrigala*, Gene expression

ß$_2$–microglobulin (ß$_2$–m) is a low molecular mass protein (~12 kDa) present in the serum as free form as well as cell surface associated form with class I major histocompatibility complex (MHC-I) (Klein 1986). Subsequently, it was also found to be associated with α-chains of CD1 molecules (Calabi and Milstein 1986) and α-chains of the Fc receptor for IgG (Simister and Mostov 1989). It helps in presenting endogenous peptides derived from proteasomal degradation of phagocytosed viral and bacterial proteins to cytotoxic T cells, and stabilization of MHC-I (Vitiello *et al.* 1990; Maffei *et al.* 1997) and helps to prevent the presentation of exogenous peptides (Rock *et al.* 1990, 1991). Thus it plays a critical role along with MHC-I in self/non-self recognition in vertebrates. The ß$_2$–m genes have been identified and characterized in several species of mammals, birds and fish. These genes are separate from the MHC gene locus (Klein 1986). ß$_2$–m is present in single copy in zebrafish (Ono *et al.* 1993), catfish (Criscitiello *et al.* 1998), tilapia (Dixon *et al.* 1993) and walleye (Christie *et al.* 2007), whereas in rainbow trout three ß$_2$–m genes have been reported expressing up to 12 allelic versions in a tetraploid manner (Magor *et al.* 2004). Haugland *et al.* (2005) reported the presence of two variants (type 1 and 2) of ß$_2$–m genes in Atlantic salmon. The expression of ß$_2$–m was detected in the head kidney, spleen, body kidney, liver and muscle tissue of the flounder (Choi *et al.* 2006). Higher expression was also reported in kidney, spleen, gills and intestine of walleye by Northern blot analysis than that in liver, heart, brain and muscle (Christie *et al.* 2007). *A. hydrophila* is one of the most devastating bacterial pathogen of fish causing massive loss to aquaculture. This paper reports for the first time the sequence diversity of ß$_2$–m in Indian carps and its tissue specific expression during *A. hydrophila* infection in mrigal (*Cirrhinus mrigala*).

Present address: [1,2]Senior Scientist, Fish Health Management Division.

MATERIALS AND METHODS

Fish: The commercially important carp species used in intensive polyculture systems viz., Indian major carps i.e. rohu (*Labeo rohita*), catla (*Catla catla*) and mrigal; medium carp (*Puntius sarana*) and minor carp (*Labeo bata*) were obtained from the farm of the Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, India, at their juvenile stages for the present study. The fish were acclimatized in the wet laboratory for a period of one week before collection of tissue. Fish were fed twice daily with

commercial pellets and water was exchanged daily to remove unused feed and fecal materials from the tanks. All the fish used in the study were apparently healthy with no external signs of bacterial, fungal or viral infections.

Complementary DNA (cDNA) synthesis: Total RNA was isolated from 100 mg liver tissue of the above 5 species using TRI reagent (Sigma) as per the procedure followed by Tripathy *et al.* (2006). The RNA samples were stored in DEPC-treated water at –20°C until used for RT-PCR. The concentration of the nucleic acid in the sample was quantified by measuring absorbance at 260 nm. The purity of the samples was also checked by measuring the ratio of OD_{260} nm and OD_{280} nm. The total RNA (5.0 µg) was used for first-strand cDNA synthesis using thermocycler (MJ Research) by incubating with 1 µl of random hexamer (100 µM) at 70°C for 5 min. The reaction was cooled at 25°C for 10 min to allow primers to anneal to the RNA after which the following components were added to the reaction in the order; 2µl of 10× buffer, 0.25 µl of RNase inhibitor (40U/µl), 2.0 µl of 100 mM dNTPs, and 1µl of MMLV-RT (200U/µl). The reagents were gently mixed and incubated for 1 h at 42 °C. The reaction was terminated by heating at 95 °C for 5 min and the cDNAs were stored at –20 °C prior to use in PCR reactions.

PCR reaction: The PCR reactions used to amplify ß$_2$–m gene and ß-actin (as house-keeping gene to check integrity of RNA) were performed using self-designed primer pairs ordered from Genie, India. The sequences of the primer pairs are shown in Table 1.

The ß-actin primer pairs were used to amplify 138 bp product whereas ß$_2$–m1 and ß$_2$–m2 were for 195–206 and 175 bp products, respectively. All amplification reactions consisted of an initial denaturation at 95°C for 3 minutes prior to 28 cycles of 95°C denaturation for 30 seconds, 55°C (59.7°C for ß$_2$–m2) annealing for 1 min, and 72°C extension for 1 min, followed by a final 72°C extension for 10 min using 1.5 units of Taq DNA polymerase. The generated PCR products (8 µl) were then analyzed by electrophoresis on a 1% agarose gel.

Cloning and sequencing of PCR products: The generated ß$_2$–m gene PCR products were purified by PCR product purification kit and subcloned into pGEMT vector as per manufacturer's instructions. Plasmid DNAs were prepared by the alkaline lysis method and cleaved with restriction enzymes to check for the positive clones (Sambrook *et al.* 1989). Three positive clones for each species PCR product carrying the desired size inserts were used for sequencing. The plasmid DNAs from the above were further purified by phenol-chloroform extraction method before sequencing.

Table 1. Sequences of the primers used in this study

Primer	Sequence (5'–3')
ß-actinF	GAC TTC GAG CAG GAG ATG G
ß-actinR	CAA GAA GGA TGG CTG GAA CA
ß$_2$–m1F	CTC CAG TCC CAA GAT TCA GGT GTA
ß$_2$–m1R	GAA GGA GAC GCT CTT GGT GAGG
ß$_2$–m2F	TCC AGT CCC AAG ATT CAG GTGT
ß$_2$–m2R	GG TGA GGT GAA ACT GCC AG

Sequencing was done with cycle sequencing kit using T7 universal primer in genetic analyser.

Nucleotide sequences of ß$_2$–m cDNAs were determined from three separate clones of each species. Mismatching bases, checked by sequencing the aberrant clone again, were found to be due to sequencing errors.

Sequence analysis and phylogeny: The nucleotide sequences of ß$_2$–microglobulin cDNA of carp species and deduced amino acid sequences were analysed using the BioEdit Sequence Alignment Editor (7.0.5.3) (Hall 1999). Multiple alignments of derived amino acid sequences were performed by the above programme using CLUSTAL W (Thompson *et al.* 1994). Phylogenetic trees were constructed by the bootstrap Neighbor-Joining method using ClustalX (1.83) (Thompson *et al.* 1997) in PHYLIP format (Felsenstein 1989) and visualized by TreeView (1.6.6) (Page 1996).

ß$_2$–m gene expression in healthy and Aeromonas hydrophila*-infected fish*: Thirty numbers of *C. mrigala* maintained in three tanks were utilized for this study. Fish from two tanks (20 numbers) were infected experimentally (i.p.) with 10^6 cfu of live *A. hydrophila* (a pathogenic isolate obtained from rohu, FHMD-AH/04) per gram body weight of fish and the fish in other tank served as control. The fish were observed for mortality for seven days. The mortality reached its plateau after 48 hours and then stopped completely. The tissues (liver, kidney, spleen, brain, muscle, testis, heart, intestine, gill and fin) from three control, three moribund and three survived fish were collected and subjected to RT-PCR following RNA extraction as mentioned earlier to observe the presence of ß$_2$–m transcripts.

RESULTS AND DISCUSSION

Carp ß$_2$–m cDNA cloning and sequencing: Two sets of primers were used to amplify the partial ß$_2$–microglobulin cDNAs of 5 different Indian carp species. The primer pair ß$_2$–m1 amplified four different carp species whereas primer pair ß$_2$–m2, which was prepared after sequencing and aligning these four carp sequences was used to amplify rohu cDNA. The primer ß$_2$–m1 produced amplicons of 195 to 206 bp whereas ß$_2$–m2 produced amplicon size of 175 bp. The partial nucleotide sequence of ß$_2$–microglobulin cDNA of mrigal is given in Fig. 1.

Table 2 shows nucleotide sequence identity matrix of five species studied here. A nucleotide sequence identity of 90.2 to 98.8% was found among the five carp species studied here considering 175 bp sequence of *L. rohita*.

Multiple alignment using CLUSTAL W of the deduced mrigal ß$_2$–m amino acid sequence with that of exotic carp species (common carp, grass carp and goldfish) along with

```
          10         20         30         40         50         60
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTCCAGTCCCAAGATTCAGGTGTACAGCCATTTTCCTGGAGAGTATGGAAAGTCGAACAC

          70         80         90         100        110        120
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCTCATCTGCCATGTGAGTGGCTTCCACCCTCCTGATATCACCATTGAACTGCTGAAGAA

          130        140        150        160        170        180
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGGCGAGATTCTCCCTGAGAGCAAGCAGACTGACCTGGCCTTCGAAAAGGGCTGGCAGTT

          190        200
....|....|....|....|....|.
TCACCTCACCAAGAGCGTCTCCTTCA
```

Fig. 1. Partial nucleotide sequence of *Cirrhinus mrigala* ß-2–microglobulin gene

```
                                10         20         30         40         50         60
                                ....|....|....|....|....|....|....|....|....|....|....|....|
Cirrhinus mrigala               SSPKIQVYSHFPGEYGKSNTLICHVSGFHPPDITIELLKNGEILPESKQTDLAFEKGWQF
Catla catla                     ..........Y..........T.....................................
Labeo rohita                    ..........Y.....................S.........................--
Puntius sarana                  ..................Q..................D...D.VV..DTQ............
Labeo bata                      ..................Q........D..............V...T...........H.
Carassius auratus               .........Y.......E.....................D..V.SNTQ............
Cyprinus carpio                 ..................E.....................D.....NTQ............
Ctenopharyngodon idella         ..........Y......E.....Y.........S........VIADAQ............
Clustal Consensus               *********::******.*** *:**.******:*:***:* ::.:::**********
                                ....|...
Cirrhinus mrigala               HLTKSVSF
Catla catla                     ....----
Labeo rohita                    --------
Puntius sarana                  ........
Labeo bata                      ........
Carassius auratus               ........
Cyprinus carpio                 ........
Ctenopharyngodon idella         ........
Clustal Consensus
```

Fig. 2. Multiple alignment of *Cirrhinus mrigala* ß-2–microglobulin translation with that of other carps. Identical (*) and similar residues (: or.) identified using CLUSTAL W are indicated. (-) denotes blank/gap. Accession numbers are: *Cirrhinus mrigala*, AM690443; *Catla catla*, AM690446; *Labeo rohita*, AM774150; *Puntius sarana*, AM690445; *Labeo bata*, AM690440; *Carassius auratus*, AM690442; *Cyprinus carpio*, AM690441 and *Ctenopharyngodon idella*, BAD86588.

the other four carp species studied here is shown in Fig. 2. Highly conserved blocks of amino acids were found in all species examined. The deduced amino acid sequences of the five carp $ß_2$–m cDNAs in the present study had 82.7 to 96.5% identity with each other. The highest identity was between the three Indian major carps viz. *Cirrhinus mrigala*, *L. rohita* and *C. catla* (96.5%) (Table 2).

Phylogenetic analysis: The phylogenetic tree based on $ß_2$–m coding sequence showed a close relationship between rohu, catla and mrigal. The Indian major, medium and minor carps could be divided into three major clusters based on the bootstrap values. The $ß_2$–m genes from *Puntius sarana* formed a major cluster with goldfish and common carp, and the minor carp *Labeo bata* clustered with Indian major carps. The grass carp was placed within a separate cluster containing *Danio rerio*. Salmonids formed a distinct cluster nearer to catfish away from the cluster of carps. These results suggest that the divergence between species might be well correlated with their evolution pattern. The results also indicated that fish $ß_2$–m proteins are more closely related to those of birds than that of mammals as reported by Choi *et al.* (2006).

Molecular mechanism of $ß_2$–m variation has been studied

Table 2. Nucleotide sequence homology (below diagonal) and amino acid sequence homology (above diagonal) of partial ß$_2$–m genes and proteins respectively of different carps (considering the length of *L. rohita* sequence)

	Cirrhinus mrigala	*Labeo rohita rohita*	*Catla catla*	*Puntius sarana*	*Labeo bata*	*Cyprinus carpio*	*Carassiu auratus*
Cirrhinus mrigala	*	0.965	0.965	0.862	0.931	0.913	0.862
Labeo rohita	0.977	*	0.965	0.827	0.896	0.879	0.827
Catla catla	0.977	0.988	*	0.827	0.896	0.879	0.827
Puntius sarana	0.937	0.920	0.920	*	0.896	0.913	0.896
Labeo bata	0.942	0.937	0.937	0.931	*	0.896	0.879
Cyprinus carpio	0.920	0.908	0.908	0.925	0.908	*	0.948
Carassius auratus	0.902	0.902	0.902	0.931	0.902	0.931	*

in many species from fishes to mammals (Shum *et al.* 1996, Magor *et al.* 2004). The present study represents the use of ß$_2$–m cDNA for the first time for the analysis of phylogenetic relationships in Indian carp species. The phylogenetic relationships among the five Indian carp species determined from ß$_2$–m cDNA sequences would reveal the genetic diversity among the species. The ß$_2$–m gene has been used previously to draw phylogenetic relationships between species (Dixon *et al.* 1993; Ono *et al.* 1993; Shum *et al.* 1996; Choi *et al.* 2006; Christie *et al.* 2007). Despite the fact that partial ß$_2$–m cDNA sequence information has been used here for constructing phylogenetic tree, this analysis can be used effectively for initial assessment of genetic variations among three different carp species for which information is scanty.

Tissue-specific expression of ß$_2$–m mRNA in healthy and Aeromonas hydrophila*-infected mrigal:* ß$_2$–microglobulin transcripts from non-induced control mrigal were detected in total RNA extracted from all the tissues studied (liver, kidney, spleen, brain, muscle, testis, heart, intestine, gill and fin) as shown in Fig. 4. The tissue-specific expression of ß$_2$–m has been studied in many fish species. For example, ß$_2$–m

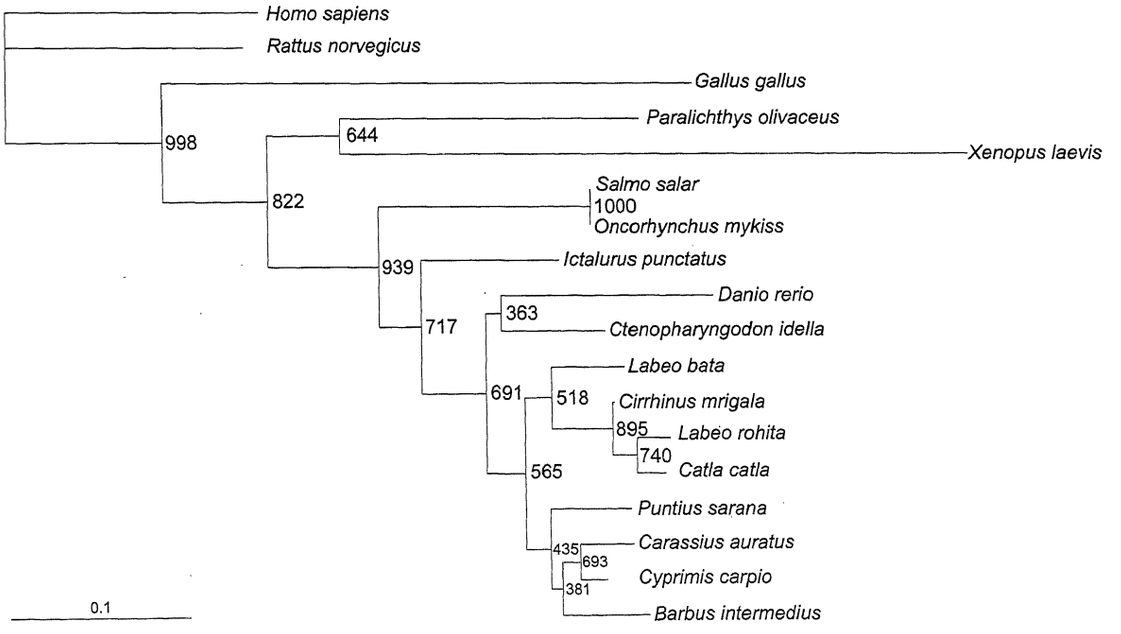

Fig. 3. A phylogenetic tree of *Cirrhinus mrigala* ß–2–microglobulin partial amino acid sequence and that of other species, with *Homo sapiens* assumed as an out-group. The tree was constructed using the neighbor-joining method. Numbers indicate bootstrap values from 1000 replicates. Accession numbers are: *Cirrhinus mrigala*, AM690443; *Catla catla*, AM690446; *Labeo rohita*, AM774150; *Puntius sarana*, AM690445; *Labeo bata*, AM690440; *Cyprinus carpio*, AM690441; *Carassius auratus*, AM690442; *Ctenopharyngodon idella*, BAD86588; *Oncorhynchus mykiss*, AAP51054; *Salmo salar*, AAG17537; *Gallus gallus*, NP 001001750; *Rattus norvegicus*, NP 036644; *Homo sapiens*, AAA51811; *Danio rerio*, NP 571238; *Barbus intermedius*, CAD44966; *Paralichthys olivaceus*, AAN40738; *Xenopus laevis*, AAF37230; and *Ictalurus punctatus*, AAC67230.

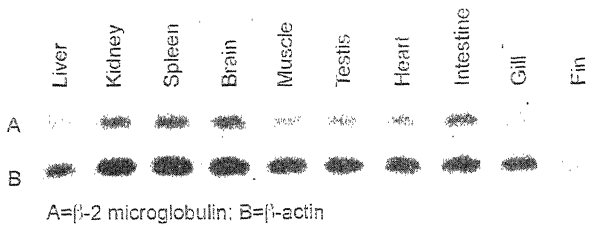

Fig. 4. Expression of ß–2–microglobulin mRNA in control mrigal (*C. mrigala*) tissues detected by RT-PCR. Corresponding ß-actin amplification for respective tissues in the same PCR reaction.

mRNA was detected in head kidney, spleen, liver, body kidney, and muscle in flounder (Choi *et al.* 2006); in head kidney, posterior kidney, spleen, gills and intestine in walleye (*Sander vitreum*) (Christie *et al.* 2007). It has been also reported in peripheral blood leucocytes in rainbow trout (Kales *et al.* 2006). The intensity of band did not increase for any tissue of mrigal in either the moribund or survived fish after *A. hydrophila* exposure (data not shown). However, further analysis of the expression profile of the $ß_2$-m gene in the mrigal genome at different time periods of bacterial infection is necessary to draw any definite conclusion.

ACKNOWLEDGEMENTS

We thank Dr N. Sarangi, Director, CIFA, Bhubaneswar for providing necessary facilities during this study and Dr P. Das, CIFA, Bhubaneswar for providing sequencing facility.

REFERENCES

Calabi F and Milstein C. 1986. A novel family of human major histocompatibility complex-related genes not mapping to chromosome 6. *Nature* **323**: 540–3.

Choi W, Lee E-Y and Choi T-J. 2006. Cloning and sequence analysis of the β_2–microglobulin transcript from flounder, *Paralichthys olivaceous*. *Molecular Immunology* **43**: 1565–72.

Christie D, Wei G, Fujiki K and Dixon B. 2007. Cloning and characterization of a cDNA encoding walleye (*Sander vitreum*) beta–2 microglobulin. *Fish and Shellfish Immunology* **22**: 727–33.

Criscitiello M F, Benedetto R, Antao A, Wilson M R, Chinchar V G, Miller N W, Clem L W and McConnell T J. 1998. β_2 Microglobulin of Ictalurid catfishes. *Immunogenetics* **48**: 339–43.

Dixon B, Stet R J M, van Erp S H M and Pohajdak B. 1993. Characterization of β_2–microglobulin transcripts from two teleost species. *Immunogenetics* **38**: 27–34.

Felsenstein J. 1989. PHYLIP-phylogeny inference package (version 3.2). *Cladistics* **5**: 164–6.

Hall T A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucliec Acids Symposium Series* **41**: 95–8.

Haugland O, Torgersen J, Syed M and Evensen O. 2005. Expression profiles of inflammatory and immune-related genes in Atlantic salmon (*Salmo salar* L.) at early time post vaccination. *Vaccine* **23**: 5488–99.

Kales S, Parks-Dely J, Schulte P and Dixon B. 2006. Beta–2–microglobulin gene expression is maintained in rainbow trout and Atlantic salmon kept at low temperatures. *Fish Shellfish Immunology* **21**: 176–86.

Klein F J. 1986. *Natural History of the Major Histocompatibility Complex*. John Wiley & Sons, New York.

Maffei A, Papadopoulos K and Harris P E. 1997. MHC class I antigen processing pathways. *Human Immunology* **54**: 91–103.

Magor K E, Shum B P and Parham P. 2004. The β_2–microglobulin locus of rainbow trout (*Oncorhynchus mykiss*) contains three polymorphic genes. *Journal of Immunology* **172**: 3635–43.

Ono H, Figueroa F, O'Huigin C and Klein J. 1993. Cloning of the β2–microglobulin gene in the zebrafish. *Immunogenetics* **38**: 1–10.

Page R D M. 1996. TreeView: an application to display phylogenetic trees on personal computers. *Computer Application in Biosciences* **12**: 357–8.

Rock K L, Gamble S R, Rothstein L E, Gramm C and Benacerraf B. 1991. Dissociation of β_2–microglobulin leads to a substantial pool of inactive class I MHC heavy chains on the cell surface. *Cell* **65**: 611–620.

Rock K L, Rothstein L E, Gamble S R and Benacerraf B. 1990. Reassociation with β_2–microglobulin is necessary for K^b class I major histocompatibility complex binding of exogenous peptides. *Proceedings of the National Academy of Sciences U.S.A.* **87**: 7517–21.

Sambrook J, Fritsch E F and Maniatis T. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor Press, New York.

Shum B P, Azumi K, Zhang S, Kehrer S R, Raison R L, Detrich H W and Parham P. 1996. Unexpected β_2–microglobulin sequence diverisity in individual rainbow trout. *Proceedings of the National Academy of Sciences U.S.A.* **93**: 2779–84.

Simister N E and Mostov K E. 1989. An Fc receptor structurally related to MHC class I antigens. *Nature* **337**: 184–7.

Thompson J D, Higgins D G and Gibson T J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673–80.

Thompson J D, Gibson T J, Plewniak F, Jeanmougin F and Higgins D G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876–82.

Tripathy S, Sahoo P K, Kumari J, Mishra B K, Sarangi N and Ayyappan S. 2006. Multiplex RT-PCR detection and sequence comparision of viruses *Mr*NV and XSV associated with white tail disease in *Macrobrachium rosenbergii*. *Aquaculture* **258**: 134–9.

Vitiello A, Potter T A and Sherman L A. 1990. The role of β_2–microglobulin in peptide binding by class I molecules. *Science* **250**: 1423–6.