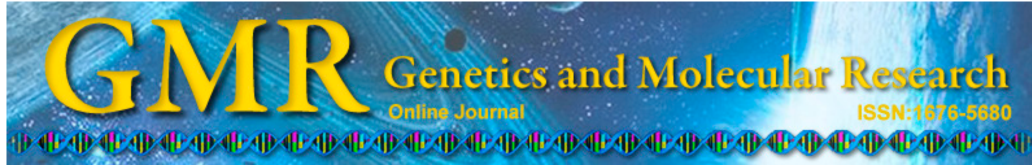

Genomic characterization and sequence diversity of the β_2-microglobulin gene in the miiuy croaker, *Miichthys miiuy*

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ABSTRACT. β_2-Microglobulin (β_2m) is related to major histocompatibility complex class I alpha chains, and forms cell-surface glycoproteins that mediate a variety of functions in immune defense. In general, β_2m has no isoforms and is not polymorphic in higher vertebrates, but polymorphisms between different alleles have been found in some fish species. In this study, full-length β_2m cDNA and genomic sequences were cloned from the miiuy croaker (*Miichthys miiuy*). The miiuy croaker β_2m gene shares many of the same characteristics as other fish species. Three exons and two introns were identified in the miiuy croaker β_2m gene; these genomic structural features are similar to those present in other fish. The deduced β_2m amino acid sequence exhibited 34.7-90.1% identity with mammal and teleost β_2m amino acid sequences. Sequence polymorphism analysis in six individuals identified three alleles that encoded two proteins, confirming that β_2m polymorphisms exist in this species. Phylogenetic

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analysis elucidated the evolutionary history of the β_2m protein among warm-blooded vertebrates and bony fish.

Key words: *Miichthys miiuy*; β_2-microglobulin; Genomic; cDNA; Sequence diversity

INTRODUCTION

Major histocompatibility complex (MHC) class I molecules are located on the surface of vertebrate nucleated cells, and are involved in presenting foreign peptide products to cytotoxic CD8$^+$ T cells by the degradation of intracellular pathogens (Srisapoome et al., 2004). They consist of one membrane-spanning α chain (heavy chain) produced by MHC genes, and one β chain (light chain) produced by the β_2-microglobuin (β_2m) gene. β_2m was first identified in human urine, and was found to be ubiquitously distributed on all nucleated cells (Berggard and Bearn, 1968; Gussow et al., 1987; Maffei et al., 1997). In mammals, β_2m is not linked to MHC loci and is not polymorphic, unlike class I heavy-chain genes (Criscitiello et al., 1998). β_2m forms a central part of the structure, and is necessary for the proper folding and cell surface display of the MHC class I molecule (Rosa et al., 1983; Vitiello et al., 1990). β_2m is an immunoglobulin superfamily protein, and its similarity to the MHC class I α3 domain is thought to have arisen by the duplication of a common ancestral gene (Burnet, 1970; Gally and Edelman, 1972). Therefore, understanding the evolution of β_2m in lower vertebrates may provide insights into the origin of the MHC (Criscitiello et al., 1998).

Dixon et al. (1993) and Ono et al. (1993) first investigated the teleost β_2m gene. To date, β_2m genes have been isolated from dozens of species of bony fish. However, most studies have focused on molecular cloning and phylogeny, and only a few have investigated its genomic structure and sequence diversity. In general, the β_2m gene contains four exons and three introns in higher vertebrates, and consists of three exons and two introns in teleosts (Parnes and Seidman, 1982; Gussow et al., 1987; Lundqvist et al., 1999; Xu et al., 2010a); however, two distinct types of β_2m have been identified in the red sea bream (*Pagrus major*) and the yellowtail (*Seriola quinqueradiata*) using expressed sequence tags (ESTs) (Kondo et al., 2010). β_2m has no isoforms and is not polymorphic in higher vertebrates, but polymorphisms between different alleles have been found in some fish species (Shum et al., 1996; Magor et al., 2004; Xu et al., 2010b). The miiuy croaker (*Miichthys miiuy*) is an important economic marine fish in China, and the MHC class Iα and class II genes in this species has already been described (Xu et al., 2010c, 2011). In order to understand the role of miiuy croaker β_2m (Mimi-β_2m) and elucidate sequence features between fish species, in this study we isolated and characterized a β_2m transcript from the miiuy croaker. We investigated the genomic representation of the gene, and studied its sequence diversity in different individuals.

MATERIAL AND METHODS

Samples, DNA and RNA isolation, and cDNA synthesis

Six miiuy croakers were obtained from the Zhoushan Fisheries Research Institute, Zhejiang Province, China. Genomic DNA was extracted from fin samples using the phenol-chloroform method. Total RNA was extracted from the spleen of adult individuals using

TRIzol reagent (Qiagen) according to the manufacturer protocol, and cDNA was synthesized using a QuantScript RT kit (Tiangen), according to the manufacturer protocol.

Primer design, amplification, and cloning

One EST sequence, similar to β_2m in *Larimichthys crocea* and other fish species, was obtained from the spleen cDNA library of the miiuy croaker by EST analysis in our laboratory (Che et al., 2014; Xu et al., 2010a). To obtain the complete cDNA sequence of the β_2m gene homolog, this EST clone was separately sequenced from both forward and reverse directions with the vector primers M13F and M13R; the sequencing was repeated three times. Full-length clone cDNA was obtained by overlapping the forward and reverse strand sequences.

To elucidate the complete Mimi-β_2m genomic organization, two primer pairs were designed to amplify introns of the Mimi-β_2m gene. Exon-intron junctions were deduced according to the known β_2m genes of other teleosts. Intron 1 was amplified with the primer pair β_2m-intron 1-F (5'-ACGAGCCGCACGCTTCTT-3') and β_2m-intron1-R (5'-TCTGATTGGCATTAGGGA -3'); intron 2 was amplified with the primer pair β_2m-intron2-F (5'-CAAACAGGACTGGCAC TT-3') and β_2m-intron2-R (5'-TAGCCGAGGACAGATGAG-3'). To investigate β_2m gene polymorphisms, the primer pair β_2m-F (5'-GAGCCGCACGCTTCTTT-3') and β_2m-R (5'-CTGCTGTAGCCGAGGAC-3') was used to amplify the complete open reading frame sequence from the cDNA template of six individuals. A polymerase chain reaction (PCR) was conducted in a final volume of 50 μL in the following manner: pre-denaturalization at 94°C for 4 min, 30 cycles of denaturation at 94°C for 40 s, annealing temperature for 40 s, increasing the extension at 72°C for 2 min to reduce artifact formation, and a final extension at 72°C for 10 min.

The PCR products were resolved by electrophoresis on 1.5% agarose gels, and the fragments of interest were excised and purified using a Gel Extraction Kit (Takara). The purified fragments were ligated into pMD-19T vectors (Takara) and cloned to TOP10 cells, according to a standard protocol. Positive clones were screened by PCR using M13+/- primers. At least three clones were sequenced per fragment using an ABI 3730xl automated sequencer (Applied Biosystems) with the M13 primer.

Sequence alignment and data analysis

Alignments of known nucleotide sequences and putative amino acid sequences of the miiuy croaker and other vertebrates were performed using the MEGA 4 software (Tamura et al., 2007). A phylogenetic tree was constructed using the amino acid sequences by the neighbor-joining method (Saitou and Nei, 1987).

RESULTS AND DISCUSSION

Structure and genomic sequence of Mimi-β2m

The full-length cDNA of β_2m, which was designated Mimi-β_2m-01, was 899-bp long (GenBank accession No. HQ695734), and included a 63-bp 5'-terminal untranslated region (UTR), a 351-bp encoding region, and a 485-bp 3'-terminal UTR with two canonical polyadenylation signals (AATAAA) and a 16-bp poly (A) tail; two putative polyadenylation signal sequences have also been found in flounder (Choi et al., 2006). The ATTTA motif, which may

be involved in rapid message degradation (Shaw and Kamen, 1986; Choi et al., 2006), was located at two positions in the Mimi-β_2m 3'-terminal UTR (Figure 1). A BLAST search revealed that the sequence obtained was similar to other teleost β_2m genes. The 351-nt encoding region coded a polypeptide with 116 amino acid (aa) residues, with a leader peptide of 19 aa at the N-terminal region. A cleavage site between the signal peptide and mature protein was predicted using signal peptide analysis by the neural network method (Nielson et al., 1997). Characteristic domains present in other species could be found in the Mimi-β_2m sequence, including a typical immunoglobulin (Ig) and MHC protein signature (YSCKVTH) at residues 79-85, in which a protein kinase C phosphorylation site was present. Two cysteine residues that formed an intra-domain disulfide bridge were highly conserved in the corresponding position in all species within the 25 and 81 sites, indicating that they encode a protein that is a member of the Ig superfamily. There was one *N*-linked glycosylation site located at residues 50-52.

Amplification of the genomic DNA that contained the intron and exon structure, resulting in a 2082-bp sequence, is show in Figure 1A. The β_2m genomic DNA consists of three exons and two introns, and has been designated Mimi-β_2m-DNA. The genomic organization of the miiuy croaker β_2m gene is very similar to that described in other fish species, but different from the β_2m genomic structure in the tongue sole (Figure 1B). In the miiuy croaker, exon 1 is 64-bp long and encodes the leader peptide, exon 2 is 273-bp long and encodes the bulk of the mature protein, and exon 3 is 14 bp long and encodes the remaining four residues of the mature protein.

Comparison and phylogenetic analysis

Alignments of the deduced amino acid sequence of the miiuy croaker β_2m gene with those of other vertebrates demonstrated that it shared many protein features. The miiuy croaker β_2m gene shares many of the same characteristics as other fish species, such as the YxCxVxH Ig motif, which is highly conserved in most vertebrates, except for the flounder (Paol), channel catfish (*Ictalurus punctatus*) (Icpu), and rainbow smelt (*Osmerus mordax*) (Osmo) (Figure 2); sequences of amino acid residues that bind to α1, α2, and α3 domains of the MHC class I heavy chain are also highly conserved. Mature β_2m starts with lysine (K) in the miiuy croaker, and the residue is conserved in other bony fish, except in *Anoplopoma fimbria* (M), *Esox lucius* (R), and *Cynoglossus semilaevis* (V). Fourteen residues of mature β_2m protein are conserved in the following aligned sequences: P_5, Y_{10}, N_{21}, C_{25}, F_{30}, P_{32}, I_{35}, F_{56}, W_{61}, F_{71}, P_{73}, C_{81}, V_{83}, and H_{85}. Mature miiuy croaker β_2m protein preserves cysteines, forming a disulfide bridge at positions 25 and 81, as in mouse and human β_2m proteins (Bjorkman et al., 1987). The miiuy croaker β_2m and other teleost β_2m are two amino acids shorter than in mammals and birds in the mature protein region. Deletions are located in the loop between the anti-parallel beta-strand (S) 6 and S7. The S1-S7 motifs are shown in Figure 2. Based on the human structure, these strands form the upper pleated sheet of the β_2m molecule. MHC class Iα chain contact residues are also located in the strands.

A phylogenetic tree was constructed using the neighbor-joining method to further analyze the evolutionary relationships of β_2m proteins in different species (Figure 3). The tree clearly showed evolutionary divergence of the β_2m gene between warm-blooded vertebrates and bony fish. Phylogenetic analysis demonstrated that the deduced amino acid sequence of the miiuy croaker β_2m gene had 90.1, 74.4, 67.8, 51.2, 49.6, 56.2, 56.2, 57.9, 56.2, 59.5, 62.0, 59.5, 60.3, 53.7, 52.9, 38.8, 49.6, 39.7, 42.1, 38.0, 34.7, and 37.2% identity with that of *Pseudosciaena crocea* (Pscr, DQ234793), *Sander vitreus* (Savi, AY734540), *Anoplopoma fimbria*

A

```
                                AGTTCATTTTCGCTCTCTACGGGACGAGCCGCACGCTTCTTTTTTTCCAAACTACCAAAGAAT   63
ATGAAGTTTGTTCTGTGCATTGCAGCTCTGGTAGCTGTCTGCTACTCAGACGACTCCAAGCACAgtaagtattattatttttatctaaag  153
 M  K  F  V  L  C  I  A  A  L  V  A  V  C  Y  S  D  D  S  K  H                                   21
atatatatacctctgtctgtgtgtgtgtgtgtgtgtgtgtgcgcgcgcgcgcgtgtatgcttaaatgatgacattttaatggagaatc  243
actagaaactgtatttgaagtaaaaatctgtttttatcctctatggctgtctgtgaactacaaactacgcacgacaggacacattatgt  333
taaataggctgtatgatttcggcagccggcaatacaaaacaaacaataaggtgtaggccgtctgtaataagttgtttgttagaaagccta  423
agagtcaggtgttaaaaagaagatttatacaacgtgttttcaattcggcggaattcccctcaacaatctggatttactttcgattttctg  513
taattccaaactgtgctgtggcagcgccctccattggcttcagctcggtacatacacatactgatgagtgttgatgaattcctacattgt  603
cagtaagaaatactgctaaaaacaaaacatgttttatgaaagagcttaagactatggcgcctttctaccattcttccattgttattatag  693
ttaatgttaaaagaagtttcatttgtattgtatcaatatatattttatagaagggaagcaattaagaacaagcaacggaaatttcaagtc 1083
aatttcttctcagcttacttaatttaaactaaccagtctaaagtcgaagcagagtccttttagtttcgatgaggtggaaaaaaacaagtg 1173
ttgctgtgacaaagtccttcaaaaatactgaagagtgtgttcttatttccctcggtcatctgcatgtaatttggatcactgtacacttta 1263
tggtagtttgacatcatttccttctctatgttgtccctagCTCCACCCAAGGTTCAGGTGTACAGCCGTGACCCTGGAAGGTTTGGCAGT 1353
                                        T  P  P  K  V  Q  V  Y  S  R  D  P  G  R  F  G  S         38
AAGAACGTCCTGATTTGTCATGTAAGTGGCTTCCACCCACCTGACATCACCATCCAGCTCATGAAGGATGAAGAGGAACTCCCTAATGCC 1243
 K  N  V  L  I  C  H  V  S  G  F  H  P  P  D  I  T  I  Q  L  M  K  D  E  E  E  L  P  N  A      68
AATCAGACTGACCTGGCCTTCAAACAGGACTGGCACTTCCATCTGACCAAGAATGTGCCCTTCACACCCCTGGATGGACAGAAGTACAGC 1333
 N  Q  T  D  L  A  F  K  Q  D  W  H  F  H  L  T  K  N  V  P  F  T  P  L  D  G  Q  K  Y  S      98
TGCAAGGTCACTCATGGGCAGAAAGTTAAAGACTATGCCTGGGgtgagttaatttgtatcagctaatatatgtgtcacattttcataacc 1423
 C  K  V  T  H  G  Q  K  V  K  D  Y  A  W                                                      112
aaaatgcatgtgatacagccaggagagaatgcattaaaaagatataataggaagaaagaaattataatattattaataatttatacgtaa 1413
aaacataatatgcttcttgctgcaggtgtgcagtataatagatagatatctaaagagacaaaaatgcaatcaaatgtaaccacatcaagg 1503
aaaaaatattcagtggtaaatgcaaatatttcttcacaatcctgacaaaatgtctatatgctttttcttctttttttacagAGCCAAACAT 1593
                                                                                E  P  N  M      116
GTAACTCATCTGTCCTCGGCTACAGCAGCGTCTATCATTCACATCTCCAATCAACAGCATCAACAGAAGGTTGGAGTTATTCCAACCTTC 1683
 *
TCACCTTTCTTTTCACGATAATAAACATCAGTACCATATGAAGAGGACTTACAATCACCATTATATTCTGAGATAATTCATGGGCTCCAG 1773
GTTATTTAGTTATCTATCTGTTTAGATTGCTAAAATGTTTTAGATTACGACTTATTTTCCACTCTTTGATACTGATAAGTGTGTTTGTAT 1863
TAAACTAATGTGGTGAGAAATGCCTTTGTGTTTTCACCTTTCTTTTTTTTAAGGCTTGAACTGTTTCTTCTACCTAAATGAGGCAATCAA 1953
ATGATAATAAAACATGAATGGGGGTTTCTTTCTTTCTTTTTGTTGTATAATTTGGATTTGCTATTTAAAGAATTTGTTCATGCCCTCTAT 2043
GAAATAAATGCCTCTTGAAAATCAAAAAAAAAAAAAAA                                                        2082
```

B

Figure 1. Genomic sequence (**A**) and schematic illustration (**B**) of the miiuy croaker β_2m gene. Exons are in uppercase and introns and untranslated regions are in lowercase. The asterisk indicates the stop codon.

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(Anfi, BT083185), *Ctenopharyngodon idella* (Ctid, AB198014), *Danio rerio* (Dare, L05383), *Barbus intermedius* (Bain, AJ507009), *Cyprinus carpio* (Cyca, L05536), *Ictalurus punctatus* (Icpu, AF016042), *O. mordax* (Osmo, BT074868), *Esox lucius* (Eslu, BT080116), *Salmo salar* (Sasa, AF180479), *Salvelinus alpinus* (Saal, EU733523), *Oncorhynchus mykiss* (Onmy, AY217450), *S. quinqueradiata* (Sequ, AB469146), *P. major* (Pama, AB469144), *Cynoglossus semilaevis* (Cyse, FJ965562), *Paralichthys olivaceus* (Paol, AF433657), *Gallus gallus* (Chicken, M84767), *Sus scrofa* (Pig, L13854), *Bos taurus* (cattle, X69084), *Mus musculus* (mouse, X01838), and *Homo sapiens* (human, AB021288), respectively. The phylogenetic tree revealed that the miiuy croaker is clustered with the large yellow croaker (*Larimichthys crocea*), which is included in the same genus. It also showed that it is easy to separate the miiuy croaker from other species.

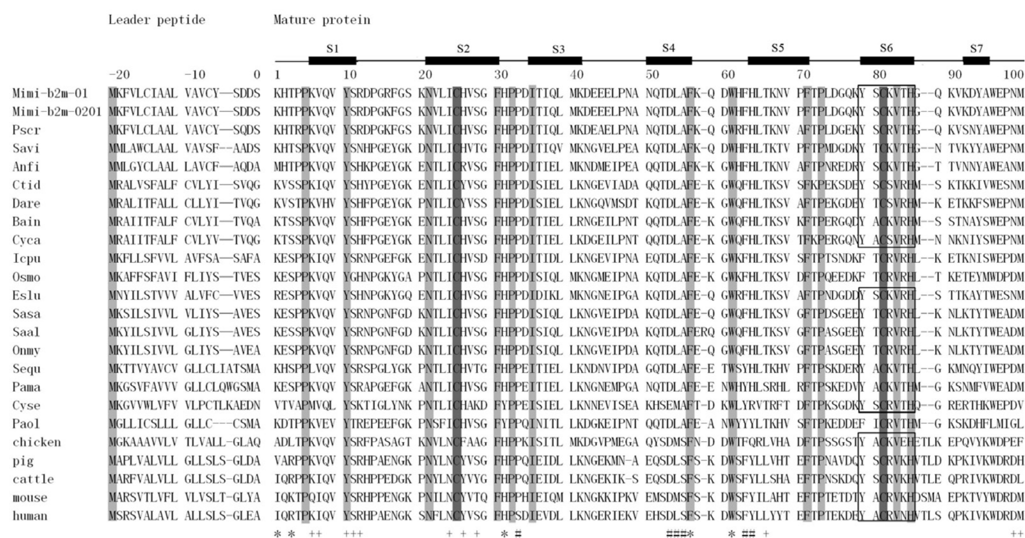

Figure 2. Alignment of the deduced amino acid sequences of the miiuy croaker β_2m gene with those of other species. Gaps used to maximize the alignment are indicated by dashes. The bold line S1 to S7 indicates the anti-parallel beta-strand, and the thin line represents the loops connecting the beta-strands. Identical amino acids and conserved cysteine sites among species are shown with grey and black backgrounds, respectively. The contact residues with alpha-1 (#), alpha-2 (*), and alpha-3(+) of class I molecules are based on the human HLA-A2 structure.

Sequence diversity analysis

Six individuals (fish A, B, C, D, E, and F) were used to analyze β_2m gene polymorphisms in the miiuy croaker. An average of 10 positive clones per individual was sequenced and 55 sequences were obtained, and irreproducible single nucleotide polymorphisms (that are a cause of polymerase errors) were excluded. A total of three different allele sequences were identified: Mimi-β_2m-01 (as described above and in Figure 4), Mimi-β_2m-0201, and Mimi-β2m-0202 (HQ695735 and HQ695736, respectively). However, alleles Mimi-β_2m-0201 and Mimi-β_2m-0202 encoded the same protein (Figure 4). Fish A only possessed allele Mimi-β_2m-0201; fish B and C had alleles Mimi-β_2m-0201 and Mimi-β_2m-0202; fish D, E, and F had

alleles Mimi-β_2m-01 and Mimi-β_2m-0202, with two β_2m mature proteins. The frequencies of Mimi-β_2m-01, Mimi-β_2m-0201, and Mimi-β2m-0202 were 21.8 (12/55), 23.6 (13/55), and 54.5% (30/55), respectively, in the 55 sequences. Alignments revealed a high degree of sequence similarity between the three sequences obtained (99.6%). Higher-vertebrate β_2m has no isoforms and is not polymorphic, but polymorphisms between different alleles have been found in some fish species (Shum et al., 1996; Magor et al., 2004; Xu et al., 2010b). In this study, three alleles were found in the miiuy croaker, which provides further evidence that confirms the above phenomenon.

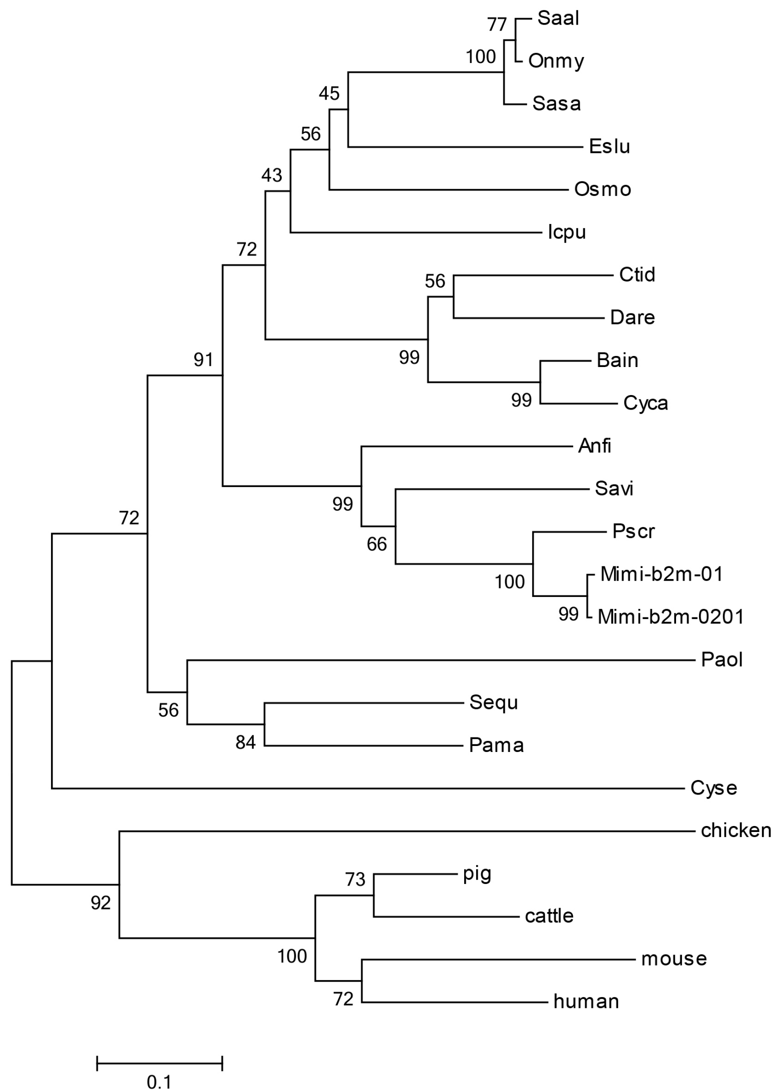

Figure 3. Phylogenetic tree of the β_2m gene from the miiuy croaker and other vertebrates, constructed using the neighbor-joining method. Numbers at each node indicate the percentage of bootstrapping (1000 replications).

```
Mimi-b2m-01    ATGAAGTTTG TTCTGTGCAT TGCAGCTCTG GTAGCTGTCT GCTACTCAGA CGACTCCAAG CACACTCCAC CCAAGGTTCA GGTGTACAGC CGTGACCCTG GAAGGTTTGG CAGTAAGAAC
Mimi-b2m-0201  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ...A...... ..........
Mimi-b2m-0202  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ...A...... ..........

Mimi-b2m-01    GTCCTGATTT GTCATGTAAG TGGCTTCCAC CCACCTGACA TCACCATCCA GCTCATGAAG GATGAAGAGG AACTCCCTAA TGCCAATCAG ACTGACCTGG CCTTCAAACA GGACTGGCAC
Mimi-b2m-0201  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Mimi-b2m-0202  .......... .......G.. .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

Mimi-b2m-01    TTCCATCTGA CCAAGAATGT GCCCTTCACA CCCCTGGATG GACAGAAGTA CAGCTGCAAG GTCACTCATG GGCAGAAAGT TAAAGACTAT GCCTGGGAGC CAAACATGTA A
Mimi-b2m-0201  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .
Mimi-b2m-0202  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .


Mimi-b2m-01         MKFVLCIAAL VAVCYSDDSK HTPPKVQVYS RDPGRFGSKN VLICHVSGFH PPDITIQLMK DEEELPNANQ TDLAFKQDWH FHLTKNVPFT PLDGQKYSCK VTHGQKVKDY AWEPNM
Mimi-b2m-0201/0202  .......... .......... .......... ....K..... .......... .......... .......... .......... .......... .......... .......... ......
```

Figure 4. Nucleotide and amino acid sequences for Mimi-β_2m alleles. Dots indicate identity with the top sequences.

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