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cDNA-derived amino acid sequences of myoglobins from nine species of whales and dolphins

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Abstract

We determined the myoglobin (Mb) cDNA sequences of nine cetaceans, of which six are the first reports of Mb sequences: sei whale (*Balaenoptera borealis*), Bryde's whale (*Balaenoptera edeni*), pygmy sperm whale (*Kogia breviceps*), Stejneger's beaked whale (*Mesoplodon stejnegeri*), Longman's beaked whale (*Indopacetus pacificus*), and melon-headed whale (*Peponocephala electra*), and three confirm the previously determined chemical amino acid sequences: sperm whale (*Physeter macrocephalus*), common minke whale (*Balaenoptera acutorostrata*) and pantropical spotted dolphin (*Stenella attenuata*). We found two types of Mb in the skeletal muscle of pantropical spotted dolphin: Mb I with the same amino acid sequence as that deposited in the protein database, and Mb II, which differs at two amino acid residues compared with Mb I. Using an alignment of the amino acid or cDNA sequences of cetacean Mb, we constructed a phylogenetic tree by the NJ method. Clustering of cetacean Mb amino acid and cDNA sequences essentially follows the classical taxonomy of cetaceans, suggesting that Mb sequence data is valid for classification of cetaceans at least to the family level.

Keywords: Myoglobin; Heterogeneity; cDNA sequence; Amino acid sequence; Sperm whale; Dolphin; Cetaceans; Phylogeny; Taxonomy

1. Introduction

Myoglobin (Mb) is a hemoprotein that contributes to intracellular oxygen storage and facilitates transcellular diffusion of oxygen (Wittenberg, 1970). Mb is typically found in mammalian skeletal or cardiac muscle tissues, where continuous oxygen flow is required for high activity of aerobic metabolism, producing the characteristic dark red color. In addition, Mb is also present in some species of invertebrates (Suzuki and Imai, 1998; Vinogradov et al., 1993).

In cetaceans, the significant contribution of Mb to oxygen storage and utilization capacities in skeletal muscle makes Mb concentration an apparent limiting factor to diving limits. Cetacean Mb concentrations range from 2 to 7 g/(100 g wet muscle) (Noren and Williams, 2000; Dolar et al., 1999), varying significantly among muscle types in adult dolphins and increasing with size and age to become 3–4 times greater in adults than in calves (Dolar et al., 1999).

Mb is typically composed of 153 amino acid residues with only three amino acid residues, CD1-Phe, E7-His and F8-His in heme cavity, that are highly conserved (Suzuki and Imai, 1998). The heme-neighboring CD1-Phe and iron-binding F8-His are strictly conserved in all globins, whereas the E7-His is replaced by Gln in a few globins of vertebrates and by Val, Leu, Tyr or Gln in a considerable number of globins of invertebrate (Suzuki and Imai, 1998). Residue E7-His is associated with ligand-binding properties and in an oxygenated form, it is capable of forming a hydrogen bond to bound dioxygen, stabilizing it. Although amino acid sequence homology among distantly

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Table 1
List of cetacean myoglobins for which amino acid sequences have been determined

Species	Common name	Accession number (reference)
Order Cetacea (whales and dolphins)		
Family **Balaenopteridae**		
Balaenoptera borealis	Sei whale	(Present study)
Balaenoptera acutorostrata	Common minke whale	P02179 (Lehman et al., 1977; present study)
Balaenoptera edeni	Bryde's whale	(Present study)
Balaenoptera physalus	Fin whale	P02180 (DiMarchi et al., 1978)
Megaptera novaeangliae	Humpback whale	P02178 (Lehman et al., 1978)
Family **Eschrichtidae**		
Eschrichtius robustus	Gray whale	P02177 (Bogardt et al., 1976)
Family **Physeteridae**		
Physeter macrocephalus	Sperm whale	P02185 (Edmundson, 1968; present study)
Family **Kogiidae**		
Kogia breviceps	Pygmy sperm whale	(Present study)
Kogia simus	Dwarf sperm whale	P02184 (Dwulet et al., 1977)
Family **Ziphidae**		
Mesoplodon stejnegeri	Stejneger's beaked whale	(Present study)
Indopacetus pacificus	Longman's beaked whale	(Present study)
Mesoplodon carlhubbsi	Hubbs' beaked whale	P02183 (Dwulet et al., 1980)
Ziphius cavirostris	Cuvier's beaked whale	P02182 (Lehman et al., 1980)
Family **Delphinidae**		
Peponocephala electra	Melon-headed whale	(Present study)
Orcinus orca	Orca	P02173 (Meuth et al., 1981)
Delphinus delphis	Common dolphin	MYDDBS (Karadjova et al., 1970)
Tursiops truncatus	Bottlenose dolphin	P02172 (Jones et al., 1976)
Globicephala melaena	Long finned pilot whale	P02174 (Jones et al., 1978)
Stenella attenuata	Pantropical spotted dolphin	(Jones et al., 1979; present study)
Family **Phocoenidae**		
Phocaena phocaena	Harbour porpoise	P02176 (Bradshaw and Gurd, 1969)

A partial amino acid sequence of myoglobin from beluga whale (*Delphinapterus leucas*) has been reported (Stewart et al., 2004).

related globins is low, their three-dimensional structure (the so-called "globin-fold") is very similar (Bolognesi et al., 1997).

Mb from sperm whale (*Physeter macrocephalus*) is one of the most thoroughly studied proteins, both structurally and functionally. Elucidation of the crystal structure of sperm whale Mb by Kendrew et al. (1958) and subsequent determination of its amino acid sequence by Edmundson (1968) were landmark findings in structural biology. A typical level of microheterogeneity was

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agctgtcggagccaggacacccggtcagcccactctcgctcttcttctcttcttcagactgtgccATGGTGCTCAGCGAG 80
                                                                  M  V  L  S  E

GGAGAATGGCAGTTGGTTCTGCACGTCTGGGCGAAGGTGGAGGCTGATGTCGCAGGCCATGGGCAGGACATCCTCATCAG 160
G  E  W  Q  L  V  L  H  V  W  A  K  V  E  A  D  V  A  G  H  G  Q  D  I  L  I  R

                                  Wh.Mb.F1
GCTCTTTAAGAGTCATCCCGAGACCCTGGAGAAATTTGACAGGTTCAAGCACCTGAAGACAGAGGCTGAGATGAAGGCCT 240
 L  F  K  S  H  P  E  T  L  E  K  F  D  R  F  K  H  L  K  T  E  A  E  M  K  A  S

CAGAGGACCTGAAGAAGCATGGCGTCACCGTGCTCACTGCCCTGGGGGCCATCCTCAAGAAGAAGGGGCATCATGAGGCG 320
  E  D  L  K  K  H  G  V  T  V  L  T  A  L  G  A  I  L  K  K  K  G  H  H  E  A

GAGCTGAAGCCCCTGGCCCAGTCGCATGCTACCAAGCACAAGATCCCCATCAAGTACCTGGAGTTCATCTCGGAAGCCAT 400
E  L  K  P  L  A  Q  S  H  A  T  K  H  K  I  P  I  K  Y  L  E  F  I  S  E  A  I

                                                  Wh.Mb.R3
CATCCACGTTCTGCACAGCAGGCACCCTGGAGACTTTGGTGCCGACGCCCAGGGGAGCCATGAACAAGGCCCTGGAACTGT 480
 I  H  V  L  H  S  R  H  P  G  D  F  G  A  D  A  Q  G  A  M  N  K  A  L  E  L  F

TCCGGAAGGACATCGCTGCCAAGTACAAGGAGCTGGGCTACCAGGGCTAAgccccacgtcccacgccccacgcccctctc 560
  R  K  D  I  A  A  K  Y  K  E  L  G  Y  Q  G  *

cccacccacctgggcagggagggagggggccttaatcttgtgtagctgtagggtttgcttctgagtgcctgctttgtttgt 640
gagaggccggtgggagatgccggaggggctgggggcagggaagggatgagggtttgttcaagtggtttcacgtgccttcc 720
tgtggggagggtcgtcagcttggaagccaggagaagcttggctctccccactgtggcccgcagagtttgggtcacactca 800
actccccttcacctaaattccaacccaattccttcccacctcaccttaaatccccaatccaaaccgtcaagctaaactc 880
cagccatgattctctgatccatcacctcaccccgtgaagaaagcagaatgtccccttgcaccgagaaggtctgggctggg 960
ttggacagccacacccagcccctggggcgcgtggtgtctggagcataagtgtgccttctcaggtgatggagcgactcacc 1040
tggcttaataaaagattcgcatgccat                                                      1067
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Fig. 1. cDNA and derived amino acid sequences of sperm whale Mb. Two consensus primers (Wh.Mb.F1 and Wh.Mb.R3) used to amplify the central region of cetacean Mb, are enclosed in boxes. The 122nd residue, for which conflicting residues Asp or Asn have been reported, is also enclosed in a box. The polyadenylation signal is underlined.

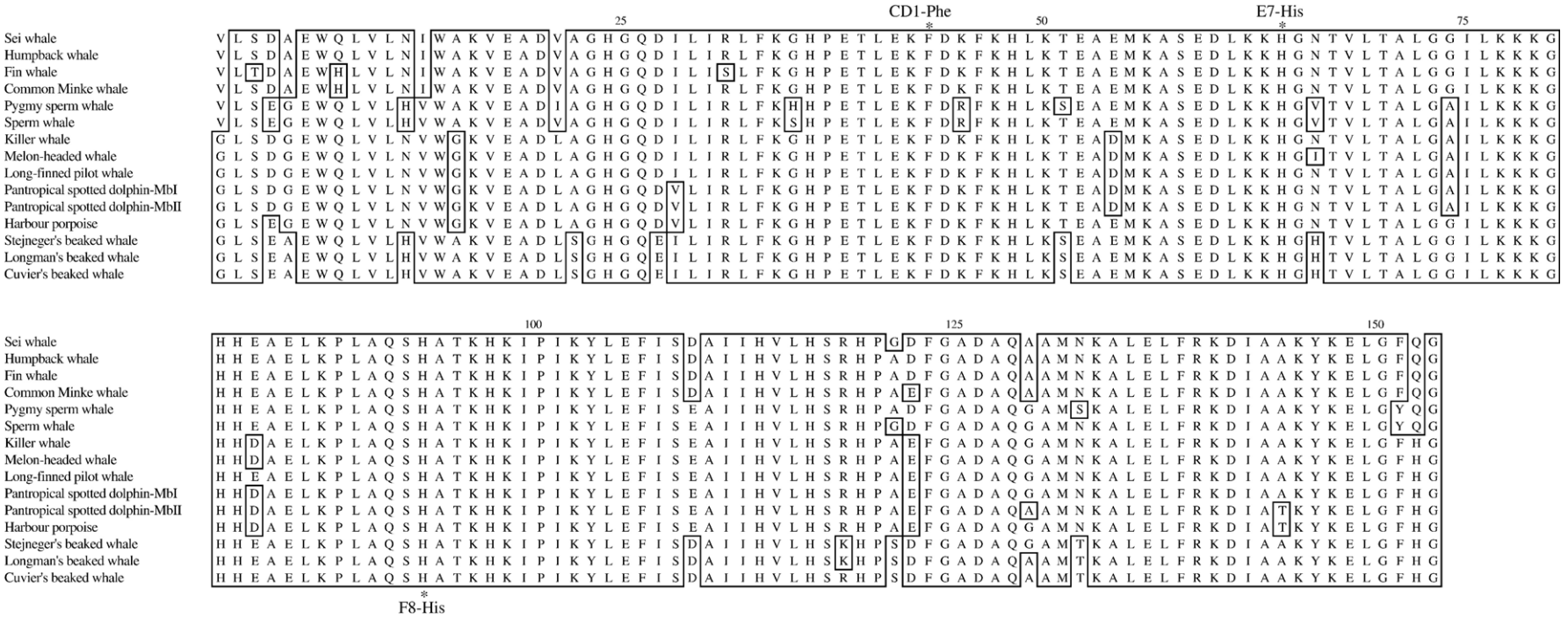

Fig. 2. Alignment of amino acid sequences of 15 cetacean myoglobins. The residues conserved in at least in half of the sequences are enclosed in the box. Three functional key residues, CD1-Phe, E7-His and F8-His, are indicated by asterisks.

demonstrated by electrophoresis and column chromatography for sperm whale Mb, with at least four components being isolated from sperm whale (Hardman et al., 1966), of which only the major Mb has been sequenced. Further, conflicting identifications of residue 122 of the chemically determined amino acid sequence of sperm whale Mb have been reported: Asp (Romero Herrera and Lehmann, 1974) or Asn (Edmundson, 1968). These problems remain to be elucidated. Functional studies of sperm whale Mb by site-directed mutagenesis have been performed using artificial cDNA designed from the amino acid sequence as a template.

In this study, we isolated mRNA of sperm whale Mb, cloned its cDNA in the pGEM plasmid, and determined the cDNA sequence for the first time. Then we compared it with the chemically determined amino acid sequence. In order to give an insight to the evolution of cetaceans, we determined the cDNA sequences from eight species of cetaceans and constructed phylogenetic trees using the Mb cDNA and amino acid sequences.

2. Materials and methods

2.1. mRNA preparation, cDNA amplification and sequence determination of cetacean Mbs

Total RNA was isolated from the skeletal muscle of cetaceans by the acid guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987). mRNA was purified from total RNA using poly(A)$^+$ isolation kit (Nippon Gene, Tokyo, Japan). Single-stranded cDNA was synthesized with Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, NJ, USA) using a lock-docking oligo-dT primer (Borson et al., 1992).

The central region of Mb cDNA was first amplified using two primers (Wh.Mb.F1: GARAARTTYGAYAARTTYAARCA and Wh.Mb.R3: TGNGCRTCNGCNCCRAANTC) designed from the consensus amino acid sequences of cetacean Mb using Ex *Taq* DNA polymerase (Takara, Kyoto, Japan) as the amplifying enzyme. PCR amplification was performed for 35 cycles, each consisting of denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C, and primer extension for 1 min at 72 °C. The amplified products were purified by agarose gel electrophoresis and subcloned into the pGEM-T Easy Vector (Promega, WI, USA). Nucleotide sequences were determined with an ABI PRISM 3100-Avant DNA sequencer using a BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems, CA, USA).

The 3′-end of the cDNA was amplified using the lock-docking oligo-dT primer and a specific primer designed from the sequence of central region. The same PCR conditions described above were used. The amplified products were purified, subcloned in pGEM and sequenced as described above.

A poly(G)$^+$ tail was added at the 3′-end of the cDNA using terminal deoxynucleotidyl transferase (Promega, WI, USA). The 5′-end of the Mb cDNA was then amplified using the oligo-dC primer (5′-GAATTC$_{18}$-3′) and a specific primer designed from sequence of the central region. The same PCR conditions as described above were used. The amplified products were purified, subcloned in pGEM and sequenced as described above.

The complete open reading frame (ORF) was then amplified using two specific primers. *KOD*$^+$ DNA polymerase (Toyobo, Tokyo, Japan) with high fidelity was used as the amplifying enzyme. PCR amplification was performed for 30 cycles, each consisting of denaturation for 15 s at 94 °C, annealing for 30 s at

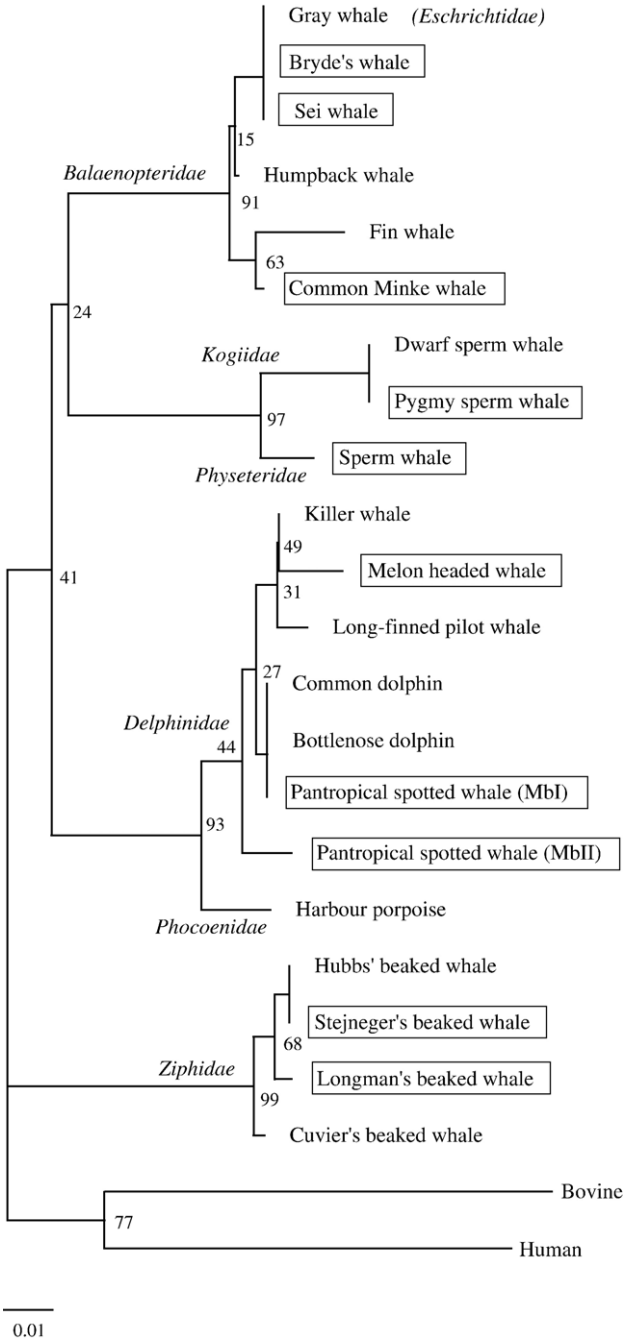

Fig. 3. Phylogenetic tree for the amino acid Mb sequences of cetaceans. The alignment of amino acid sequences, calculation of difference matrix with "blosum" method, and the NJ tree construction were done using a default setting of the program available on DDBJ homepage (http://www.ddbj.nig.ac.jp/Welcome-j.html). The cDNA-derived Mb sequences, determined in this study, are enclosed in boxes. The family names are indicated along the branches. The numbers represent bootstrap values.

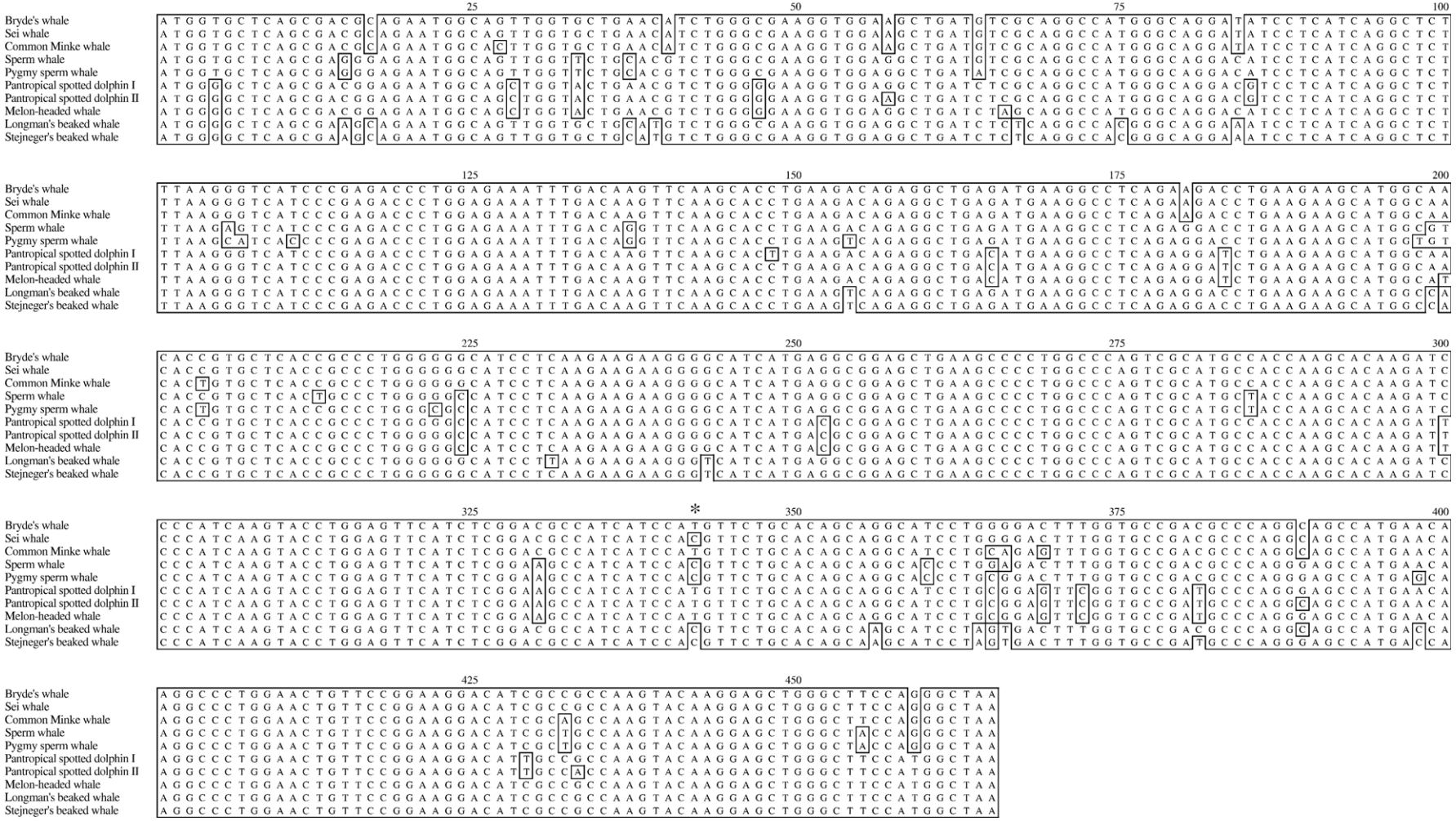

Fig. 4. Alignment of 15 cetacean Mb ORF nucleotide sequences. The nucleotides conserved at least in half of the sequences are enclosed in the box. The third position of codon 114 is indicated by an asterisk.

60 °C, and primer extension for 50 s at 68 °C. The amplified products were purified, subcloned in pGEM and sequenced as described above.

2.2. Alignment of cDNA and amino acid sequences of cetacean Mbs and construction of phylogenetic tree

Multiple alignments of cetacean Mb sequences were made with the ClustalW program using default settings available on the DDBJ homepage (http://www.ddbj.nig.ac.jp/Welcome-j.html). Phylogenetic trees were constructed by the neighbor-joining (NJ) method available on the DDBJ homepage. Bootstrap values were calculated from 1000 replications of the alignment. Amino acid sequences were taken from SwissProt, DDBJ and GenBank. Human and bovine Mb sequences were used as the outgroup sequences.

3. Results and discussion

Composite Mb cDNA sequences were constructed from three fragments amplified separately by PCR for nine cetacean species shown in Table 1. To confirm the sequences, the ORF region was amplified again using a high fidelity-DNA polymerase, cloned in pGEM, and sequenced independently from at least 3 clones. For each of sperm whale and pantropical spotted dolphin, ten clones were sequenced. Except for the sequence of pantropical spotted dolphin, no sequence heterogeneity was found among the other Mbs.

We determined the Mb cDNA sequences of nine cetaceans for the first time. The sequences will be deposited in the DDBJ database. As a representative sequence, the 1067-bp Mb cDNA of sperm whale consisted of a 5′ untranslated region (65 bp), an ORF coding for a 154-amino acid residue protein (462 bp), and a 3′ untranslated region (540 bp) (Fig. 1). Of the cDNA-derived amino acid sequences determined in this study, six from sei whale, Bryde's whale, pygmy sperm whale, Stejneger's beaked whale, Longman's beaked whale, and melon-headed whale are the first reports of Mb sequences, and three from sperm whale, common minke whale, and pantropical spotted dolphin confirm the chemical amino acid sequences determined previously.

Sperm whale Mb is one of the most well studied proteins. The cDNA-derived amino acid sequence in our study agreed with that determined by Romero Herrera and Lehmann (1974), and we confirmed that residue 122 was Asp, and not Asn. Furthermore, considerable heterogeneity has been reported in the protein forms of sperm whale Mb. However, in this study, we failed to detect such heterogeneity from cDNA sequences of ten independent clones from one individual; all were identical. Therefore, it is suggested that the minor components of sperm whale Mb are not as abundant as presumed based on elution profiles of ion-exchange chromatography in Mb preparation (Hardman et al., 1966) and that some of the diverse components might be derived from post-transcriptional modification of the major Mb molecule.

The Mb cDNA-derived amino acid sequences from common minke whale and pantropical spotted dolphin agreed with the previously determined chemical amino acid sequences. However, we found that there are two types of Mb in the skeletal muscle of pantropical spotted dolphin: Mb I with the same amino acid sequence as that deposited in the protein database, and Mb II, which differs at two amino acid residues (position 130: Gly for Mb I and Ala for Mb II, and position 145: Ala for Mb I and Thr for Mb II). These two forms of Mb appear to exist in the skeletal muscle of *Stenella attenuata* in approximately equal proportion: of the ten clones examined, five corresponded to Mb I and the remaining five to Mb II.

So far, 20 amino acid Mb sequences from cetaceans have been determined, a total of 14 by the group led by Gurd and

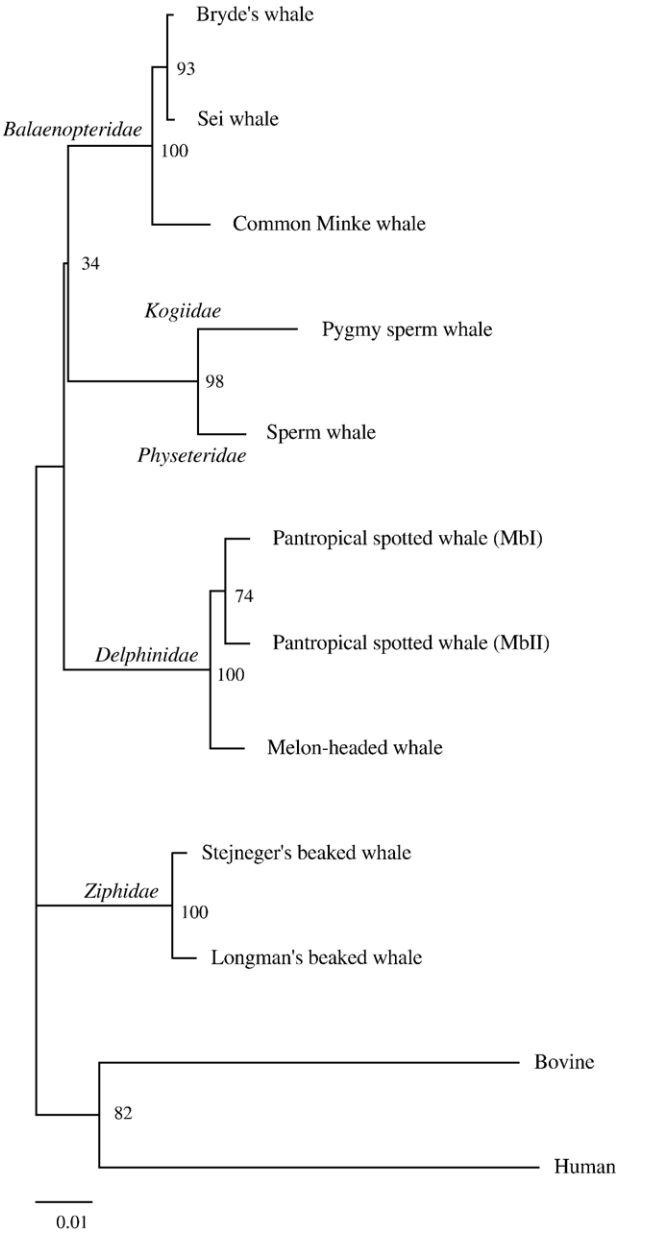

Fig. 5. Phylogenetic tree of Mb cDNA sequences (ORF region) of cetaceans. The NJ tree was constructed using a default setting of the program available on DDBJ homepage (http://www.ddbj.nig.ac.jp/Welcome-j.html). The family names are indicated along the branches. The numbers represent the bootstrap values.

seven with new data in this study (see Table 1). An alignment of 15 representative amino acid sequences of cetacean Mb shown in Fig. 2 shows that the key residues, CD1-Phe, E7-His and F8-His, typical for Mbs and hemoglobins, have been conserved in all cetaceans. Of the 153 amino acid residues in the alignment, 124 are identical, leaving 29 informative positions. At the amino acid level, four patterns of Mb sequences with groupings of identical sequences emerged: saddleback dolphin, bottlenose dolphin and pantropical spotted dolphin; Stejneger's beaked whale and Hubbs' beaked whale; pygmy and dwarf sperm whales; and sei whale, Bryde's whale and gray whale. The NJ tree in Fig. 3 indicates that the clustering of amino acid sequences of cetacean Mbs follows essentially the classical taxonomy at the family level: Balaenopteridae (Bryde's whale, Sei whale, humpback whale, fin whale and common minke whale), Physeteridae (sperm whale), Kogiidae (pygmy and dwarf sperm whales), Delphinidae (Orca, melon-headed whale, long finned pilot whale, common dolphin, bottlenose dolphin and pantropical spotted dolphin), Phocoenidae (Harbour porpoise) and Ziphidae (Hubbs' beaked whale, Stejneger's beaked whale, Longman's beaked whale and Cuvier's beaked whale). This suggests that Mb sequence analysis is at least valid for classification of cetaceans at the family level. In addition, the tree in Fig. 3 is essentially in agreement with those based on other taxonomies of cetaceans inferred from molecular characters of retroposon, DNA sequences of mitochondrial control region, nuclear actin intron, and nuclear α-lactoalbumin, and from amino acid sequences of 12 proteins coding by complete mitochondrial DNA, although several points of controversy have arisen (Sharon and Jimmy, 1998; Nikaido et al., 2001; Palumbi and Cipriano, 1998; Lyrholm et al., 1996; Rychel et al., 2004; Arnason et al., 2004; Dalebout et al., 2004; Arnason, 2005). However, it should be emphasized that the bootstrap values in Fig. 3 are too low to draw any significant insights on the relationships among the families, except for the close relationship among Delphinidae and Phocoenidae, which is supported by a bootstrap value of 93%. In contrast, the cluster of Balaenopteridae uncharacteristically contains the Mb sequence of the gray whale (Eschrichtidae). This placement may be a result of the low resolution of the NJ tree based on Mb amino acid sequences or the difficulty of the identifying orthologous Mb, such as in the case of pantropical spotted dolphin in which two or more Mb are expressed concomitantly. A tree constructed with the maximum-likelihood method using the same data set gave much lower resolution, even at a family level, than that given using the NJ method (data not shown).

An alignment of Mb ORF cDNA sequences of cetaceans determined in this study (Fig. 4) showed that of the 462 nucleotides in the alignment, 407 are identical, leaving 55 informative positions. An NJ tree (Fig. 5) constructed from the ORF alone is similar to that constructed from the amino acids of Fig. 3. Although the amino acid sequences of sei whale and Bryde's whale are identical, the cDNA sequence of Mb distinguishes them with only a single silent nucleotide difference (T or C at the third position of codon 114 indicated by asterisk in Fig. 4), suggesting a very close relationship between the two species. Thus, Mb cDNA sequence data appears to be a valid classification tool for cetaceans at the species level. The tree constructed with the maximum-likelihood method using the same data set gave a similar topology to that using the NJ method (data not shown).

In this paper, we reported ten cDNA sequences of Mbs from nine cetacean species, including the first report of the cDNA sequence of sperm whale Mb, one of the best studied proteins. Our sequence data, together with other molecular data, contribute significantly to the understanding of cetacean evolution.

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