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Evolutionary changes of the importance of olfaction in cetaceans based on the *olfactory marker protein* gene

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ABSTRACT

Odontocetes and mysticetes are two extant suborders of cetaceans. It is reported that the former have no sense of olfaction, while the latter can smell in air. To explain the ecological reason why mysticetes still retain their sense of smell, two hypotheses have been proposed — the echolocation-priority hypothesis, which assumes that the acquisition of echolocation causes the reduction of the importance of olfaction, and the filter-feeder hypothesis, which assumes that olfactory ability is important for filter-feeders to locate their prey because clouds of plankton give off a peculiar odor. The olfactory marker protein (OMP) is almost exclusively expressed in vertebrate olfactory receptor neurons, and is considered to play important roles in olfactory systems. In this study, full-length open reading frames of *OMP* genes were identified in 6 cetacean species and we analyzed the nonsynonymous to synonymous substitution rate ratio based on the maximum likelihood method. The evolutionary changes of the selective pressures on *OMP* genes did fit better to the filter-feeder hypothesis than to the echolocation-priority hypothesis. In addition, no pseudogenization mutations are found in all five odontocetes *OMP* genes investigated in this study. It may suggest that OMP retains some function even in 'anosmic' odontocetes.

1. Introduction

Olfactory marker protein (OMP) is a highly abundant small cytoplasmic protein encoded by an intronless *OMP* gene (Danciger et al., 1989; Margolis, 1972). Expression of the *OMP* gene is highly restricted to mature olfactory chemosensory neurons, and is considered to play an important role in the olfactory signal-transduction cascade across vertebrate species (Danciger et al., 1989; Margolis, 1980; Reisert et al., 2007). Several studies reported that OMP-knockout mice show considerably reduced ability to respond to odor stimuli (Buiakova et al., 1996; Youngentob and Margolis, 1999; Youngentob et al., 2001), but the biochemical function of OMP remains largely elusive.

Amniotes that have undergone a transition from the terrestrial to aquatic environment generally have reduced olfactory capacity (Kishida and Hikida, 2010), and aquatic cetaceans are known to have reduced their sense of olfaction (Dehnhardt, 2002). *Olfactory receptor* (*OR*) genes are highly reduced in cetacean genomes, especially in odontocetes (Hayden et al., 2010; Kishida et al., 2007; McGowen et al., 2008). Furthermore, modern odontocetes have no nervous system structures that mediate olfaction, i.e., no olfactory bulb or olfactory tract (Oelschläger and Oelschläger, 2008), suggesting that odontocetes have lost their sense of olfaction altogether. In contrast to odontocetes, mysticetes were shown in a recent study to have fully-equipped olfactory nervous system structures and histologically complex olfactory bulbs, indicating that they can smell in air (Thewissen et al., 2011). To explain the ecological reason why olfaction is present in mysticetes but absent in odontocetes, it has widely been considered that the acquisition of echolocation causes a reduction of the importance of olfaction (echolocation-priority hypothesis) (Cave, 1988; Hoch, 2000). However, mysticetes can smell in air, but not underwater (Thewissen et al., 2011), meaning that mysticete olfaction cannot be compensated for by the acquisition of echolocation, i.e., an underwater sonar system. Recently, another hypothesis has been proposed that olfaction is important for filter-feeders to locate their prey because clouds of plankton, especially krill, give off a peculiar odor on the surface of the sea (filter-feeder hypothesis) (Thewissen et al., 2011). Fig. 1 shows the evolutionary changes of the importance of olfaction each hypothesis predicts.

No cetacean *OMP* genes have been reported to date. However, as described earlier, odontocetes have no tissues in which the *OMP* gene is known to be expressed, and it is an interesting question whether the *OMP* gene still has a function in odontocetes or not. In addition, the evolutionary pathways of *OMP* genes may reflect the evolutionary changes of the importance of olfaction. In this study,

Abbreviations: bp, base pair(s); ML, maximum likelihood; MYA, million years ago; OMP, olfactory marker protein; OR, olfactory receptor; ORF, open reading frame; PCR, polymerase chain reaction.

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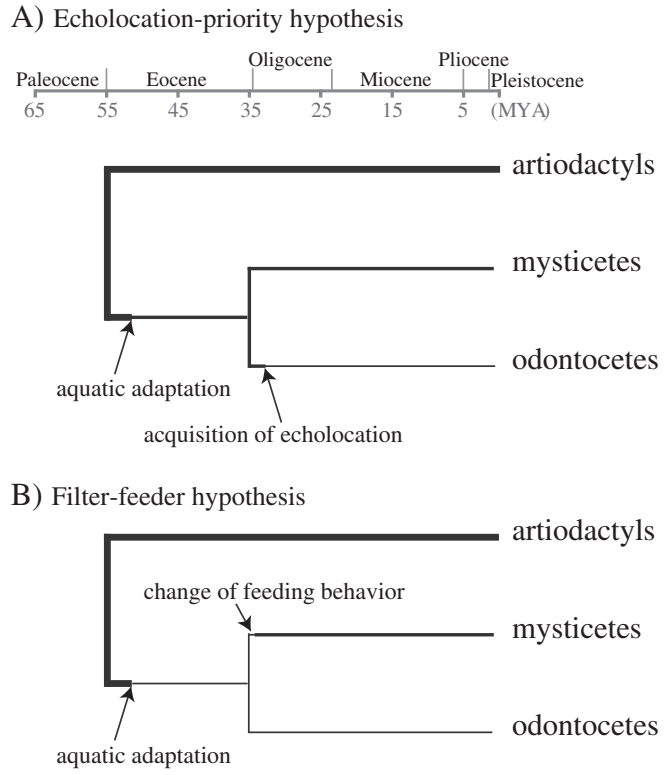

Fig. 1. The predicted evolutionary changes of the importance of olfaction based on the echolocation-priority hypothesis (A)/the filter-feeder hypothesis (B). Thinner branches indicate less importance of olfaction. Geological scale bar is provided above, and the periods of the evolutionary events follow Uhen (2007). The echolocation-priority hypothesis predicts that the importance of olfaction has been reduced in the odontocete branch since the acquisition of echolocation, while olfaction is as important for mysticetes as for their aquatic ancestors. On the other hand, the filter-feeder hypothesis predicts that the olfaction is as useless for odontocetes as for their aquatic ancestors, while the importance of olfaction has been increased in the mysticete branch since they have become filter-feeders.

full-length open reading frames (ORFs) of *OMP* genes were identified in 6 cetacean species (5 odontocetes and a mysticete) and we analyzed the nonsynonymous to synonymous substitution rate ratio ω (d_n/d_s) based on the maximum likelihood (ML) method.

2. Materials and methods

2.1. Amplification and sequencing of whale OMP genes

Muscle tissues of Baird's beaked whale *Berardius bairdii* and short-finned pilot whale *Globicephala macrorhynchus* were purchased from a fish market in Japan, and genomic DNA was extracted following the protocol described by Kishida et al. (2007). Genomic DNA samples of dwarf sperm whale *Kogia sima*, Dall's porpoise *Phocoenoides dalli* and minke whale *Balaenoptera acutorostrata*, which were prepared in Kishida et al. (2007), were used in this study. A set of primers, OMP_full_5 (5′-ACGGTGGAGGCGGCAGCAGCAA-3′) and OMP_full_3 (5′-AGGGTAGCAGCAGGCAGCTGCCA-3′), was employed in PCR reactions to amplify the full-length sequences of the ORF of *OMP* genes. Sequences of the PCR products were determined directly on an ABI3130 automated sequencer using BigDye terminator v3.1 (Applied Biosystems). The procedures we followed to design the OMP_full_5 and OMP_full_3 primers are provided as Supplementary methods. Minke whale, dwarf sperm whale, beaked whale, porpoise and pilot whale *OMP* sequences are available in the DDBJ/EMBL/GenBank databases under the following accession numbers, respectively: AB626889, AB626890, AB626891, AB626892 and AB642168.

2.2. OMP genes of bottlenose dolphin and terrestrial mammals

OMP gene sequences of human *Homo sapiens* (GenBank ID: BC069115), mouse *Mus musculus* (GenBank ID: U02557), dog *Canis lupus* (GenBank ID: XM_844636) and cow *Bos taurus* (GenBank ID: XM_865027) were retrieved from the GenBank database. The draft genome assembly of the bottlenose dolphin *Tursiops truncatus* was downloaded from the Ensembl genome browser release58 (http://www.ensembl.org/). The dolphin *OMP* gene sequence was searched for using the FASTA3.5 program (Pearson and Lipman, 1988) and the cow *OMP* gene was used as a query. The positions of the initiation and termination codons were judged by comparison with the human, mouse, dog and cow *OMP* gene sequences.

2.3. Sequence analyses

The *OMP* genes thus obtained were aligned manually (Fig. 2). The nonsynonymous to synonymous substitution rate ratio ω provides an indication of the changes of selective pressures as follows: higher ω ratios indicate relaxation of purifying selection, and $\omega > 1$ suggests positive selection (Yang, 2006). The CODEML program in the PAML4.4 package (Yang, 2007) was used to analyze changes of selective pressure based on widely-accepted phylogenetic relationships (human, mouse, (dog, (cow, (minke whale, (beaked whale, porpoise))))). Several models shown in Table 1 were compared. In all models, the transition/transversion rates were not fixed and the F3×4 model was used for codon usage biases. Likelihood ratio tests were performed to compare between models, and the significance of differences was evaluated by calculating twice the log-likelihood difference assuming that it follows a χ^2 distribution, with the number of degrees of freedom equal to the difference in the numbers of free parameters between models. The method of Zhang et al. (1997), in which the numbers of nonsynonymous and synonymous substitution sites in a particular branch were compared directly with those of non-synonymous and synonymous sites which were not changed, was applied to examine the significant existence of positive/purifying selection. In this method, the numbers of nonsynonymous sites and substitutions, and synonymous sites and substitutions were estimated by the method of Nei and Gojobori (1986) based on the ancestral nucleotide sequences inferred by the Bayesian method (Yang et al., 1995). Numbers of nonsynonymous and synonymous sites were also estimated by the ML method (Goldman and Yang, 1994). We also modified the method of Zhang et al. (1997) to examine whether selective pressure on a particular branch can be considered as homogeneous in comparison with that on a compared branch. In this method, the numbers of nonsynonymous and synonymous substitutions were compared directly between these two branches (test of homogeneity of nonsynonymous/synonymous change ratios).

3. Results

The length of *OMP* is highly conserved among mammalian species with the exception of three odontocetes (dwarf sperm whales, pilot whales and bottlenose dolphins). Sperm whales lack 4 amino acids (12 bp) located at the end of the first α-helix, and delphinid whales (pilot whales and dolphins) lack 5 amino acids (15 bp) located at the third β-strand (Fig. 2). Sperm whale, pilot whale and dolphin *OMP* sequences were excluded from ML analyses because these gaps reduce the computational regions from this short-length gene. The ω ratios, estimated based on the free-ratio model (allowing ω ratios to vary along different branches), are shown in Fig. 3(A). Interestingly, the ω ratios were much higher not only in the odontocete branches, but also in an ancestral branch named 'Eocene whale branch' [a tree branch which represents the lineage that includes the common ancestors of mysticetes and odontocetes. Most of this branch has been in the Eocene Epoch (Fig. 1)], in comparison with