Fixed genetic differences between two species of finless porpoises, genus *Neophocaena* (Cetacea, Mammalia), with lack of genetic divergence between two subspecies of the narrow-ridged finless porpoise, *N. asiaeorientalis*: revealed from cytochrome *b* sequence analyses

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ABSTRACT

We obtained complete cytochrome *b* gene sequences of 12 *Neophocaena asiaeorientalis sunameri* specimens, collected from fishery markets in Korea, and these sequences were compared to the corresponding partial (402 bp) and complete (1,140 bp) haplotypes of the genus *Neophocaena*, obtained from GenBank, in order to examine genetic divergences both between *N. asiaeorientalis* and *N. phocaenoides* and between *N. a. asiaeorientalis* and *N. a. sunameri*. From a maximum likelihood tree with partial cytochrome *b* sequences of the two *Neophocaena* species we first detected two clades, corresponding to the two species of *Neophocaena*, with average genetic distance of 1.64% and four fixed site differences. We concluded that our sequencing results from cytochrome *b* sequences are more informative in the examination of genetic divergences in *Neophocaena* than the previous molecular results based on *F*-statistics with the control region and nuclear markers. In addition, from our complete cytochrome *b* sequence analysis we recognized lack of genetic divergence between the two subspecies of *N. asiaeorientalis*, and we concluded that *N. a. sunameri* from East Asian Sea is a synonym of *N. a. asiaeorientalis* from the Yangtze River. In future, further genetic analyses with additional specimens of *Neophocaena* are necessary to confirm our findings.

Keywords: stock identity, genetics, taxonomy, cytochrome *b* gene, *Neophocaena*, East Asia

INTRODUCTION

Mead and Brownell (2005) noted that the geographic distribution of the finless porpoise (*Neophocaena phocaenoides* Cuvier, 1829) extends from Indo-Pacific to Japan, including coastal waters and some rivers, and they recognized three subspecies (*N. p. phocaenoides*, *N. p. asiaeorintalis*, and *N. p. sunameri*). However, Pilleri and Gihr (1975) reported that the genus *Neophocaena* comprises three distinct species, *N. phocaenoides*, *N. aisaorientalis*, and *N. sunameri*, and Jefferson and Wang (2011) reclassified finless porpoises as two distinct species from their review on the previous studies of finless porpoises with morphological and molecular characters: the Indo-Pacific finless porpoise, *N. phocaenoides*, and the narrow-ridged finless porpoise, *N. asiaeorientalis*, with two subspecies (Yangtze finless porpoise, *N. a. asiaeorientalis*, and East Asian finless porpoise, *N. a. sunameri*).

Molecular genetic studies for taxonomic reconsideration have become widespread during the past decade, and mitochondrial DNA (mtDNA) is a highly sensitive genetic marker suitable for studies of closely related taxa or populations of a variety of species (Sunnucks, 2000). Any obvious groupings within the genus *Neophocaena* were not recognized in the previous molecular studies with finless porpoises from traditional phylogenetic trees, and to recognize population subdivisions *F*-statistics were utilized on the basis of mtDNA control region (Yoshida *et al*., 2001; Yang *et al*., 2008), microsatellites and control region (Wang *et al*., 2008; Li *et al*., 2011), microsatellites (Chen *et al*., 2010), and nuclear introns (Ju *et al*., 2012).

Regarding evolutionary rates, the cytochrome *b* gene vary at a slower rate than the control region (Lopez *et al*., 1997), and the utility of using DNA barcoding approaches with mtDNA cytochrome *b* sequences was demonstrated for the discrimination between the two mongoose species of the genus *Herpestes* (Bennett *et al*., 2011) and among delphinid cetacean species (Amaral *et al*., 2007a). Li *et al*. (2011) obtained cytochrome *b*

sequences of *N. a. sunameri* from Yellow Sea in China, but they did not perform any further genetic analyses with these cytochrome *b* sequences. Thus, it is necessary to examine whether or not population divergences among finless porpoises are revealed from conventional phylogenetic trees with conservative cytochrome *b* gene sequences.

In this study, we used 12 *N. a. sunameri* specimens, collected from fishery markets in Pohang, Korea, and obtained their complete cytochrome *b* gene sequences. These sequences were compared to the corresponding partial (402 bp) and complete (1,140 bp) sequences of *Neophocaena*, obtained from GenBank, in order to examine genetic divergences both between two species of *Neophocaena* (*N. asiaeorientalis* and *N. phocaenoides*) and between two subspecies of *N. asiaeorientalis* (*N. a. asiaeorientalis* and *N. a. sunameri*).

MATERIALS AND METHODS

For this analysis, we collected 12 specimens (specimen nos. 2507-2509, 2511, 2513-2515, 2538-2540, 2542, and 2548) of *N. a. sunameri* from fishery markets in Pohang, Korea, which were caught by fisheries 'by-catch' in the year 2013, as given in Table 1. Small pieces of muscle were taken and preserved in a deep freezer.

Total cellular DNA was extracted using a genomic DNA extraction kit (Intron Co., Seoul, Korea). From muscle samples, total cellular DNA was extracted using a Genomic DNA extraction kit (Intron, Daejeon, Korea). The cytochrome *b* gene was PCR-amplified using the primers CB-out1 and CB-out2 (Cassens *et al*., 2000). PCR thermal cycle for cytochrome *b* sequence was as follows: 94℃for 5 min; 94℃for 1 min, 55℃for 1 min, 72℃for 1 min (32 cycles); 72℃for 5 min. To remove primer and unincorporated nucleotides, the amplified product was purified using a DNA PrepMate kit with a silica-based matrix (Intron Co.). The purified PCR products were analyzed with an automated DNA Sequencer (Perkin Elmer 377) at Bioneer Co. (Seoul, Korea).

The complete sequences (1,140 bp) of the cytochrome *b* gene were obtained from 12 *N. a. sunameri* in Korea, and these sequences were compared to the corresponding complete nine haplotypes of two subspecies in *N. asiaeorientalis*, obtained from GenBank, as given in Table 2. In addition, from the complete cytochrome *b* gene sequences of *N. asiaeorientalis*, obtained from this study and GenBank, partial cytochrome *b* sequences (402 bp; site nos. 12-413) were obtained and analyzed together with the corresponding five partial sequences of *N. phocaenoides*, obtained from GenBank, as listed in Table 2.

Sequence alignment, detection of parsimonious informative sites, model selection, calculation of nucleotide distances, and tree constructions with 1,000 bootstrapped replications were conducted using MEGA5 (Tamura *et al*., 2011). The Jukes-Cantor (JC) model, which showed the lowest Bayesian information criterion scores, was selected, and maximum likelihood trees were constructed. *Balaenoptera physalus* (NC001321) and *Delphinus delphis* (AF084084) were used as outgroups.

RESULTS

The complete nine cytochrome *b* haplotypes were obtained from 12 *N. a. sunameri* specimens, as shown in Table 1. Within 18 haplotypes of *N. asiaeorientalis* (nine haplotypes from this study and nine haplotypes from GenBank), 30 sites (2.63%) were variable, and 15 sites (1.32%) were parsimonious informative. The average JC distance among nine haplotypes of *N. a. sunameri* from Korea was 0.44%.

A maximum likelihood tree with complete 18 cytochrome *b* haplotypes from *N. asiaeorientalis* is shown in Figure 1, and the 18 haplotypes from the Yangtze River, Yellow Sea (China), and East Sea (Korea) formed one clade (Gp 1), with within group average JC distance of 0.57%. In addition, one haplotype (HM137098) of *N. a. asiaeorientalis* from the Yangtze River was identical to one haplotype (CB02KoreaS) of *N. a. sunameri* from Korea, and another haplotype (HM137092) of *N. a. asiaeorientalis* from the Yangtze River was identical to another haplotype (HQ108397) of *N. a. sunameri* from Yellow Sea. Additionally, HQ108415 from Yellow Sea was identical to CB04KoreaS from East Sea, and HQ108420 from Yellow Sea was identical to CB08KoreaS from East Sea.

Another maximum likelihood tree with partial 23 cytochrome *b* haplotypes (402 bp) of two *Neohpocaena* species from India, China, and Korea is shown in Figure 2, and two clades (Gps 1 and 2) were recognized: the 18 haplotypes of *N. asiaeorientalis* from China and Korea (Gp 1) were distinct from the five haplotypes of *N. phocaenoides* from India and Arabia Sea (Gp 2), with average JC distance of 1.64% and four fixed site difference (site nos. 60, 145, 261, and 408).

DISCUSSION

Jefferson and Wang (2011) reported that the sharing of mtDNA control region haplotypes and nuclear DNA alleles between the two species of finless porpoises is a common result amongst the previous molecular studies with the genus *Neophocaena*. Li *et al*. (2011) and Ju *et al.* (2012) could not find any obvious groupings in the two species of finless porpoises from conventional phylogenetic trees based on nuclear intron, microsatellite, and

mtDNA control region sequences, and they distinguished the two species by using *F*-statistics. Additionally, Wang *et al*. (2008) noted that the shared DNA in *Neophocaena* was due to insufficient time since divergence to allow complete lineage sorting that would result in fixed genetic differences.

The nuclear genes vary at a slower rate than mtDNA sequences (Steppan *et al*., 2005), and mtDNA cytochrome *b* gene is more conservative than mtDNA control region (Lopez *et al*., 1997), whereas the variability of microsatellites is often so high that it is possible to address issues such as discrimination at the individual level (Wan *et al*., 2004). In addition, the cytochrome *b* gene has several advantages when compared to the control region in phylogenetic analysis of the genus *Delphinus* (Amaral *et al*., 2007b).

From our study based on the partial cytochrome *b* sequences of *Neophocaena* (Fig. 2), we first detected two clades, corresponding to the two species of *N. asiaeorientalis* and *N. phocaenoides*, with average JC distance of 1.64% and four fixed site differences. Thus, we considered that our results based on the cytochrome *b* sequences are more informative for investigating genetic divergences in *Neophocaena* than the previous results on the basis of the control region and other genetic markers, and that complete lineage sorting have occurred in the cytochrome *b* gene of the two *Neophocaena* species because the time after divergence was long enough to result in fixed genetic differences between the two species, although further genetic analyses with specimens throughout distribution range of the two species are necessary to confirm our findings.

Jefferson and Wang (2011) noted that the Yangtze River finless porpoise *N. a. asiaeorientalis* is considered to be distinct in the previous morphological and molecular studies from East Asian finless porpoise, *N. a. sunameri*, although shared haplotypes and alleles between the two subspecies of *N. asiaeorientalis* were revealed. Li *et al*. (2011) and Ju *et al*. (2012) used *F*-statistics with microsatellite, mtDNA control region, and nuclear intron markers to distinguish two subspecies of *N. asiaeorientalis*. In this study based on the complete cytochrome *b* sequences of *N. asiaeorientalis* (Fig. 1), we found lack of genetic divergence between *N. a. asiaeorientalis* from the Yangtze River (Gp 1, in part) and *N. a. sunameri* from Yellow Sea and East Sea in East Asia (Gp 1, the rest). Additionally, each of two *N. a. asiaeorientalis* haplotypes was identical to each of two *N. a. sunameri* haplotypes.

Huelsenbeck *et al*. (1996) reported that a classification should be the product of all available characters distributed as widely and evenly as possible over the organisms studied. Jefferson and Wang (2011) noted that there is still some uncertainty about *N. a. asiaeorientalis*' isolation in the Yangtze River proper, and Pilleri and Gihr (1975) reported that finless porpoises from Japan and China have been considered as same subspecies in a previous morphometric analysis. Additionally, we considered that our results with the cytochrome *b* sequences are more informative for analyzing population divergences of finless porpoises than the previous results with other genetic markers, as mentioned above. Thus, we concluded that *N. a. sunameri* is a synonym of *N. a. asiaeorientalis*, although further genetic analyses with more specimens throughout distribution range of *N. asiaeorientalis* are needed to confirm our findings.

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Table 1. Specimen number and mitochondrial DNA complete (1140 bp) cytochrome *b* haplotypes of 12 *Neophocaena asiaeorientalis sunameri* specimens, collected from fishery markets at Pohang in Korea. Among the 12 sequences nine haplotypes were identified.

Specimen number (complete cytochrome *b* haplotype)
2507 (CBO1KoreaS); 2508, 2513, 1538, and 2548 (CBO2KoreaS); 2509 (CBO3KoreaS); 2511 (CBO4KoreaS); 2514 (CBO5KoreaS); 2515 (CBO6KoreaS); 2539 (CBO7KoreaS); 2540 (CBO8KoreaS); and 2542 (CBO9KoreaS)

Table 2. GenBank identification of 14 cytochrome *b* haplotypes in the genus *Neophocaena*, used in this study. The 14 haplotypes from GenBank were complete[1] (1140 bp) nine haplotypes of two subspecies in *N. asiaeorientalis* and partial[2] (402 bp) five sequences of *N. phocaenoides*.

Species name	Locality	Accession number (complete[1] or partial[2] cytochrome *b* haplotype)
N.a.asiaeorientalis	Yangzte River	HM137084[1], HM137092[1], HM137098[1], and HM137100[1]
N.a.sunameri	Yellow Sea	HQ108395[1], HQ108397[1], HQ108415[1], HQ108419[1], and HQ108420[1]
N.phocaenoides	India	EF203442[2], EF203444[2], and EF203438[2]
〃	Arabia Sea	DQ364692[2] and DQ364691[2]

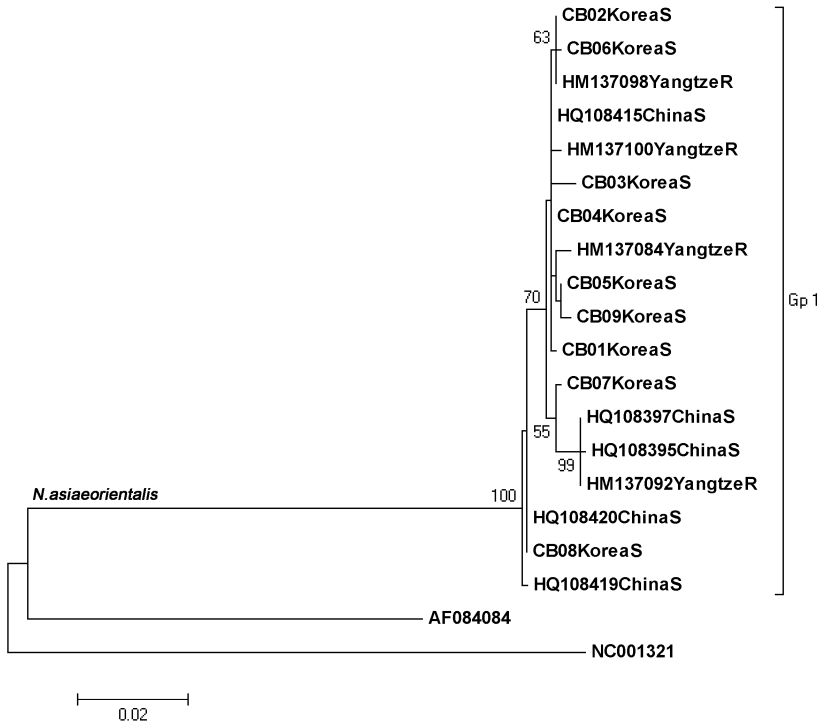

Fig. 1. A maximum likelihood tree with complete (1,140 bp) 18 cytochrome *b* haplotypes of *Neophocaena asiaeorientalis.* Nine haplotypes of *N. a. sunameri* from Korea were obtained from this study, as given in Table 1, and nine haplotypes of *N. asiaeorientalis* were obtained from GenBank, as listed in Table 2. The tree was constructed with 1,000 bootstrapped replications, and the bootstrap values >50% are reported at the internodes. Location name follows accession number in the nine haplotypes, obtained from GenBank, and *Balaenoptera physalus* (NC001321) and *Delphinus delphis* (AF084084) were used as outgroups.

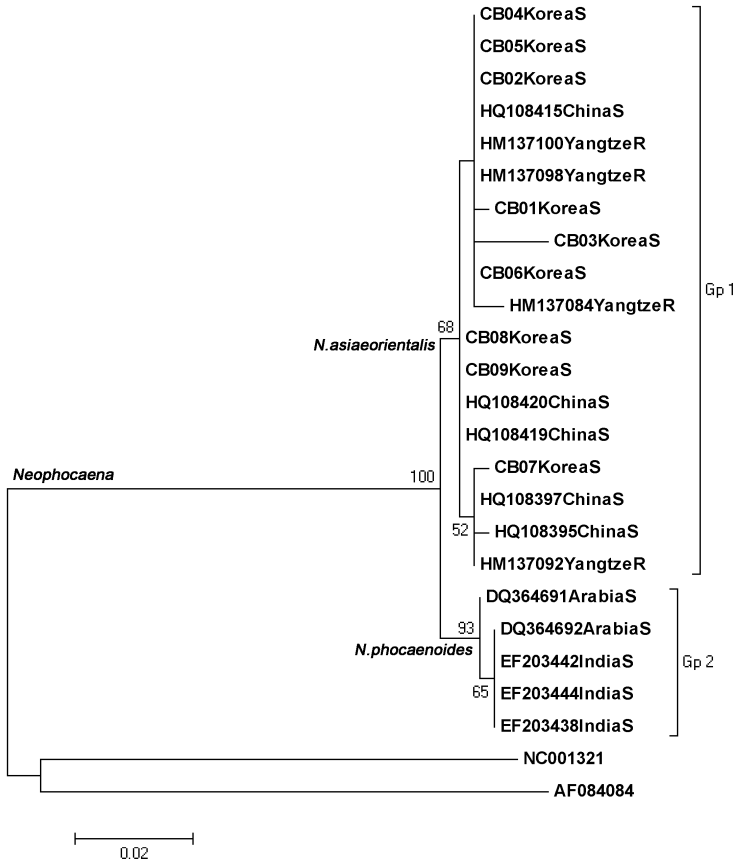

Fig. 2. A maximum likelihood tree with partial (402 bp) 23 cytochrome *b* haplotypes of two species in the genus *Neophocaena*. Nine haplotypes of *N. a. sunameri* from Korea were obtained from this study, as given in Table 1, and nine haplotypes of *N. asiaeorientalis* and five haplotypes of *N. phocaenoides* were obtained from GenBank, as listed in Table 2. The tree was constructed with 1,000 bootstrapped replications, and the bootstrap values >50% are reported at the internodes. Location name follows accession number in 14 haplotypes, obtained from GenBank, and *Balaenoptera physalus* (NC001321) and *Delphinus delphis* (AF084084) were used as outgroups.