Preliminary study on genetic differences between two species of finless porpoises, genus *Neophocaena*, with lack of genetic divergence between two subspecies of the narrow-ridged finless porpoise, *N. asiaeorientalis*: cytochrome *b* sequence analyses

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ABSTRACT

Using samples from bycaught finless porpoises, cytochrome *b* sequences were analysed and phylogenetic trees were constructed. The aims were to: (1) determine genetic divergences within the genus *Neophocaena*; (2) examine interspecific divergences between *N. asiaeorientalis* and *N. phocaenoides*; and (3) examine intraspecific divergence between *N.a. asiaeorientalis* and *N.a. sunameri.* For this purpose, complete cytochrome *b* sequences for 12 *N.a. sunameri* specimens, collected from fishery markets at Pohang in southeastern Korea, were obtained, and these sequences were compared to the corresponding partial (402bp) and complete (1,140bp) sequences of *Neophocaena*, obtained from GenBank. From a maximum likelihood tree with the partial sequences of the two *Neophocaena* species, two clades were detected, corresponding to the two species, with average genetic distance of 1.64%, four fixed site differences (1.00%), and a G^{st} value of 0.64, although we did not examine the specimens from Southeast Asia and contiguous South China Sea. Furthermore, from the complete sequences, we recognised a lack of genetic divergence between the two subspecies of *N. asiaeorientalis*, with a G^{st} value of 0.06 and two pairs of identical sequences between them, indicating that our results do not support current subspecies classification. Thus, we newly found that our cytochrome *b* sequencing results are useful for the examination of interspecific and intraspecific divergences in *Neohpocaena*, although further genetic analyses with additional specimens of *Neophocaena* across its distributional range are necessary to confirm the findings in this study.

KEYWORDS: GENETICS; TAXONOMY; BYCATCH; SEA OF JAPAN; ARABIAN SEA; INDIAN SEA; EAST CHINA SEA; NORTHERN HEMISPHERE; FINLESS PORPOISE

INTRODUCTION

Mead and Brownell (2005) noted that the geographic distribution of the finless porpoise (*Neophocaena phocaenoides* Cuvier, 1829) extends from the Indo-Pacific Ocean to Japan, including coastal waters and some rivers, and they recognised three subspecies (*N.p. phocaenoides*, *N.p. asiaeorientalis*, and *N.p. sunameri*). However, Pilleri and Gihr (1975) had previously reported that the genus *Neophocaena* comprises three distinct species, *N. phocaenoides*, *N. asiaeorientalis*, and *N. sunameri.* Jefferson and Wang (2011) reclassified finless porpoises as two distinct species from the 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 sensitive genetic marker suitable for studies of closely related taxa or populations of a variety of species (Sunnucks, 2000). To examine population subdivisions of *Neophocaena*, *F*-statistics were utilised on the basis of the mtDNA control region (Yang *et al.*, 2008; Yoshida *et al.*, 2001), nuclear microsatellites and mtDNA control region (Li *et al.*, 2011; Wang *et al.*, 2008), nuclear microsatellites (Chen *et al.*, 2010), and nuclear introns (Ju *et al.*, 2012). Although Li *et al.* (2011) obtained cytochrome *b* sequences of *N.a. sunameri* from the Yellow Sea, they did not perform any further genetic analyses with these cytochrome *b* sequences. Thus cytochrome *b* sequences and phylogenetic trees have not been used to determine genetic divergences within the genus *Neophocaena*.

Regarding evolutionary rates, the cytochrome *b* gene varies at a slower rate than the control region (Lopez *et al.*, 1997), and the use of DNA barcoding approaches with mtDNA cytochrome *b* sequences was demonstrated in the discrimination between the two mongoose species of the genus *Herpestes* (Bennett, 2011) and among delphinid cetacean species (Amaral *et al.,* 2007a). Thus, it is valuable to examine whether or not population subdivisions between finless porpoises are revealed by examining the 'conservative' cytochrome *b* gene sequences and conventional phylogenetic trees.

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

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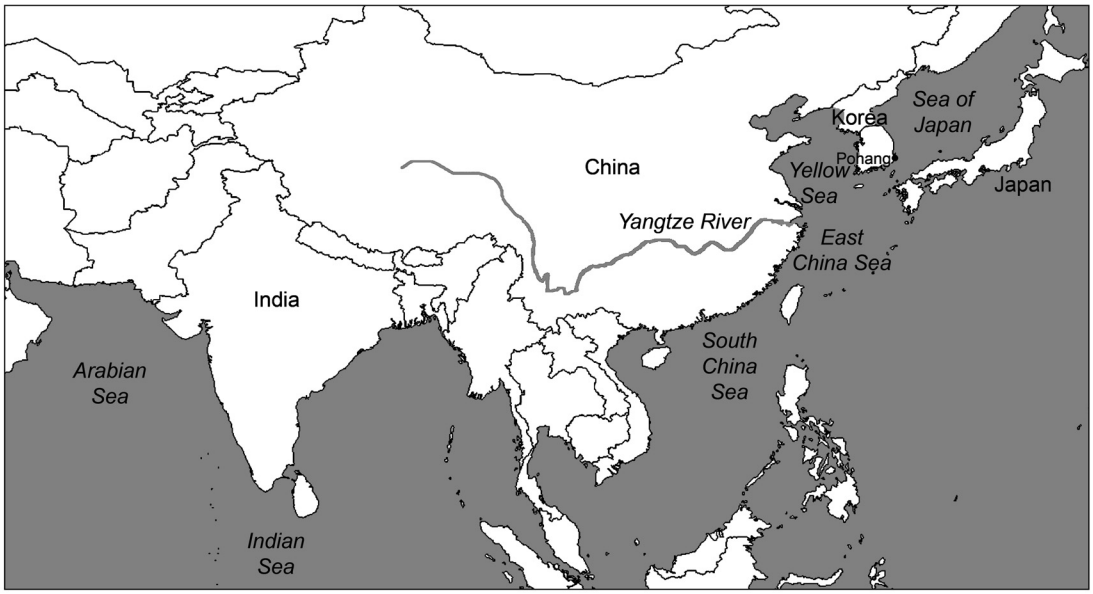

Fig. 1. A map covering the distributional range of two species of genus *Neophocaena*, with collection site of *N. asiaeorientalis sunameri* at Pohang in southeastern Korea. *N. phocaenoides* ranges from the Arabian Sea to the South China Sea, whereas *N.a. asiaeorientalis* is found in the Yangtze River, and *N.a. sunameri* inhabits in East China Sea, Yellow Sea, and the waters of Korea and Japan, including the Sea of Japan (East Sea).

subspecies of *N. asiaeorientalis* (*N.a. asiaeorientalis* and *N.a. sunameri*).

MATERIALS AND METHODS

For this analysis, 12 specimens (specimen nos. 2507-09, 2511, 2513-15, 2538-40, 2542, and 2548) of *N.a. sunameri* were collected from fishery markets at Pohang in southeastern Korea. They were caught from the Sea of Japan (East Sea) as bycatch in 2013, as given in Table 1. A map detailing the distributional range of the two species of *Neophocaena*, with the collection site of *N. asiaeorientalis sunameri* at Pohang in south-eastern Korea is shown in Fig. 1. Small pieces of muscle tissue were taken and preserved in a deep freezer.

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°C for 5 minutes; 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute (32 cycles); and 72°C for 5 minutes. 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 analysed with an automated DNA Sequencer (Perkin Elmer 377) at Bioneer Co. (Seoul, Korea).

Table 1

Specimen number and cytochrome *b* complete haplotypes of 12 *Neophocaena asiaeorientalis sunameri* specimens, collected from fishery markets at Pohang in southeastern Korea. Among the 12 sequences nine haplotypes were identified.

Specimen number	Cytochrome *b* complete haplotype
2507	CB01Korea
2508, 2513, 1538, and 2548	CB02Korea
2509	CB03Korea
2511	CB04Korea
2514	CB05Korea
2515	CB06Korea
2539	CB07Korea
2540	CB08Korea
2542	CB09Korea

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 ten complete sequences of two subspecies in *N. asiaeorientalis*, obtained from GenBank, as given in Table 2. In addition, from the cytochrome *b* complete sequences of *N. asiaeorientalis*, obtained from this study and GenBank, cytochrome *b* partial sequences (402 bp; site nos. 12-413) were obtained and analysed 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, tree constructions with 1,000 bootstrapped replications, and estimation of coefficient of evolutionary differentiation (G^{st}) were conducted using MEGA5 (Tamura *et al.*, 2011). The Jukes-Cantor (JC) model, which showed the lowest Bayesian information criterion scores, was selected by the program, and maximum likelihood trees were constructed. Fin whale, *Balaenoptera physalus* (NC001321) and common dolphin, *Delphinus delphis* (AF084084) were used as outgroups.

RESULTS

Nine cytochrome *b* complete haplotypes were obtained from 12 *N.a. sunameri* specimens, as shown in Table 1, and these nine haplotypes were deposited under the accession numbers from KJ472895 to KJ472903. Within 19 haplotypes of *N. asiaeorientalis* (9 haplotypes from this study and 10 haplotypes from GenBank), 31 sites (2.72%) were variable,

Table 2

GenBank identification of 15 cytochrome *b* haplotypes in the genus *Neophocaena*, used in this study. The ten haplotypes of *N. asiaeorientalis* were complete[1] (1,140bp) sequences and five haplotypes of *N. phocaenoides* were partial[2] (402bp) sequences.

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]
	East China Sea	NC021461[1]
N. phocaenoides	Indian Sea	EF203442[2], EF203444[2], and EF203438[2]
	Arabian Sea	DQ364692[2] and DQ364691[2]

and 16 sites (1.40%) were parsimonious informative. The average JC distance among nine haplotypes of *N.a. sunameri* from Korea was 0.44%.

A maximum likelihood tree with 19 cytochrome *b* complete haplotypes of *N. asiaeorientalis* from four regions in East Asia is shown in Fig. 2, and the 19 haplotypes from East China Sea, Yellow Sea (China), the Yangtze River, and Sea of Japan formed one clade (Gp 1), with within group average JC distance of 0.57% and a G^{st} value of 0.06. In addition, one haplotype (HM137098) of *N.a. asiaeorientalis* from the Yangtze River was identical to one haplotype (CB02Korea) of *N.a. sunameri* from the Sea of Japan, and another haplotype (HM137092) of *N.a. asiaeorientalis* from the Yangtze River was identical to another haplotype (HQ108397) of *N.a. sunameri* from the Yellow Sea. Additionally, HQ108415 from the Yellow Sea was identical to CB04Korea from the Sea of Japan and HQ108420 from the Yellow Sea was identical to CB08Korea from the Sea of Japan.

Another maximum likelihood tree with 24 cytochrome *b* partial haplotypes (402bp) of two *Neohpocaena* species from the Arabian Sea, Indian Sea, East China Sea, Yellow Sea, and Sea of Japan is shown in Fig. 3, and two clades (Gps 1 and 2) were recognised: the 19 haplotypes of *N.*

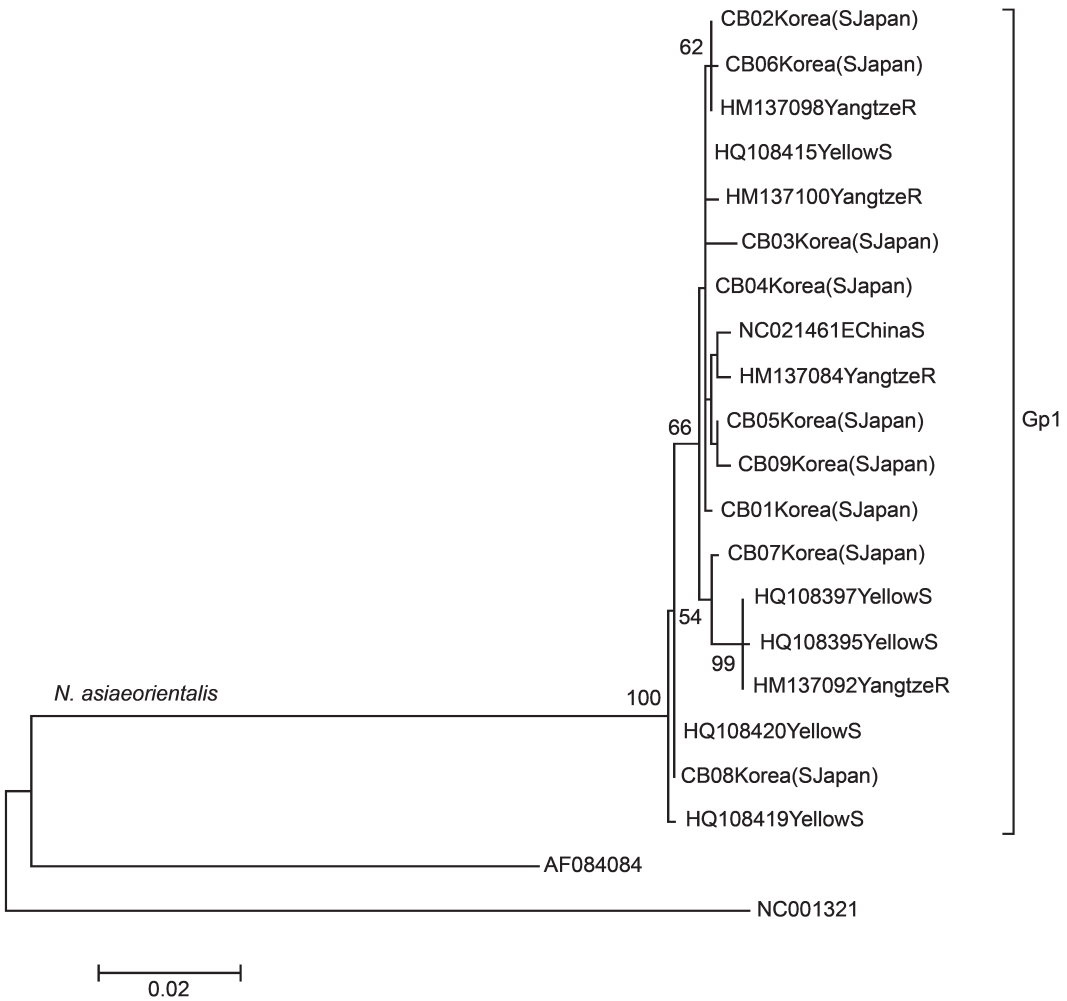

Fig. 2. A maximum likelihood tree with 19 cytochrome *b* complete haplotypes (1,140 bp) of *N.asiaeorientalis*. Nine haplotypes of *N.a. sunameri* from Korea were obtained in this study, as given in Table 1 and 10 haplotypes of *N. asiaeorientalis* were obtained from GenBank, as listed in Table 2. The tree was constructed with 1,000 bootstrapped replications, and the bootstrap values >50% are reported at the internodes. Location name follows haplotype name or accession number in each haplotype, obtained from this study and GenBank. Fin whales, *B. physalus* (NC001321) and common dolphins, *Delphinus delphis* (AF084084) were used as outgroups.

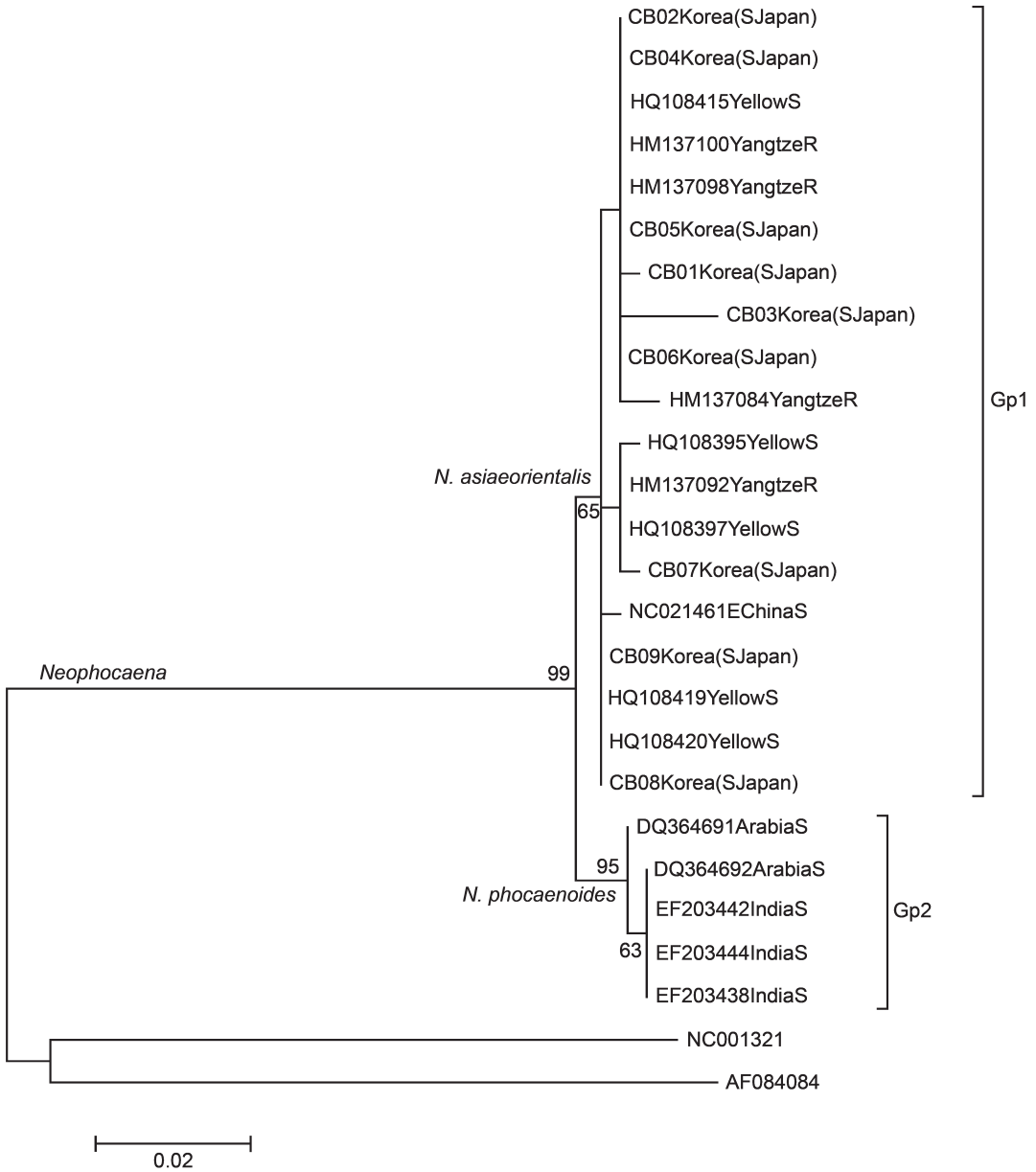

Fig. 3. A maximum likelihood tree with 24 cytochrome *b* partial (402 bp) haplotypes of two species in the genus *Neophocaena*. Nine haplotypes of *N.a. sunameri* from Korea were obtained from this study, as given in Table 1, and ten haplotypes of *N. asiaeorientalis* and five haplotypes of *N. phocaenoides* were obtained from GenBank, as listed in Table 2. Fin whales, *B. physalus* (NC001321) and common dolphins *Delphinus delphis* (AF084084) were used as outgroups.

asiaeorientalis from the four regions of East China Sea, Yellow Sea, the Yangtze River, and Sea of Japan (Gp 1) were distinct from the five haplotypes of *N. phocaenoides* from the Indian and Arabian Seas (Gp 2), with average JC distance of 1.64%, and four fixed site differences (1.00%) at site numbers. 60, 145, 261, and 408, and a G^{st} value of 0.64.

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 was 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, so they distinguished between 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.

From our study based on cytochrome *b* partial sequences (Fig. 3), it was found that *Neophocaena* comprises two clades (Gps 1 and 2), corresponding to the two species of *N. asiaeorientalis* and *N. phocaenoides*, with average JC distance of 1.64%, four fixed site differences (1.00%), and a G^{st} value of 0.64, although the specimens from Southeast Asia and the contiguous South China Sea were not examined.

The nuclear genes vary at a slower rate than mtDNA sequences (Steppan *et al.*, 2005), and the mtDNA cytochrome *b* gene is more conservative than the mtDNA control region (Lopez *et al.*, 1997). 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 was used as one of the barcoding genes (Bennett, 2011) and it has several advantages when compared to the control region in phylogenetic analysis of the genus *Delphinus* (Amaral *et. al*., 2007b). Furthermore, the G^{st} value is equivalent to the F^{st} value (Halliburton, 2004), and the F^{st} value above 0.25 indicates 'very great' genetic differentiation and between them, whereas differentiation is negligible when F^{st} is as small as 0.05 or even less (Wright, 1978).

Cytochrome *b* sequences were used (not analysed by former researchers to discriminate the two species of *Neophocaena*) and two genetically distinct species were found (Fig. 3; Gps 1 and 2), indicating that the cytochrome *b* gene is a useful marker to distinguish the two species. However, further genetic analyses with specimens throughout the distribution range of the two species (especially the specimens from Southeastern Asia and contiguous South China Sea) are necessary to confirm our findings. It was also considered that complete lineage sorting has 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.

In morphological and molecular studies of the East Asian finless porpoise, *N.a. sunameri*, Jefferson and Wang (2011) noted that the Yangtze River finless porpoise, *N.a. asiaeorientalis,* is distinct from it, although they noted that obvious distinction between the two subspecies was not revealed by previous analyses. Li *et al.* (2011) and Ju *et al.* (2012) used *F*-statistics with microsatellite, mtDNA control region, and nuclear intron markers to distinguish between two subspecies of *N. asiaeorientalis*. However, Yang *et al*. (2002) noted that the differentiation between the Yangtze and Yellow Sea populations was not significant from the control region analysis.

In this study, based on cytochrome *b* complete sequences of *N. asiaeorientalis* (Fig. 2), a lack of genetic divergence was found between *N.a. asiaeorientalis* from the Yangtze River (Gp 1, in part) and *N.a. sunameri* from the East China Sea, Yellow Sea, and Sea of Japan (Gp 1, the rest), with a G^{st} value of 0.06 and two pairs of identical sequences between them. 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 the same subspecies in a previous morphometric analysis.

The sequencing results presented here do not support the current classification, recognising two subspecies within *N. asiaeorinetalis.* In future, genetic analyses with more specimens of *Neophocaena* across its distribution range are needed to confirm the findings of this study.

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