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POPULATION STRUCTURE OF FINLESS PORPOISES (*NEOPHOCAENA PHOCAENOIDES*) IN COASTAL WATERS OF JAPAN BASED ON MITOCHONDRIAL DNA SEQUENCES

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To clarify population structure of finless porpoises (*Neophocaena phocaenoides*) in Japan, we examined mitochondrial DNA sequences of 174 animals. All individuals except for a female were collected in 5 geographically discrete coastal waters where Japanese porpoises are mainly distributed: Sendai Bay–Tokyo Bay, Ise–Mikawa Bays, Inland Sea–Hibiki Nada, Omura Bay, and Ariake Sound–Tachibana Bay. We analyzed 345 base pairs of the control region for all animals and detected 10 haplotypes. Two of those were shared by animals from > 1 area, whereas the other 8 were each found only in 1 area. The most common haplotype in Ise–Mikawa Bays and Ariake Sound–Tachibana Bay was not found at other locations. Analysis of the frequency distribution of haplotypes quantified genetic differentiation, and measurements of gene flow indicated limited dispersal of animals among locations. We conclude that finless porpoises in each of the 5 locations belong to distinct populations.

Key words: control region, finless porpoise, Japan, mitochondrial DNA, *Neophocaena phocaenoides*, population structure

The finless porpoise (*Neophocaena phocaenoides*) is a small, toothed cetacean inhabiting a narrow band of shallow waters along coastlines of tropical and temperate Asia from the Persian Gulf east to Japan (Kasuya 1999). These waters are areas of intensive human activity, and the porpoise has been threatened by direct or indirect take, habitat degradation, and pollution. A rapid decline in population size has been reported in some areas (Reeves et al. 1997), so the porpoise is listed on Appendix I of the Convention on International Trade in Endangered Species (CITES).

In eastern Asia, the finless porpoise is distributed along the Chinese coast and the western and southern coast of the Korean Peninsula and throughout much of the Japanese Archipelago. Occurrence of animals has not been confirmed in waters surrounding Tsushima and Iki Islands between Korea and Japan (Shirakihara et al. 1992; see Fig. 1), which suggests that exchange of porpoises is infrequent between Korean and Japanese waters. In Japan, frequent sightings of finless porpoises are restricted to 5 geographically discrete coastal waters (Sendai Bay–Tokyo Bay, Ise–Mikawa Bays, Inland Sea–Hibiki Nada, Omura Bay, and Ariake Sound–Tachibana Bay; Fig. 1), and animals seldom occur in other areas (Shirakihara et al. 1992, 1994). Geographic variation has been reported in timing of parturition (Shirakihara et al. 1993) and external (Shirakihara 1993) and skull (Yoshida

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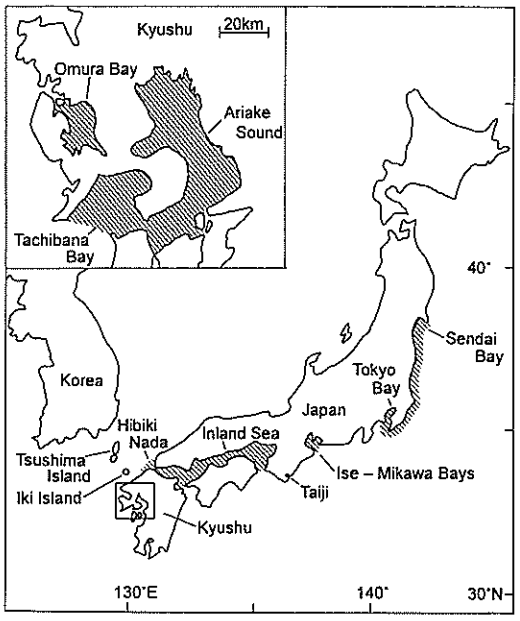

FIG. 1.—Five coastal waters where finless porpoises are mainly distributed in Japan: Sendai Bay–Tokyo Bay, Ise–Mikawa Bays, Inland Sea–Hibiki Nada, Omura Bay, and Ariake Sound–Tachibana Bay, from which specimens were collected. A female taken incidentally near Taiji also was examined.

et al. 1995) morphology among the locations. Yoshida et al. (1995) suggested that finless porpoises in Japanese coastal waters were subdivided into 5 small populations corresponding to the 5 geographic concentrations.

Although finless porpoises are not a target for commercial fisheries in Japan, animals have been incidentally taken by fishing nets (Kasuya 1999; Shirakihara et al. 1993). Further information on population structure is necessary for effective conservation. Mitochondrial DNA (mtDNA) has been used as a marker to identify population structure in a number of cetacean species (Baker and Palumbi 1997). We analyzed mtDNA control region sequences of finless porpoises collected in the coastal waters of Japan to clarify population structure.

MATERIALS AND METHODS

Sample collection.—Tissue samples were collected from 174 finless porpoises between 1980 and 1999. Muscle, liver, or skin was obtained from 161 deceased animals and skin biopsies were obtained from 2 live individuals. Blood was collected from 11 live animals kept in aquaria. Of the deceased animals, 155 were incidentally caught in fishing nets or found beachcast and the remaining 6 porpoises died in aquaria. One animal is deposited in the Ibaraki Nature Museum (INM-1–001763) and 11 are in the National Science Museum, Tokyo (NSMT M29891, 30114, 30120, 30132, 32428, 32429, 32444, 32457, 32465, 32520, and 32521). Muscle, liver, and skin were frozen at −20°C and blood samples were stored frozen in vacuum glass tubes with ethylenediaminetetraacetic acid (EDTA)-2Na at the National Research Institute of Far Seas Fisheries.

All individuals (except for 2 females) were obtained within 5 geographically discrete coastal waters where finless porpoises are mainly distributed in Japan: Sendai Bay–Tokyo Bay, Ise–Mikawa Bays, Inland Sea–Hibiki Nada, Omura Bay, and Ariake Sound–Tachibana Bay (Shirakihara et al. 1992, 1994; Fig. 1). Thus, we conducted pairwise comparisons of mtDNA sequences among the 5 locations. Of the 2 females mentioned above, 1 was born in captivity from a mother from the Inland Sea (Japanese Association of Zoological Gardens and Aquariums 1996). The mother's tissue was not available to this study. The calf was added to sequence comparisons as an animal from the Inland Sea. Another female, which was incidentally taken near Taiji located outside the 5 locations (Fig. 1), was excluded from the population comparisons.

Extraction of DNA, polymerase chain reaction, and sequencing.—From the tissues, total cellular DNA was extracted by standard phenol–chloroform procedure. That procedure consisted of digestion with proteinase K (100 μg/ml) in TEK buffer (50 mM Tris-HCl, 10 mM EDTA, 1.5% KCl, pH 7.5) and 1.0% sodium dodecyl sulfate at 60°C for ≥ 2 h followed by phenol–chloroform extraction and precipitation of total DNA with ethanol. The polymerase chain reaction (PCR) amplifications were performed in 25 μl volumes of Tris buffer (67 mM, pH 8.8) containing 2 mM $MgCl_2$, 1 mM of each deoxynucleotide triphosphate, 1 μM of each primer, 0.6 units of AmpliTaq™ (Applied Biosystems, Fos-

ter City, California) DNA polymerase, and 50–100 ng of template DNA. The control region was amplified using primers t-PRO (5′-CCTCCCTAAGACTCAAGGAA-3′) shortened from Árnason et al. (1993) and Primer-2 (5′-GAAGAGGGATCCCTGCCAAGCGG-3′) described in Hori et al. (1994). The temperature profile was 0.5 min at 96°C, 0.5 min at 60°C, and 1.5 min at 72°C for 30 cycles. Amplified fragments were purified with a Geneclean II® Kit (Bio101, LaJolla, California) and subjected to direct sequencing with the ABI PRISM® Cycle Sequencing Kits (Applied Biosystems, Foster City, California) for 26 cycles (at 96°C for 0.5 min, 50°C for 0.25 min, and 60°C for 4 min), using the same primers as in the PCR reaction.

Data analysis.—Sequences were aligned using the program Clustal W 1.7 (Thompson et al. 1994). Geographic and sexual heterogeneity in frequency distribution of haplotypes was tested by the randomized chi-square test (Roff and Bentzen 1989). To test the significance of observed chi-square values, 1,000 randomizations were executed for each pairwise comparison using the program ROFF (Matsuishi 1992). The sequential Bonferroni method (Rice 1989) was used to adjust critical values ($P < 0.05$) for multiple comparisons.

The DNA polymorphism was measured as the average number of nucleotide differences per site between 2 sequences within 1 sampling area (nucleotide diversity) and as the number of net nucleotide substitutions per site between areas (nucleotide divergence—Nei 1987). The population genetic structure was quantified from Wright's *F*-statistics (F_{ST}—Wright 1951) using the program Arlequin (Schneider et al. 1997). Significances of the observed pairwise F_{ST}-values were determined by comparison with 1,000 random permutation tests followed by the sequential Bonferroni method. Gene flow (Nm) was evaluated from pairwise F_{ST}; that is, $Nm = (1/F_{ST} - 1)/2$ (Slatkin 1985). To visualize relationships among haplotypes detected, a minimum spanning network was constructed using the number of pairwise nucleotide differences with the program MINSPNET provided by L. Excoffier (Department of Anthropology, University of Geneva, Geneva, Switzerland).

Results

A total of 345 base pairs of mtDNA control region was sequenced for all 174 animals. We found 10 unique haplotypes (types a–j). Number of nucleotide substitutions between them ranged from 1 to 6. All substitutions were transitional changes, and no insertions or deletions were observed. Sequences of the 10 haplotypes have been deposited in GenBank under accession numbers AF193543–193552.

The frequency distribution of haplotypes found in each of the 5 locations (Table 1) indicated that no sexual heterogeneity occurred. A female collected near Taiji possessed type c, which was obtained only from Ise–Mikawa Bays. Indeed, the distribution did not differ between males and females ($\chi^2 = 5.14$, $P = 0.40$) in Ariake Sound–Tachibana Bay, where 6 of 10 haplotypes were detected (Table 1). Thus, we tested geographic heterogeneity by combining sexes. Two of the 10 haplotypes (b and d) were shared by animals from > 1 area, whereas the other 8 haplotypes were each found only in 1 area. Such haplotypes were most common in 2 locations: type c in Ise–Mikawa Bays and type f in Ariake Sound–Tachibana Bay. The frequency distribution of haplotypes differed ($\chi^2 = 22.00$–121.00, $P < 0.05$) among the 5 locations except for a comparison between Inland Sea–Hibiki Nada and Omura Bay ($\chi^2 = 0.87$, $P = 0.10$).

Estimates of nucleotide diversity within each of the 5 locations indicated that in Sendai Bay–Tokyo Bay and Ariake Sound–Tachibana Bay, values (0.314% and 0.408%) were higher than estimates from the other 3 locations (0.000–0.054%; Table 2). Nucleotide divergence between the 5 locations was evaluated from 0.002% between Inland Sea–Hibiki Nada and Omura Bay to 0.828% between Inland Sea–Hibiki Nada and Ariake Sound–Tachibana Bay. Estimates were higher than values for harbour porpoises (*Phocoena phocoena*—Wang et al. 1996), which is a closely related species, except for between Inland Sea–Hibiki Nada and Omura Bay; however, nucleotide diversity was lower in 3 areas: Ise–Mikawa Bays, Inland Sea–Hibiki Nada, and

TABLE 1.—Frequency distribution of mitochondrial DNA control region haplotypes (a–j) of finless porpoises in 5 coastal waters where animals are mainly distributed in Japan.[a]

Area	Haplotypes										Total
	a	b	c	d	e	f	g	h	i	j	
Sendai Bay–Tokyo Bay	7 (7, 0)[b]	7 (3, 4)	0	0	0	0	0	0	0	0	14 (10, 4)
Ise–Mikawa Bays	0	4 (4, 0)	52 (28, 21)[c]	0	0	0	0	0	0	0	56 (32, 21)[c]
Inland Sea–Hibiki Nada	0	0	0	27 (10, 17)	3 (1, 2)	0	0	0	0	0	30 (11, 19)
Omura Bay	0	0	0	8 (6, 2)	0	0	0	0	0	0	8 (6, 2)
Ariake Sound–Tachibana Bay	0	0	0	9 (5, 4)	0	46 (28, 18)	6 (3, 3)	2 (0, 2)	1 (1, 0)	1 (0, 1)	65 (37, 28)

[a] A female collected near Taiji located outside the above 5 waters is excluded.
[b] Male, female.
[c] Sex was unknown for 3 animals.

Omura Bay. The estimated F_{ST}-values ranged from −0.013 between Inland Sea–Hibiki Nada and Omura Bay to 0.893 between Ise–Mikawa Bays and Omura Bay (Table 3). Among the 5 locations, observed values were positive and different ($P < 0.05$) from those calculated with 1,000 randomizations, except for a comparison between Inland Sea–Hibiki Nada and Omura Bay. The estimate of gene flow was infinity between Inland Sea–Hibiki Nada and Omura Bay, whereas estimates were lower among all other locations (Table 3).

The minimum spanning network of 10 haplotypes indicated that haplotypes were divided into 2 primary clusters (Fig. 2). One cluster (f–h) was found only in Ariake Sound–Tachibana Bay, whereas the other (a–e, i, and j) was constituted of haplotypes obtained from the 5 locations.

Discussion

Dispersal of finless porpoises seems to be limited among coastal waters of Japan. The most common haplotype in Ise–Mikawa Bays and Ariake Sound–Tachibana Bay was not found at other locations (Table 1). The frequency distribution of haplotypes was significantly different among 5 coastal waters except for a comparison between Inland Sea–Hibiki Nada and Omura Bay. Furthermore, level of genetic differentiation was significant, and gene flow was evaluated as extremely low among the 5 locations except for a comparison between Inland Sea–Hibiki Nada and Omura Bay (Table 3). Analysis of our results suggests that finless porpoises in Japan are subdivided into ≥4 populations: Sendai Bay–Tokyo Bay, Ise–Mikawa Bays, Inland Sea–Hibiki Nada and Omura Bay, and Ariake Sound–Tachibana Bay.

We could not find genetic differentiation between animals of Inland Sea–Hibiki Nada and Omura Bay, perhaps because of low statistical power resulting from the small sample size of Omura Bay. Indeed, past studies suggest rare movements of porpoises between Hibiki Nada and Omura Bay;

TABLE 2.—Nucleotide diversity (on the diagonal) and nucleotide divergence (below the diagonal) estimated for mitochondrial DNA control region sequences of finless porpoises collected in 5 coastal waters of Japan (×100).[a]

		Area				
	Area	1	2	3	4	5
1	Sendai Bay–Tokyo Bay	0.314				
2	Ise–Mikawa Bays	0.114	0.039			
3	Inland Sea–Hibiki Nada	0.428	0.294	0.054		
4	Omura Bay	0.426	0.292	0.002	0.000	
5	Ariake Sound–Tachibana Bay	0.508	0.621	0.828	0.825	0.408

[a] For comparison, harbour porpoises (*Phocoena phocoena*) in coastal waters of eastern North America have nucleotide diversity of 0.341–0.434 and nucleotide divergence of 0.000–0.011 (Wang et al. 1996).

no reports exist of porpoise occurrence in coastal regions between the waters (Shirakihara et al. 1992, 1994). For animals in Omura Bay, long-term persistence is possibly threatened. Deterioration of food supply has been suggested (Shirakihara 1993), and their estimated abundance is much lower (187 individuals—Yoshida et al. 1998) than populations in the other waters: 1,952 in Ise–Mikawa Bays (T. Miyashita et al., in litt.), 4,900 in Inland Sea (Kasuya and Kureha 1979), and 3,093 in Ariake Sound–Tachibana Bay (Yoshida et al. 1997). For conservation purposes, porpoises in Omura Bay should be treated separately from animals in Inland Sea–Hibiki Nada until sufficient information on population structure is accumulated. We conclude that finless porpoises in Japanese waters are subdivided into 5 small populations and conservation plans should be developed for each. That conclusion was derived from the maternally inherited mtDNA sequences. Although we obtained no evidence for a sex-biased dispersal by males (Greenwood 1980), which has been observed in many mammalian species (e.g., harbour porpoises—Wang et al. 1996), it would be desirable to conduct further analyses using nuclear markers, which are transmitted by both sexes.

We analyzed population structure of finless porpoises by combining animals from Sendai Bay–Tokyo Bay, which runs from north to south along Japan's eastern coast. This is because past study indicates a continuous distribution of porpoises in these waters (Shirakihara et al. 1992). However, we found geographic separation of haplotypes with all animals with haplotype a collected in Sendai Bay whereas 7 porpoises from coastal waters near Tokyo Bay possessed haplotype b. Our small sample size may obscure overlapping haplotypes, but the possibility that porpoises in these waters are subdivided into 2 populations should be tested. Further study is recommended for

TABLE 3.—Wright's *F*-statistics (above the diagonal) and gene flow (Nm; below the diagonal) evaluated among finless porpoises in 5 coastal waters of Japan.

		Area				
	Area	1	2	3	4	5
1	Sendai Bay–Tokyo Bay		0.571*	0.763*	0.672*	0.563*
2	Ise–Mikawa Bays	0.376		0.869*	0.893*	0.723*
3	Inland Sea–Hibiki Nada	0.155	0.076		−0.013	0.734*
4	Omura Bay	0.245	0.060	∞		0.685*
5	Ariake Sound–Tachibana Bay	0.388	0.191	0.181	0.230	

* Significant difference of observed *F*-statistics at 5% level with sequential Bonferroni correction.

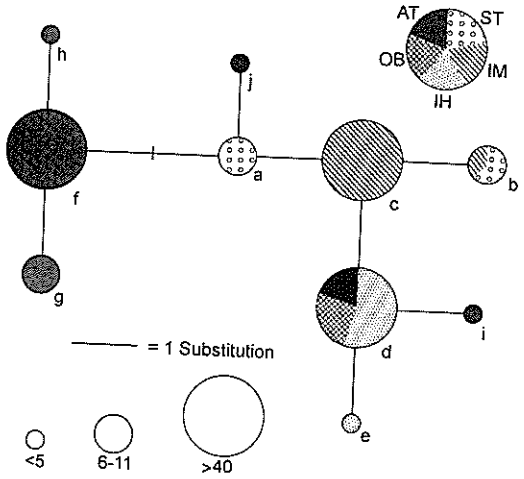

FIG. 2.—Minimum spanning network of 10 mitochondrial DNA control region haplotypes (a–j) from finless porpoises in Japanese coastal waters. Size of circles is proportional to number of animals represented by that haplotype. ST, Sendai Bay–Tokyo Bay; IM, Ise–Mikawa Bays; IH, Inland Sea–Hibiki Nada; OB, Omura Bay; AT, Ariake Sound–Tachibana Bay.

animals in Sendai Bay–Tokyo Bay to clarify their population structure using nuclear genetic markers from individuals from central locations.

Finless porpoises in 4 of the 5 ranges possess unique haplotypes. If animals are incidentally taken far from their normal ranges, we may be able to determine their origin. A female finless porpoise taken near Taiji located outside the 5 normal ranges of Japanese porpoises (Fig. 1) possessed haplotype c. That haplotype was dominant in Ise–Mikawa Bays and was not found in any other waters (Table 1), so this individual was thought to have come from Ise–Mikawa Bays. Such analyses may provide further information on gene flow in this species.

Our present study reveals strong philopatry of finless porpoises. Little evidence exists of frequent intermingling of animals between Omura Bay and Ariake Sound–Tachibana Bay, which are only 60 km apart. Coastal waters between Omura Bay and Ariake Sound–Tachibana Bay possess rocky and steep bottom. Habitats of Japanese finless porpoises consist of a dominance of sandy or muddy bottoms and offshore extension of shallow regions with depth of < 50 m, whereas rocky or steep waters are not inhabited by this species (Shirakihara et al. 1992). These geographic environments possibly restrict animal movement between neighboring ranges. The existence of 3 finless porpoise populations has been proposed in coastal waters in China (Gao and Zhou 1993); however, population structure of porpoises should be examined in detail throughout Asian waters.

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